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Hypoxia modulates CCR7 expression in head and neck cancers

Haneen A. Basheer,^{1,2} Edvinas Pakanavicius,³ Patricia A. Cooper,¹ Steven D. Shnyder,¹ Lisette Martin,⁴ Keith D. Hunter,⁴ Victoria Vinader,¹ Kamyar Afarinkia^{1*}

¹*The Institute of Cancer Therapeutics, University of Bradford, Bradford, BD7 1DP, United Kingdom.*

²*Faculty of Pharmacy, Zarqa University, PO Box 132222, Zarqa 13132, Jordan.*

³*Division of Biosciences, University College London, London WC1E 6BT, United Kingdom.*

⁴*School of Clinical Dentistry, University of Sheffield, Sheffield, S10 2TA, United Kingdom.*

Correspondence should be addressed to KA (k.afarinkia@brad.ac.uk)

Highlights

- Under hypoxia, the expression of CCR7 is elevated in both *in vitro* and *in vivo* models.
- There is a correlation between HIF-1 α and CCR7 in all head and neck clinical stages.
- Hypoxia contributes to a metastatic phenotype in HNC by upregulating CCR7.

Abstract

Background: The chemokine receptor CCR7 is expressed on lymphocytes and dendritic cells and is responsible for trafficking of these cells in and out of secondary lymphoid organs. It has recently been shown that CCR7 expression is elevated in a number of cancers, including head and neck cancers, and that its expression correlates to lymph node (LN) metastasis. However, little is known about the factors that can induce CCR7 expression in head and neck cancers.

Method: We compared the protein expression and functional responses of CCR7 under normoxia and hypoxia in head and neck cancer cell lines OSC-19, FaDu, SCC-4, A-253 and Detroit-562 cultured as monolayers, spheroids, and grown *in vivo* as xenografts in balb/c mice. In addition, we analysed the correlation between hypoxia marker HIF-1 α and CCR7 expression in a tissue microarray comprising 80 clinical samples with various stages and grades of malignant tumour and normal tissue.

Results: Under hypoxia, the expression of CCR7 is elevated in both *in vitro* and *in vivo* models. Furthermore, in malignant tissue, a correlation is observed between hypoxia marker HIF-1 α and CCR7 across all clinical stages. This correlation is also strong in early histological grade of tumours.

Conclusion: Hypoxia plays a role in the regulation of the expression of CCR7 and it may contribute to the development of a metastatic phenotype in head and neck cancers through this axis.

Keywords: CCR7, head and neck cancer, hypoxia, HIF-1 α , tumour grade, tumour stage

Abbreviations: APES = 3-aminopropyltriethoxysilane; DAPI = 4',6-diamidino-2-phenylindole; HIF = hypoxia inducible factor; HNC = head and neck cancer; LDH-A = lactate dehydrogenase A; LN = lymph nodes; TMA = tissue microarrays.

Introduction

Tumours occurring in the anatomical regions of the head and neck are the sixth most common types of cancer worldwide, with over 500,000 new cases and over 350,000 deaths reported every year. [1] A particularly unwelcome hallmark of head and neck cancers is the high incidence of metastasis, particularly to regional lymph nodes. In fact, by the time of diagnosis, nearly half of head and neck cancers have already metastasised to neck lymph nodes and one in ten has metastasised to distant organs. [2]

It is now well established that the chemotactic signalling axis involving chemokines CCL19 and CCL21 acting on cell surface chemokine receptor CCR7 plays an important role in lymph node (LN) metastasis in a number of cancers. In normal physiology, CCR7 is expressed on lymphocytes and mature dendritic cells. The activation of CCR7 by CCL21 and CCL19 released from the secondary lymphoid organs enables these cells to migrate against the chemokine gradient in and out of the lymph nodes. It has been hypothesised that cancer cells adopt this chemotactic axis, through upregulating the expression of CCR7; thus enabling them to invade the lymphatic system. [3] So far, the significance of the CCL19/CCL21-CCR7 axis in LN metastasis has been shown in pancreatic, [4] colorectal, [5] gastric, [6] melanoma [7], breast [8-9] and in the head and neck cancers. [10-21] In particular, these studies have shown that CCR7 expression is elevated in a wide range of oral and oropharyngeal clinical tumour tissue; that the elevation in CCR7 expression is associated with a metastatic phenotype [13] and correlates with the potential for cervical LN metastasis in a number of oral cancers; [12-18] and that CCR7 expression is a negative prognostic factor in many different head and neck cancers. [19-21] Moreover, intracellular pathways which are stimulated as a result of the activation of CCR7 are being identified,[10, 22-26] and we are now beginning to gain an understanding of the mechanisms by which this axis contributes to the biology of the tumour cell.

However, whilst the role of CCR7 axis in the expansion of head and neck cancers is apparent, with the exception of tumour-associated inflammation, [10] relatively little known of the factors which promote the upregulation of CCR7 expression on tumour cells.

It is well established that as tumours grow, poor and irregular development of blood vessels leads to the emergence of regions within the tumour lacking in oxygen (hypoxia) and nutrients. Whilst the level of oxygenation in tumours can be quite varied, [27] more physiologically relevant, “severe” hypoxia in particular is known to have a wide ranging and

profound influence on the tumour microenvironment, and to promote malignancy and metastasis in cancers, [28-29] including head and neck cancers [30,31] through a diverse number of pathways. Interestingly, one of those pathways involves the upregulation of chemotactic receptors, for example CXCR4, [32-36] on tumour cells which in turn increase their metastatic potential.

In view of the recent evidence linking CCR7 expression with hypoxia in breast [35] and lung [36] cancers, we hypothesised that hypoxia may also be a factor in promoting CCR7 expression in head and neck cancers. Therefore in this study we investigated whether CCR7 expression correlates with the emergence of hypoxia in head and neck cancers.

Materials and Methods

Cells and cell culture

All cell lines, except OSC-19, were obtained from European Collection of Cell Cultures (ECACC) UK, American Type Culture Collection (ATCC) or National Cancer Institute (NCI) Developmental Therapeutics Programme. OSC-19 was obtained from Japanese Collection of Research Bioresources (JCRB).

Reagents and methods

Recombinant CCL19 (catalogue number 361-MI-025) and CCL21 (catalogue number 366-6C-025) were purchased from R&D Systems (Abingdon, UK). For flow cytometry, CCR7 Alexa Fluor® 488 conjugated anti-human CCR7 antibody (catalogue number 353205, Biolegend) (2:100 dilution) and the corresponding isotype matched control antibody (catalogue number 400233, Biolegend) (2:100 dilution) were used. In Immunohistochemistry, deparaffinised 5 µm-thick xenograft or tissue sections were incubated with CCR7 primary antibody (rabbit monoclonal IgG; Abcam, ab32527) (1:1000 dilution) for 1 h room temperature; whilst for HIF-1α (Anti-HIF-1α antibody, Abcam, catalogue number ab51608, 1:100 dilution) and Ki-67 (1:100 dilution) detection, the antibodies were incubated overnight at 4°C. A vectastain ABC kit with 3,3-diaminobenzidine (DAB) as chromogen was used to visualise the proteins. For cellular immunofluorescence, cells were permeabilised using 0.1% Triton X-100 before treatment with CCR7 and Ki-67 primary antibodies and were detected using fluorescent secondary antibody (Alexa Fluor® 546 Donkey Anti-Rabbit IgG, life technologies, catalogue number A10040) for 1 h in the dark. For pimonidazole detection, spheroids were treated with pimonidazole for 2 h prior to fixation in Bouin's Solution and paraffin embedding.

Hypoxyprobe™-1 Fitc conjugated antibody was added for 2 h at 37 °C. Cell nuclei were visualised using blue fluorescent dye DAPI. Scratch assays were performed in a 24 well plate with cells in media containing 2% serum. Images were captured at 0 h and an appropriate time to observe a significant difference between the two control experiments with and without added chemokine. These times were 24 h (SCC-4), 18 h (OSC-19), 24 h (FaDu), 42 h (DLD-1), 21 h (SW-480) and 14 h (PC-3) and migrated areas were measured using ImageJ software (NIH, USA). [37]

Xenografts

Balb/c immunodeficient nude female mice (Envigo, Loughborough, U.K.), aged between 6 and 12 weeks were used. Throughout the study, all mice were housed in air-conditioned rooms in facilities approved by the United Kingdom Home Office to meet all current regulations and standards. All procedures were carried out under a United Kingdom Home Office Project License (PPL 40/3670). 1×10^6 Tumour cells in 100 μ L of cell culture medium were injected into both abdominal flanks of the mouse, and tumour growth was then monitored using calliper measurements. Once tumours reached a volume of 150 mm^3 , mice were euthanised, and the tumours excised and fixed in 10% neutral buffered formalin for 24 h before being processed for paraffin embedding. 5 μ m-thick tissue sections were then collected on to 3-aminopropyltriethoxysilane (APES) coated glass slides and allowed to dry at 37°C overnight.

Tissue microarray

Head and neck tissue microarrays slides (consecutive slides from block HN803c) were purchased from Insight Biotechnology Limited (Wembley, UK). The microarrays were prepared by the US Biomax Inc. (<https://www.biomax.us/>) and information on tumour extent (T), regional lymph nodes (N) and distant metastasis (M) was provided, along with patient clinical characteristics (see **Supplementary Information S1**). Biomax collects human tissues are under HIPPA approved protocols with the donor providing informed consent.

Analysis

General: All data is presented as the mean \pm standard deviation of at least 3 independent experiments. Graph construction and statistical analysis was performed using GraphPad Prism 7.03. Differences among groups were assessed using the ANOVA test. Differences

between two groups were assessed using a *t* test. P values were calculated to determine statistical significance of the results. *P < 0.05 and **P < 0.01 were deemed significant.

Evaluation of CCR7 and HIF-1 α staining in microarrays: H-score system [38] was used for the evaluation of the staining level of CCR7 and HIF-1 α . Briefly, tumour cells were scored according to a pre agreed staining level intensity (**Supplementary Information S2**), and the H-score was subsequently generated by adding the percentage of strongly stained cell (x3), the percentage of moderately stained cell (x2), and the percentage of weakly stained cell (x1), over a possible range of 0–300. The scores were independently obtained by two expert pathologists (KDH and LM) and the correlation between the two independent scores was above 0.9. For statistical analysis, the mean of the two values was calculated and used. Statistical analyses for clinical samples between H-scores of CCR7 and HIF-1 α for each clinical stage (1, 2, 3 and 4) and lymph node metastasis were performed using Pearson correlation test and the significance of the results is represented by P value. Statistical differences between the H-score for CCR7 or HIF-1 α expression between tumour and non-malignant cells were evaluated using Mann-Whitney U test. Association between high expression and clinical characteristics was evaluated using Fisher exact test. High expression levels were assigned for H-score \geq 150 as the mid-way between the highest (300) and lowest (0) H-scores. *P < 0.05 and **P < 0.01 were deemed significant. Statistical tests on clinical samples were performed using Python 2.7 SciPy 0.19.

Results and Discussions

CCR7 is expressed in head and neck cancer cell lines and the receptor is functionally active

We first set out to determine the expression of CCR7 in various head and neck cancer cell lines cultured as monolayers using immunofluorescence. We chose OSC-19 (metastatic tongue), FaDu (pharynx), SCC-4 (tongue), A-253 (submaxillary salivary gland) and Detroit-562 (metastatic pharynx). For comparison, the expression of CCR7 in a number of other cancer cells lines was also assessed. In all five head and neck cancer cell lines, we detected the presence of CCR7. (**Figure 1A**) As the cells are permeabilised, antibody can access and react with both cytoplasmic and membranous CCR7. Indeed, we observed that in the head and neck cancer cell lines, expression of CCR7 was mostly membranous (except SCC-4), yet we observed cytoplasmic expression of CCR7 in both COLO205 and SW480 (not shown) colorectal cancer cell lines. (**Figure 1A**)

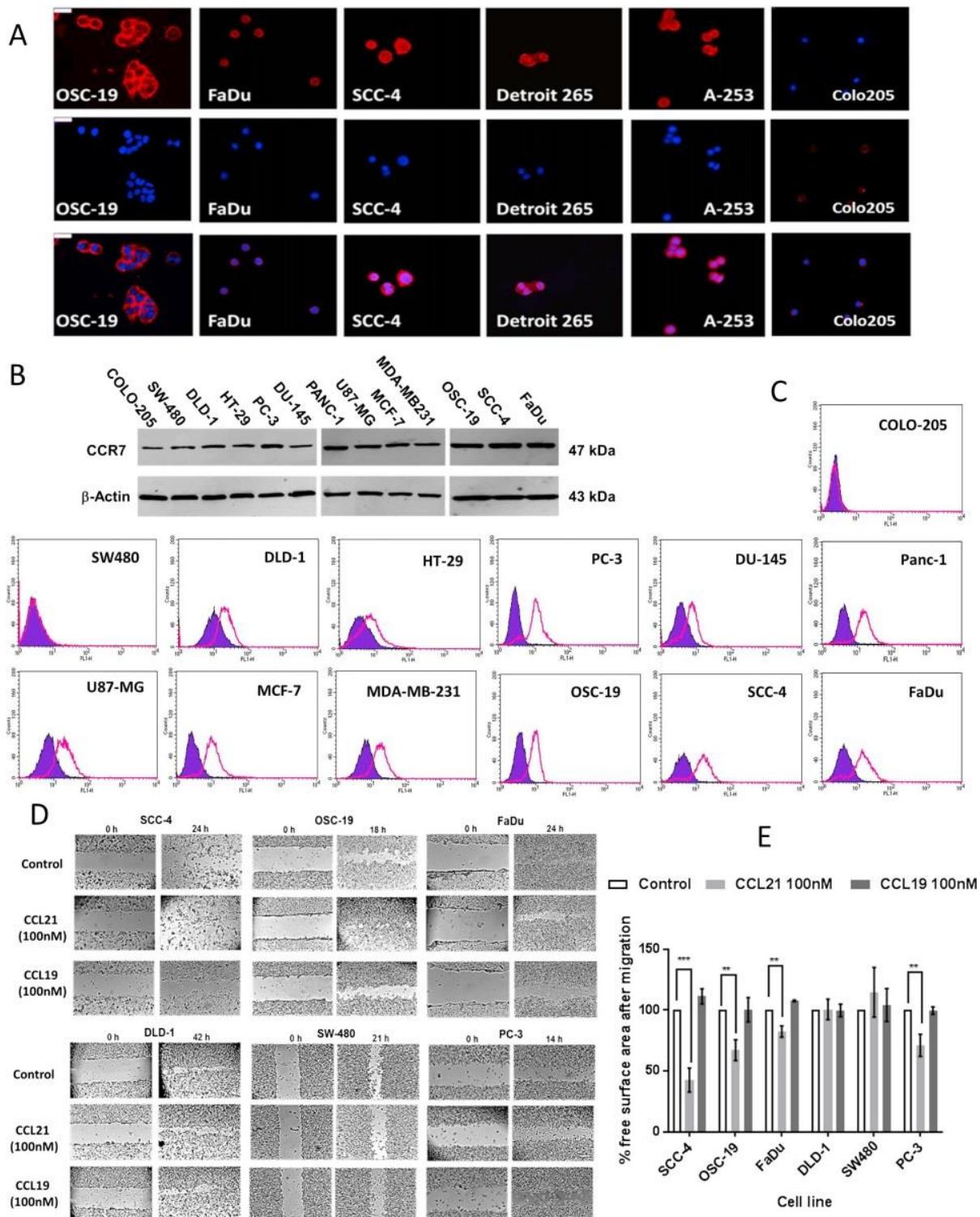


Figure 1: Expression and functional activity of CCR7 in cell lines: **(A)** Expression of CCR7 in head and neck cancer cell lines by immunofluorescence where blue represent staining for DAPI and red represent staining for CCR7. **(B)** Expression of CCR7 is detected in cancer cell lines by Western blot. **(C)** Expression of CCR7 in cancer cell lines by flow cytometry where the filled purple trace is the isotype control and cyan trace is the expression of CCR7. **(D)** Representative scratch assays. **(E)** Enhancement of cell motility in different cell lines upon treatment with CCL21 (100nM) and CCL19 (100nM) ($n = 3$). Two tailed T test P values are considered statistically different when ($P < 0.05$). SCC4 *** $p=0.0005$ (CCL21), $p=0.0647$ (CCL19); OSC19 ** $p=0.026$ (CCL21), $p=0.9686$ (CCL19); FADU ** $p=0.0031$ (CCL21), $p=0.076$ (CCL19); DLD1 $p=0.9234$ (CCL21), $p=0.9064$ (CCL19); SW480 $p=0.2838$ (CCL21), $p=0.6270$ (CCL19); PC3 ** $p=0.0052$ (CCL21), $p=0.7871$ (CCL19).

Using Western blot technique, CCR7 was ubiquitously detected in a wide range of cancer cell lines (**Figure 1B**). To quantify the levels of CCR7, we used flow cytometry and measured the expression of membranous protein using isotype antibody as control for non-specific binding (**Figure 1C**). There was general agreement between Western blot and flow cytometry results, although interestingly, not in all cell lines (e.g. SW480 and COLO205). A similar observation was previously reported by Na *et al.* [39] which showed that defects in the signal peptide domain of CCR7 can lead to the accumulation of cytoplasmic protein, which is unable to translocate to the cell membrane. Since Western blot analysis is carried out on whole cell lysates, it may have overestimated the relative presence of membranous CCR7 receptor in SCC-4 cell line.

In addition to demonstrating that the above head and neck cancer cell lines express CCR7, we also confirmed that the receptors are functionally active. In a scratch assay for OSC-19, FaDu and SCC-4, cell motility was increased in the presence of CCL21, a ligand for CCR7 (**Figure 1D**). Interestingly, the cell lines' response to CCL19 was less pronounced. This is consistent with reports from other laboratories that have shown CCL21 is better at inducing migration [40] and probably reflects the difference in the intracellular pathways activated by the two ligands. [41] In control experiments, the colon cell line DLD-1 and SW480 which express little CCR7, showed no increase in motility in the presence of CCL21 whilst CCR7 expressing prostate PC-3 cell line did (**Figure 1D, 1E**).

Whilst our results showed that head and neck cancer cell lines grown as monolayers express CCR7, we wanted to ensure that the CCR7 expression is maintained when the cells are grown as aggregates or as xenografts *in vivo*. There is evidence that cells can change their expression profiles when grown as multicellular spheroids or if xenoplanted in mice. For instance, ovarian cancer cell lines are known to change the expression pattern of several proteins. [42] In head and neck cancers similarly, the significance of tumour microenvironment to factors that influence disease progression has also been highlighted. [43]

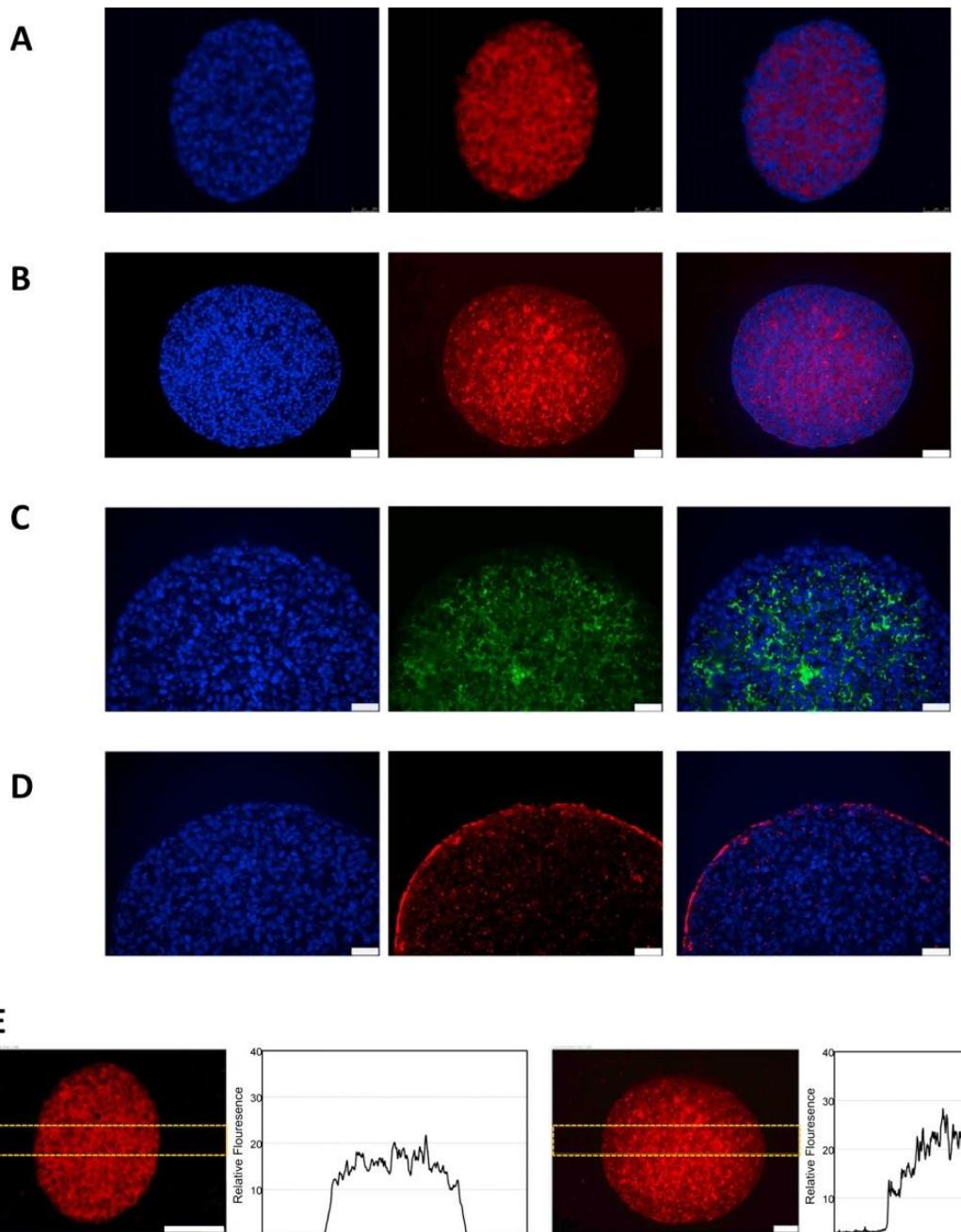


Figure 2: Expression and functional activity of CCR7 in multicellular spheroids: (A) Expression of CCR7 in a small spheroid where blue represents staining for DAPI and red represents staining for CCR7. Scale bar = 25 μ m at 20x magnification (B) Expression of CCR7 in a larger spheroid shows more red staining in the core. Scale bar = 50 μ m at 40x magnification (C) Presence of hypoxia in the core of larger spheroid is shown by immunofluorescence staining for pimonidazole adducts. (D) Proliferating rim of larger spheroid is shown by immunofluorescence staining for Ki-67. (E) Distribution of CCR7 as detected by immunofluorescence is uniform in smaller spheroid but elevated in the core of larger spheroid.

To determine the expression of CCR7 in *in vitro* 3D models, OSC-19 cells were grown as multicellular spheroids, formalin fixed and embedded in paraffin before sections were taken and analysed by immunofluorescence. The results (**Figure 2A**) clearly show that CCR7 expression is preserved in the cells within the spheroid. Furthermore, OSC-19, FaDu, A-253, and Detroit-562 cells were injected subcutaneously into mice and grown as tumours,

excised, formalin fixed and embedded in paraffin before sections were taken and analysed by immunohistochemistry. Again, we observed that mostly membranous CCR7 expression is preserved in the cells within the different xenoplanted tumour tissues. The cytoplasmic expression of CCR7 was strongest in FaDu derived xenograft tissue. (**Figure 3**) Expression of CCR7 was also confirmed in clinical tissue (*vide infra*).

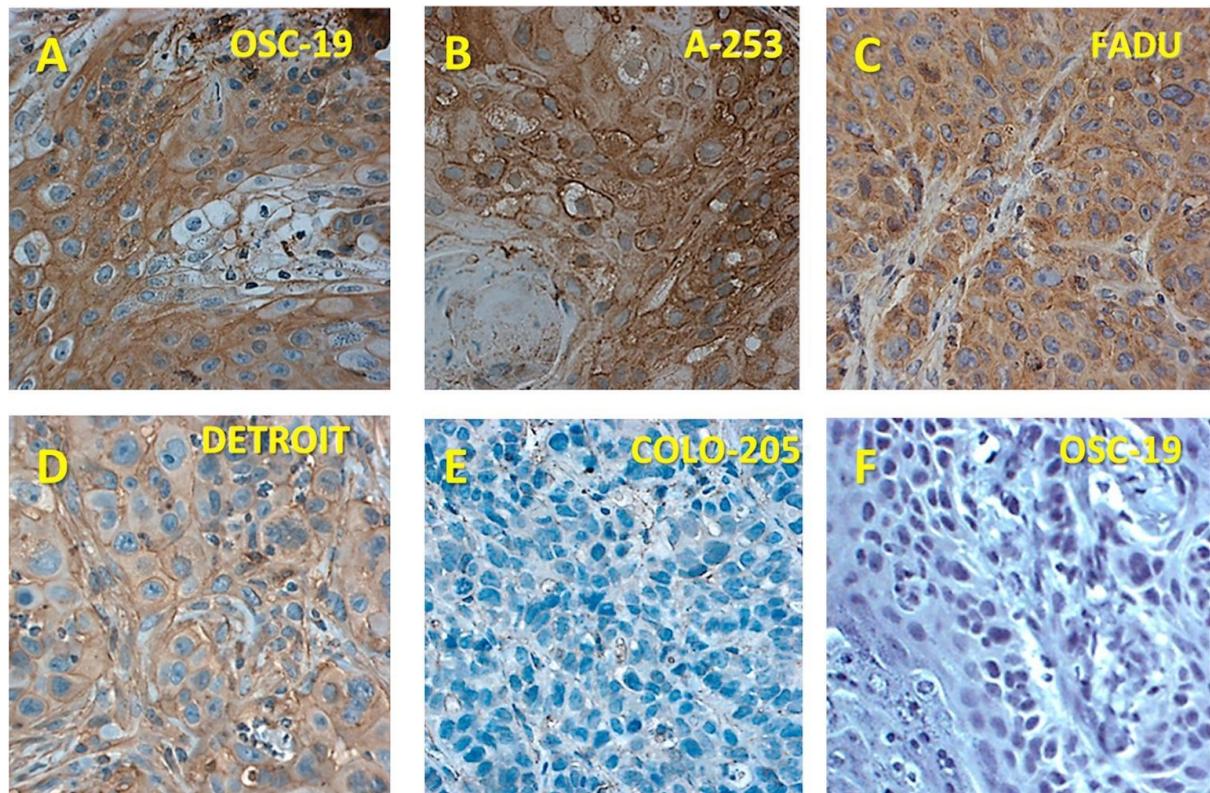


Figure 3. Expression of CCR7 in xenografts derived from different head and neck cell lines Protein expression (brown) is identified by biotinylated-labelled antibody. Basophilic structure of cell (blue) was counter stained with haematoxylin solution. **A**, OSC-19; **B**, A-253; **C**, FaDu; **D**, Detroit-256; **E**, COLO205. Negative control for OSC-19 is also shown, **F**. Scale bar = 100 μ m at 40x magnification

CCR7 protein expression increases under hypoxia in both *in vitro* and *in vivo* models

We next turned our attention to the role of hypoxia in CCR7 receptor expression. We quantitatively compared the cell surface expression of CCR7 in OSC-19 cells incubated under hypoxic and normoxic conditions. We found that all head and neck cancer cell lines, except SCC-4, increased their CCR7 expression under hypoxic conditions compared to cells incubated under normoxia. (**Figure 4A-B**) In particular, OSC-19 cells increased their CCR7 expression by 2.5 fold as measured by flow cytometry.

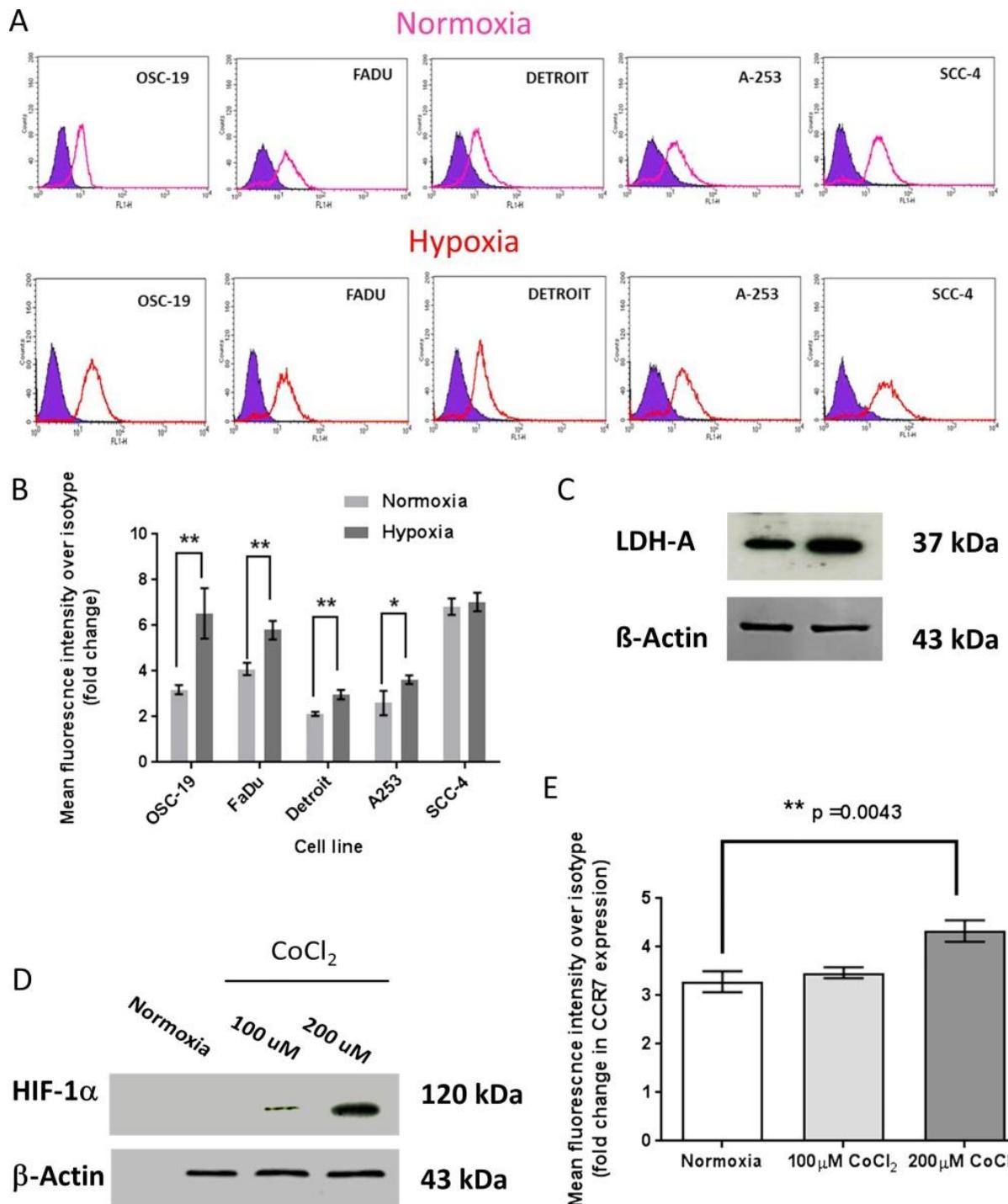


Figure 4. (A) Flow cytometry histograms showing relative expression of CCR7 under normoxia and under physiologic hypoxia (0.1% O₂) (B) bar chart showing relative expression of CCR7 in different head and neck cancer cell lines. All expression levels are normalised to isotype (n = 3). Two tailed T test P values are considered statistically different when (P < 0.05): OSC19 **p=0.0067; FADU **p=0.0038; Detroit **p=0.0032; A253 *p=0.0351; SCC4 p=0.5537. (C) Levels of lactate dehydrogenase-A (LDH-A), a downstream indicator of HIF-1α mediated hypoxic response is elevated in OSC-19 cells under hypoxia. (D) Treatment of OSC-19 cells with CoCl₂ induces HIF-1α. (E) Treatment of OSC-19 cells with CoCl₂ results in an increase in CCR7 expression (n=3), 100μM CoCl₂ p = 0.2574, **, 200μM CoCl₂ **p=0.0043.

A well-known feature of hypoxia in cells is the induction of transcription factors HIF-1 and HIF-2, with the former being the more commonly studied, as well as downstream proteins

such as lactate dehydrogenase A (LDH-A). [44,45] HIFs upregulate various cellular proteins in response to hypoxia, in order to compensate for the lack of available oxygen to the cell. [45,46] HIF-1 is comprised of two subunits: HIF-1 β , which is constitutively expressed and HIF-1 α , levels of which are suppressed by oxygen sensing proteins under normoxia. Under hypoxia, however, intracellular levels of HIF-1 α increase, which in complex with HIF-1 β leads to transcriptional changes in the cell. Although there are non-hypoxic mechanisms leading to the elevation of intracellular levels of HIF-1 α , [47,48] detection of elevated HIF-1 α or HIFs' downstream proteins such as LDH-A [44] is generally considered to be an indicator of hypoxia. Indeed, we showed that in OSC-19 cells, levels of LDH-A are elevated under hypoxia. (**Figure 4C**)

Stabilisation of HIF-1 α can also be achieved by addition of CoCl₂ to cell culture medium to mimic hypoxia. [49] So we next looked at the relationship between CCR7 and chemically induced (CoCl₂) hypoxia. Indeed, addition of CoCl₂ to OSC-19 cell culture medium, resulted in a dose dependent increase in HIF-1 α . (**Figure 4D**) A parallel increase in levels of CCR7 expression in the same cells was similarly detected by flow cytometry. (**Figure 4E**)

Of course, whilst exposure of cell monolayers to low oxygen conditions, or the use of CoCl₂ to mimic hypoxia are useful models, neither realistically recapitulates the conditions experienced by cancer cells in tissue. In this context, multicellular spheroids provide a convenient *in vitro* tool which can better emulate the conditions experienced by cancer cells which are within a matrix. For example, since gas perfusion through cell layers is restricted, an oxygen gradient is established towards the core of spheroids which mimics hypoxia in tumours. Generally speaking 200 μ m is considered the depth where oxygen levels fall to that seen in severe hypoxia (around 0.1% O₂), therefore spheroids with a radius greater than that are expected to have a hypoxic core. [50]

Indeed, analysis of the CCR7 expression in small and larger spheroids demonstrates that in the latter, the expression of the protein is higher in the hypoxic core compared to the oxygenated rim. When sections from a 230 μ m diameter OSC-19 spheroid, which was formalin-fixed and embedded in paraffin, were stained for CCR7 by immunofluorescence, we observed a uniform distribution of fluorescence intensity. However, when sections from a 500 μ m diameter OSC-19 spheroid were stained for CCR7 by immunofluorescence, the intensity of fluorescence in the core of the spheroids was relatively higher than in the periphery. (**Figure 2B, 2E**) To confirm the presence of hypoxia in the larger spheroid core,

the spheroids were treated with pimonidazole [51] prior to fixing and embedding in paraffin. Cellular proteins which react with pimonidazole were visualised by immunofluorescence which clearly showed more fluorescence in the spheroid core confirming existence of hypoxia. (Figure 2C) In addition, a marker of proliferation, Ki-67 [52] was more strongly detected as predicted in the non-hypoxic rim of the spheroid. (Figure 2D)

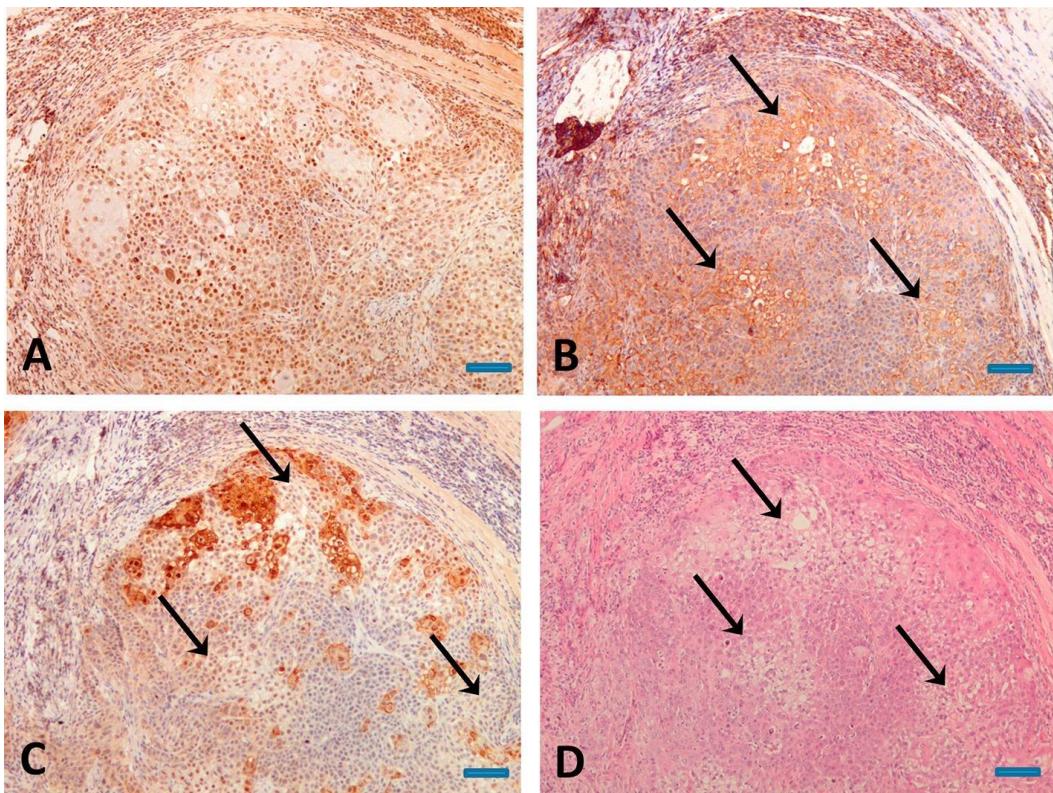


Figure 5. Staining of sequential sections from the A-253 xenografted issues for Ki-67, (A); CCR7, (B); HIF-1 α , (C); and haematoxylin/eosin (H&E), (D). Scale bar = 100 μ m

Having shown a correlation between hypoxia and CCR7 expression in 2D and 3D *in vitro* models, we next turned our attention to *in vivo* models. We had previously shown (above) that head and neck cancer cell lines maintain expression of CCR7 when grown as xenografts in mice. Therefore, we set out to analyse the correlation between the expression of CCR7, and hypoxia in xenotransplanted tissue from head and neck cancer cell line A-253 as an example. (Figure 5) Sequential tissue sections were immunolabelled for CCR7, HIF-1 α as an indicator of hypoxia, Ki-67 as a marker for proliferating cells, as well staining with haematoxylin and eosin (H&E) to assess tumour morphology. (Figure 5A) Analysis of the tissue sections showed immunolocalisation of CCR7 across the tumour, but labelling intensity was higher in the vicinity of the hypoxic regions as indicated by HIF-1 α nuclear

localisation. (**Figure 5B and C**) The elevation of CCR7 in the hypoxic/necrotic region of the xenografted tumour was also confirmed with H&E staining. (**Figure 5B and D**)

Correlation between CCR7 protein expression with tumour grade and HIF-1 α in clinical tissue

Having shown a correlation between CCR7 and HIF-1 α *in vitro* and *in vivo*, we turned our attention to the analysis of the correlation between the two in clinical tissue. Consecutive slides from tissue microarrays (TMAs) with 80 patient cores (**Table 1** and **Supplementary Information S1**) were labelled with HIF-1 α and CCR7, and were scored independently for both using the H-score system, with the correlation (R) between the two independent scorers being >0.9. We first established that CCR7 expression is elevated in malignant tissues compared to non-malignant samples using the Mann-Whitney U test (P = 0.007). (**Figure 6A**) In contrast, we did not observe a correlation between malignancy and HIF-1 α expression. Interestingly, we found a correlation between higher expression of CCR7 (H-score > 150) with the clinical stage of cancer, and this is also strong in stage 1 compared to non-malignant tissues. (**Table 1**) In contrast, we found no correlation between higher expression of HIF-1 α (H-score > 150) and any clinical stages (**Table 1**). Similarly, we observed an association between expression of both CCR7 and HIF-1 α in histological grade 1 tumours compared with non-malignant tissue, with P = 0.0001 and 0.018 respectively for CCR7 and HIF-1 α . (**Table 2**)

| CCR7 | | | HIF-1 α | | | |
|---------------|---------|----------|----------------|---------|----------|-------|
| | Low (n) | High (n) | P | Low (n) | High (n) | P |
| non-malignant | 9 | 2 | | 9 | 2 | |
| Stage 1 | 0 | 6 | P=0.002** | 2 | 4 | P=0.1 |
| Stage 2 | 12 | 11 | P=0.14 | 18 | 5 | P=1 |
| Stage 3 | 8 | 11 | P=0.06 | 17 | 2 | P=0.6 |
| Stage 4 | 5 | 8 | P=0.05* | 10 | 3 | P=1 |
| LN metastasis | 3 | 5 | P=0.07 | 6 | 2 | P=1 |

Table 1: Association of high expression of CCR7 or HIF-1 α with head and neck cancer in different stages of the disease.

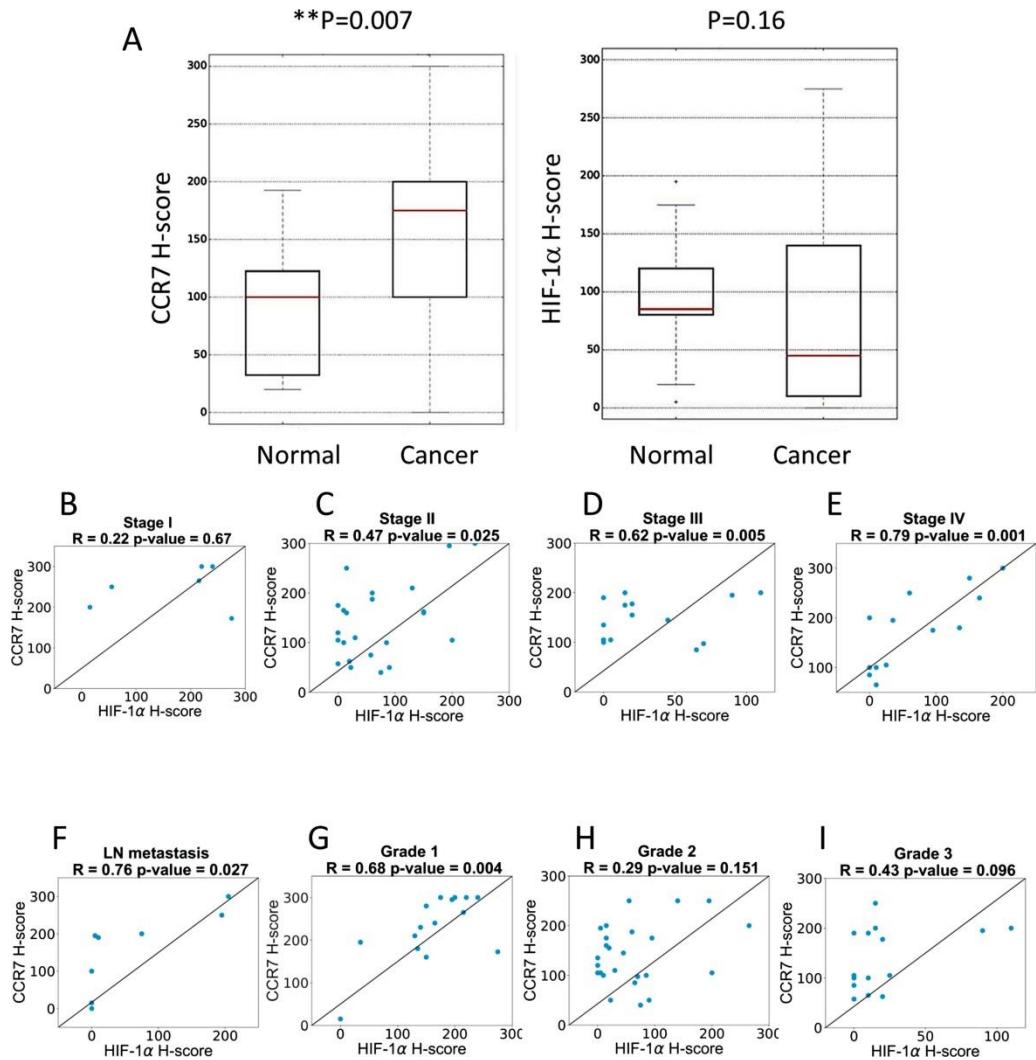


Figure 6. (A) Comparison of the range of H-score for CCR7 and HIF-1 α between non-malignant and malignant tissue samples show that CCR7 expression is elevated in malignant compared to non-malignant tissue (red line represent median value of H-scores in each case). (B-E) Analysis of the relative expression of CCR7 and HIF-1 α in each individual sample shows a correlation between the two for each clinical stage as well as for lymph node metastases (F-I). Analysis of the relative expression of CCR7 and HIF-1 α in each individual sample showed a correlation between the two in individual samples for all grades; with the highest correlation observed in grade 1.

An analysis of the relative expression of CCR7 and HIF-1 α in each individual tissue sample revealed a correlation (R) between the two for each clinical stage (Figure 6B-E), as well as for lymph node metastases (Figure 6F). Similarly, we observed a correlation between the relative expression of CCR7 and HIF-1 α in individual samples for all grades; with the highest correlation observed in grade 1 ($R=0.68$, $P=0.004$) (Figure 6G-I) suggesting that the level of differentiation of tumours may be relevant for the relationship between CCR7 and HIF-1 α . Our findings are concordant with two previous studies in breast [35] and lung [36] cancer which also reported a correlation between the CCR7 and HIF-1 α expression, and studies

which associate another important chemokine receptor, CXCR4, to hypoxia and HIF-1 α [31, 32]. They support the hypothesis that hypoxia, particularly in the early stages of tumour development, may be involved in promoting the invasion and migration of head and neck cancer cells through CCR7 axis.

| Grade | CCR7 | | | HIF-1 α | | |
|---------------|------------|-------------|------------|----------------|-------------|----------|
| | Low (n) | High (n) | P | Low (n) | High (n) | P |
| non-malignant | 9 | 2 | | 9 | 2 | |
| Grade 1 | 1 | 15 | P=0.0001** | 5 | 11 | P=0.018* |
| Grade 2 | 11 | 15 | P=0.26 | 23 | 3 | P=0.6 |
| Grade 3 | 9 | 7 | P=0.23 | 16 | 0 | P=0.16 |

Table 2: Association of high expression of CCR7 or HIF-1 α with head and neck cancer in different histological grades of the disease.

Conclusions

We have shown that in 2D monolayers, 3D multicellular spheroids, xenotransplanted cells and clinical tissue, there is a positive correlation between hypoxia and CCR7 expression. In clinical samples in particular, the correlation between hypoxia and CCR7 in the all clinical stages suggests the two are related.

Hypoxia is a key feature of head and neck tumours, is widely observed in the clinical setting, and is associated with resistance to radiotherapy, reduced therapeutic response, and a poorer clinical outcome. [53,54] The role of hypoxia in head and neck tumours is undoubtedly complex and acts through multiple pathways. However, understanding the mechanisms by which hypoxia can promote the tumour progression, provides opportunities to combat its negative impact on the disease. [55-59] Our results strongly associate hypoxia with the emergence of the expression of CCR7, complementing earlier observations by Ferris who has shown that release of CCL21 and CCL19 into the tumour microenvironment by invading lymphocytes can increase CCR7 expression through an autocrine loop. [10] Taken together, both results confirm a central role for the CCR7 axis in head and neck

tumours. In addition, in view of CCR7 being a negative prognostic factor for lymph node (LN) metastasis, [19-21] our findings suggests that hypoxia is likely to contribute to the development of a metastatic phenotype in head and neck cancers through upregulation of CCR7 signalling. Therefore, inhibition of CCR7 signalling by small molecule CCR7 antagonists [60] may prove to be a very useful adjunct therapy to be used in combination with existing treatments for head and neck cancers.

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Author contribution

Experimental work was carried out by HAB. Statistical analysis was carried out by EP. Xenografts were prepared by PAC and SDS. Scoring of the clinical tissue was carried out independently by LM and KDH. VV and KA designed experiments. Manuscript was written by KA and all authors reviewed the manuscript.

Competing financial interest statement

Authors declare no competing financial interests.

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Supplementary information

Supplementary Information S1: Characteristics of microarrays clinical sample

| position | gender | age | organ | pathology | grade | stage | TNM | type |
|----------|--------|-----|-------------|---|-------|-------|--------|-----------|
| A1 | F | 61 | Oral cavity | Squamous cell carcinoma of palate | 1 | I | T1N0M0 | Malignant |
| A2 | M | 61 | Lip | Squamous cell carcinoma of lower lip | 1 | I | T1N0M0 | Malignant |
| A3 | M | 77 | Lip | Squamous cell carcinoma of lower lip | 1 | I | T1N0M0 | Malignant |
| A4 | F | 75 | Oral cavity | Squamous cell carcinoma of upper jaw | 1 | I | T1N0M0 | Malignant |
| A5 | M | 67 | Oral cavity | Squamous cell carcinoma of right palate | 1 | II | T2N0M0 | Malignant |
| A6 | M | 50 | Tongue | Squamous cell carcinoma of root of tongue | 1 | III | T3N1M0 | Malignant |
| A7 | M | 66 | Pharynx | Squamous cell carcinoma of pharynx | 1 | IVA | T3N2M0 | Malignant |
| A8 | M | 49 | Gingiva | Squamous cell carcinoma of right gingiva | 1 | IVA | T4N0M0 | Malignant |
| A9 | M | 55 | Pharynx | Squamous cell carcinoma of epiglottis | 1 | IVA | T4N0M0 | Malignant |
| A10 | M | 57 | Pharynx | Squamous cell carcinoma of oropharynx | 1 | IV | T4N0M0 | Malignant |
| B1 | M | 74 | Nose | Squamous cell carcinoma of root of nose | 2 | II | T2N0M0 | Malignant |
| B2 | M | 90 | Cheek | Squamous cell carcinoma of cheek | - | II | T2N0M0 | Malignant |
| B3 | M | 72 | Larynx | Squamous cell carcinoma of larynx | 1 | II | T2N0M0 | Malignant |
| B4 | F | 61 | Oral cavity | Squamous cell carcinoma of right mandible | 1 | II | T2N0M0 | Malignant |
| B5 | M | 50 | Larynx | Squamous cell carcinoma of larynx | 1 | III | T2N1M0 | Malignant |
| B6 | M | 67 | Larynx | Squamous cell carcinoma of larynx | 2 | III | T3N1M0 | Malignant |
| B7 | M | 58 | Larynx | Squamous cell carcinoma of larynx | 2 | III | T3N1M1 | Malignant |
| B8 | M | 52 | Nose | Squamous cell carcinoma of nasopharynx | 2 | I | T1N0M0 | Malignant |
| B9 | M | 60 | Tongue | Squamous cell carcinoma of tongue | 2 | II | T2N0M0 | Malignant |
| B10 | M | 60 | Tongue | Squamous cell carcinoma of tongue | 2 | II | T2N0M0 | Malignant |
| C1 | M | 67 | Larynx | Squamous cell carcinoma of throat | 3 | II | T2N0M0 | Malignant |
| C2 | M | 48 | Cheek | Squamous cell carcinoma of cheek | 1 | II | T2N0M0 | Malignant |
| C3 | M | 70 | Oral cavity | Squamous cell carcinoma of right mandible | 2 | II | T2N0M0 | Malignant |
| C4 | M | 43 | Oral cavity | Squamous cell carcinoma of right mandible | 2 | II | T2N0M0 | Malignant |
| C5 | M | 56 | Pharynx | Squamous cell carcinoma of pharynx | 2 | II | T2N0M0 | Malignant |
| C6 | M | 45 | Cheek | Squamous cell carcinoma of right cheek | - | II | T2N0M0 | Malignant |
| C7 | M | 47 | Larynx | Squamous cell carcinoma of larynx | 2 | II | T2N0M0 | Malignant |
| C8 | M | 61 | Pharynx | Squamous cell carcinoma of epiglottis | - | II | T2N0M0 | Malignant |
| C9 | M | 50 | Larynx | Squamous cell carcinoma of larynx | 2 | II | T2N0M0 | Malignant |
| C10 | F | 38 | Lip | Squamous cell carcinoma of lower lip | 2 | II | T2N0M0 | Malignant |
| D1 | M | 47 | Larynx | Squamous cell carcinoma of larynx | 3 | III | T2N1M0 | Malignant |
| D2 | F | 50 | Pharynx | Squamous cell carcinoma of hypopharynx | 2 | III | T2N1M0 | Malignant |
| D3 | M | 71 | Pharynx | Squamous cell carcinoma of hypopharynx | 2 | III | T2N1M0 | Malignant |
| D4 | M | 44 | Pharynx | Squamous cell carcinoma of hypopharynx | 2 | III | T2N1M0 | Malignant |
| D5 | M | 61 | Pharynx | Squamous cell carcinoma of epiglottis | 2 | III | T2N1M0 | Malignant |
| D6 | M | 54 | Oral cavity | Squamous cell carcinoma of left upper jaw | 2 | III | T3N0M0 | Malignant |
| D7 | M | 61 | Pharynx | Squamous cell carcinoma of hypopharynx | 3 | III | T3N0M0 | Malignant |
| D8 | M | 58 | Larynx | Squamous cell carcinoma of larynx | 3 | III | T3N0M0 | Malignant |

| | | | | | | | | |
|-----|---|----|-------------|--|---|-----|--------|------------|
| D9 | M | 63 | Pharynx | Squamous cell carcinoma of epiglottis | 2 | III | T3N1M0 | Malignant |
| D10 | M | 71 | Pharynx | Squamous cell carcinoma of epiglottis | 3 | III | T3N1M0 | Malignant |
| E1 | M | 51 | Pharynx | Squamous cell carcinoma of left pharynx | 2 | III | T3N1M0 | Malignant |
| E2 | M | 55 | Larynx | Squamous cell carcinoma of larynx | 2 | III | T3N1M0 | Malignant |
| E3 | M | 75 | Oral cavity | Squamous cell carcinoma of maxillary sinus | 2 | IV | T4N0M0 | Malignant |
| E4 | M | 56 | Pharynx | Squamous cell carcinoma of epiglottis | 1 | IVA | T4N0M0 | Malignant |
| E5 | M | 57 | Larynx | Squamous cell carcinoma of larynx | - | IVA | T4N0M0 | Malignant |
| E6 | M | 49 | Larynx | Squamous cell carcinoma of larynx | 3 | IVA | T4N1M0 | Malignant |
| E7 | F | 69 | Cheek | Squamous cell carcinoma of right cheek | 2 | I | T2N0M0 | Malignant |
| E8 | M | 29 | Larynx | Squamous cell carcinoma of larynx | - | II | T2N0M0 | Malignant |
| E9 | F | 56 | Lip | Squamous cell carcinoma of oral lip | 2 | II | T2N0M0 | Malignant |
| E10 | M | 50 | Oral cavity | Squamous cell carcinoma of maxillary sinus | 2 | II | T2N0M0 | Malignant |
| F1 | M | 50 | Tongue | Squamous cell carcinoma of tongue | 3 | II | T2N0M0 | Malignant |
| F2 | M | 64 | Larynx | Squamous cell carcinoma of larynx | 3 | II | T2N0M0 | Malignant |
| F3 | M | 49 | Larynx | Squamous cell carcinoma of larynx | 3 | III | T2N1M0 | Malignant |
| F4 | F | 42 | Nose | Squamous cell carcinoma of nasal sinus | 3 | III | T3N0M0 | Malignant |
| F5 | M | 50 | Larynx | Squamous cell carcinoma of larynx | 3 | III | T3N1M0 | Malignant |
| F6 | M | 58 | Larynx | Squamous cell carcinoma of larynx | 3 | IV | T2N2M0 | Malignant |
| F7 | M | 75 | Nose | Squamous cell carcinoma of nasal sinus | 3 | IV | T4N0M0 | Malignant |
| F8 | M | 48 | Pharynx | Squamous cell carcinoma of hypopharynx | 3 | IV | T4N0M0 | Malignant |
| F9 | M | 74 | Pharynx | Squamous cell carcinoma of hypopharynx | - | IV | T4N0M0 | Malignant |
| F10 | M | 65 | Larynx | Squamous cell carcinoma of larynx | 3 | IV | T3N2M0 | Malignant |
| G1 | F | 32 | Oral cavity | Carcinoma sarcomatodes of maxillary sinus | - | II | T2N0M0 | Malignant |
| G2 | F | 40 | Lymph node | Metastatic mucoepidermoid carcinoma | - | - | - | Metastasis |
| G3 | M | 67 | Lymph node | Metastatic mucoepidermoid carcinoma | - | - | - | Metastasis |
| G4 | M | 45 | Lymph node | Metastatic mucoepidermoid carcinoma | - | - | - | Metastasis |
| G5 | M | 47 | Lymph node | Metastatic squamous cell carcinoma | 1 | - | - | Metastasis |
| G6 | M | 48 | Lymph node | Metastatic squamous cell carcinoma | 2 | - | - | Metastasis |
| G7 | M | 53 | Lymph node | Metastatic squamous cell carcinoma | 3 | - | - | Metastasis |
| G8 | F | 45 | Lymph node | Metastatic squamous cell carcinoma | 2 | - | - | Metastasis |
| G9 | F | 52 | Lymph node | Metastatic acinic cell carcinoma | - | - | - | Metastasis |
| G10 | M | 38 | Tongue | Adjacent normal tongue tissue | - | - | - | NAT |
| H1 | M | 28 | Tongue | Tongue tissue | - | - | - | Normal |
| H2 | F | 27 | Tongue | Tongue tissue | - | - | - | Normal |
| H3 | M | 48 | Tongue | Tongue tissue | - | - | - | Normal |
| H4 | F | 42 | Tongue | Adjacent normal tongue tissue | - | - | - | NAT |
| H5 | F | 15 | Tongue | Tongue tissue | - | - | - | Normal |
| H6 | F | 19 | Tongue | Tongue tissue | - | - | - | Normal |
| H7 | M | 35 | Tongue | Tongue tissue | - | - | - | Normal |
| H8 | F | 18 | Tongue | Tongue tissue | - | - | - | Normal |
| H9 | F | 19 | Tongue | Tongue tissue | - | - | - | Normal |
| H10 | M | 28 | Tongue | Tongue tissue | - | - | - | Normal |

Supplementary Information S2: Representative images from immunohistochemical staining of the tumour microarray for expression level of CCR7 (A-D) and HIF-1 α (E-H) in head and neck tumours. (A and E) strong staining; (B and F) medium staining; (C and G) weak staining; (D and H) control.

