Tumour cells express functional lymphatic endothelium-specific hyaluronan receptor in vitro and in vivo: Lymphatic mimicry promotes oral oncogenesis?

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Abstract
Lymphatic metastasis represents the main route of tumour cell dissemination in oral squamous cell carcinoma (OSCC). Yet, there are no FDA-approved therapeutics targeting cancer-related lymphangiogenesis to date. The lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1), a specific lymphatic marker, is associated with poor survival in OSCC patients. In this study, we present a potential novel mechanism of lymphatic metastasis in OSCC—lymphatic mimicry (LM), a process whereby tumour cells form cytokeratin1/LYVE-1+, but podoplanin-negative, mosaic endothelial-like vessels. LM was detected in one-third (20/57; 35.08%) of randomly selected OSCC patients. The LM-positive patients had shorter overall survival (OS) compared to LM-negative group albeit not statistically significant. Highly-metastatic tumour cells formed distinct LM structures in vitro and in vivo. Importantly, the siRNA-mediated knockdown of LYVE-1 not only impaired tumour cell migration but also blunted their capacity to form LM-vessels in vitro and reduced tumour metastasis in vivo. Together, our findings uncovered, to our knowledge, a previously unknown expression and function of LYVE-1 in OSCC, whereby tumour cells could induce LM formation and promote lymphatic metastasis. Finally, more detailed studies on LM are warranted to better define this phenomenon in the future. These studies could benefit the development of targeted therapeutics for blocking tumour-related lymphangiogenesis.

Introduction
Oral squamous cell carcinoma (OSCC) is one of the most common cancers in the head and neck region arising anywhere in the oral cavity. Unfortunately, despite recent advances in cancer management, the overall 5-year overall survival (OS) rate remains stagnant at around 50%1. The poor prognosis of OSCC patients is mainly attributed to the invasiveness of OSCC cells and their ability to swiftly disseminate to regional lymph nodes2. Thus, there is an urgent need to better understand the mechanisms behind OSCC metastasis, and to identify novel druggable targets that can improve the survival of OSCC patients.

Vascularisation is a crucial event during tumour development and metastasis3,4. Intratumoural vasculature has long been thought to be formed by endothelial cells alone. However, the seminal work of Maniotis and colleagues showed that aggressive uveal melanoma cells were able to acquire endothelial cell behaviour by generating de novo vessel-like networks independently of existing vascular endothelial cells5. This novel paradigm, which is termed vascular mimicry (VM), has sparked an enormous interest in the field of cancer research5,6. Thenceforth,
myriad studies have reported intriguing aspects of VM in different types of cancer (reviewed in Hendrix et al.)\(^6\). Interestingly, the transcriptional signature of VM-forming tumour cells revealed remarkable phenotypic plasticity (i.e. stemness), which facilitates transdifferentiation into other cell types\(^5,7\). Of particular importance, current antiangiogenic therapy remains ineffective on VM, thereby paving the way for more selective and personalised approaches\(^5\). Recently, the VM channels were shown to represent a promising prognostic target and therapeutic approach in head and neck squamous cell carcinomas (HNSCC)\(^8\).

Lymphatic vessels in the tumour microenvironment are the main route of dissemination in carcinomas including HNSCC, where tumour cells can preferentially metastasise to several hundred of regional lymph nodes\(^9,10\). Furthermore, lymphatic vessel density has been shown to predict metastasis-free survival in OSCC patients better than blood microvessel density, and hence also for guiding future therapeutic approaches\(^11\). Mirroring angiogenesis, tumour cells were also shown to secrete lymphangiogenic factors that facilitate lymphangiogenesis and metastasis to sentinel lymph nodes\(^12\). Importantly, the discovery of specific markers for lymphatic endothelial cells (LEC), such as the lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE-1) and podoplanin (i.e. D2-40), has made it possible to distinguish between lymphatic and blood vessels\(^10,13\). Of note, LYVE-1 bears a high degree of specificity for lymphatic vessels, and it has been an essential component of many important studies on tumour-induced lymphangiogenesis\(^13,14\). Moreover, LYVE-1 is strongly associated with nodal metastasis in OSCC, and its antibody was able to inhibit the development and progression of primary breast tumours\(^15,16\).

Based on the well-investigated concept of VM, we aimed to test our hypothesis that tumour cells can attain a LEC-like phenotype and form lymphatic vessel-like structures (i.e. lymphatic mimicry, LM) in OSCC tissue to facilitate tumour growth and metastasis. For this purpose, LYVE-1 was adopted in our study as a lymphatic marker using clinical samples, in vivo and in vitro experimental approaches. We also examined whether LM expresses other LEC markers such as D2-40.

Results and discussion
The CK\(^+\)/LYVE-1\(^+\) vessel-like structures are identified in OSCC tumours

First, we examined the presence of lymphatic vessel-like structures (i.e. LM) in primary OSCC tumours (\(n = 57\)) using specific tumour and LEC markers (CK and LYVE-1, respectively). The following criteria were set to identify the LM phenomenon in tumour tissues: (1) intratumoral vessel- or capillary-like structures; (2) LM lining is positive for OSCC tumour marker (CK\(^+\)) staining; and (3) positive for LEC marker (LYVE-1\(^+\)) staining. Interestingly, the OSCC tissues contained vessel-like structures lined by CK\(^+\)/LYVE-1\(^+\) cells as depicted in (Fig. 1a). In addition, some CK\(^+\)/LYVE-1\(^+\) cells were also seen as a few “hot spots” in nests of densely packed tumour cells, where LYVE-1 immunoreactivity was observed in the tumour cell membrane and cytoplasm (Fig. 1b). These LM structures were observed in 20/57 (35.08%) OSCC patients (Fig. 1c).

We then assessed whether LM structures express other LEC markers such as D2-40, which represents, together with LYVE-1, the most commonly used LEC markers in HNSCC\(^10\). Also, we tested the status of CD44, which enhances tumour aggressiveness by promoting tumour cell plasticity and VM\(^17\). Using the multiplexed immunohistochemistry (miHIC), normal lymphatic vessels were LYVE-1\(^+\)/D2-40\(^−\) (Fig. 1d). However, LM structures were strongly CK\(^+\)/LYVE-1\(^+\)/CD44\(^+\) but entirely lacking D2-40 immunoreactivity (Fig. 1e).

The prognostic value of LM status in OSCC patients

To investigate the clinical relevance of LM expression with the survival and the clinicopathological parameters of OSCC patients, we divided the patients (\(n = 57\)) into two groups based on the LM status: positive (LM\(^+\)) and negative (LM\(^−\)) groups. Interestingly, the estimated OS of the LM\(^+\) group (58 months) was noticeably shorter compared to the LM\(^−\) group (80 months) (Supplementary Fig. 1). Likely due to a small sample size, such difference did not however reach a statistical significance (\(P = 0.351\)). Yet, the correlation between LM\(^+\) status with younger age group (\(P = 0.016\)) and higher clinical T-stage (\(P = 0.077\)) represented interesting hints (Supplementary Table 1). The investigators were blinded to the clinical data of the patients during the experiment and when assessing the outcome by using samples with coded labels, with no reference to any respective group.

Tumour cell lines express LYVE-1

To confirm that the detected intratumoral LYVE-1-immunoreactivity was derived from tumour cells and not from other cells (e.g. vicinal LEC), we first quantified LYVE-1 mRNA in OSCC cells using the highly sensitive droplet-digital PCR (ddPCR). The ddPCR reactions with at least \(13 \times 10^3\) droplets per sample were accepted for further analysis. The absolute quantification analysis revealed that OSCC cells express LYVE-1 gene. LYVE-1 copies were detected in the cells with high-metastatic potential (i.e. HSC-3; 5 copies/\(\mu\)l) and also in the low-metastatic SCC-25 cells (2 copies/\(\mu\)l) (Fig. 2a). LYVE-1 protein was also detected in both OSCC cell lines by immunoblotting (Fig. 2b). We then utilised immunofluorescence staining to assess whether monolayered OSCC cells express LYVE-1 immunoreactivity. As expected, LYVE-1 protein was detected in the cytoplasm...
Fig. 1 (See legend on next page.)
of both OSCC cell lines (Fig. 2c). Additionally, the expression of LYVE-1 in multiple keratinocyte and cancer cell lines was also studied (supplementary data). We found that these cell lines differentially express LYVE-1 in vitro when cultured as monolayers (Supplementary Fig. 2a, b) or in a 3D organotypic myoma model (Supplementary Fig. 2c).

The high-metastatic HSC-3 cells form LYVE-1+ vessel-like structures in vitro and in vivo
After having confirmed the presence of LYVE-1 in OSCC cell lines, we investigated the ability of these cells to create vessel-like structures on a 3D matrix. Interestingly, when seeded on Matrigel, the high-metastatic HSC-3 formed well-defined, interconnected, ‘vessel-like’ network that resembled the early stages of endothelial cell tubulogenesis (Fig. 3a, b). In contrast, the low-metastatic SCC-25 cells failed to form any consistent structures and remained as single cells, although both cell lines were cultured under identical experimental conditions (Fig. 3c). Of note, such vessel-like cell networks were reminiscent of the LM structures in OSCC patients and exhibited stronger LYVE-1-immunoreactivity compared with the monolayer cell cultures (Fig. 3d). To investigate the ability of HSC-3 cells to form such LM structures in vivo, cell suspension was injected into the lateral tongue border of BALB/c nude male mice (n = 18), followed by double-labelled IF on the harvested xenograft sections. Surprisingly, CK+/LYVE-1+ vessel-like structures, similar to those observed in patient samples, were detected in 8/18 (44.44%) of the xenograft sections, of which three were metastatic tumours (Fig. 3e).

LYVE-1 knockdown impedes the ability of HSC-3 to form vessel-like structures on Matrigel
Given the crucial role of LYVE-1 in inducing lymphangiogenesis18, we next aimed to determine its putative role in the formation of LM in OSCC. To this end, we employed siRNA-mediated knockdown approach in HSC-3 cell line, while SCC-25 cells were excluded as they failed to form similar vessel-like networks. qPCR and western blot analyses showed a clear attenuation of LYVE-1 in the silenced group (Fig. 4a, b). HSC-3 cells transfected with siRNA LYVE-1 (siLYVE-1) or siControl were cultured on Matrigel for 24 h. Strikingly, siLYVE-1 treated cells showed a clear impairment in their ability to form vessel-like structures when compared to siControl cells, suggesting a pivotal role of LYVE-1 in LM formation in OSCC cells (Fig. 4c and Supplementary videos 1–3).

LYVE-1 knockdown shows moderate, non-significant, impact on HSC-3 cell viability
To confirm that the impaired ability of siLYVE-1 cells to form vessel-like structures is not due to cell death, we utilised resazurin dye assay to assess the effect of LYVE-1 siRNA knockdown on HSC-3 cell proliferation and...
viability. Results revealed that transfection with LYVE-1 siRNA caused a modest, non-significant, decrease on the growth of HSC-3 cells ($P > 0.05$; Fig. 4d).

**OSCC cell migration and invasion are suppressed by LYVE-1 knockdown**

It was recently shown that endogenous LYVE-1 can facilitate transluminal cell migration$^{19}$. Thus, we assessed the influence of LYVE-1 on the tumour cell migration, a crucial metastatic feature, by employing the IncuCyte scratch-wound healing assay on siLYVE-1 and siControl OSCC cells. Notably, siLYVE-1-knocked down cells showed a remarkable and consistent reduction in their directed migration (i.e. relative wound density) compared to the siControl cells ($P < 0.05$; Fig. 4e, f). Then, we evaluated the pro-invasion potential of LYVE-1 by employing the 3D organotypic myoma model$^{20}$. We found that siLYVE-1 cells had smaller invasion depth and invasion area compared with the siControl cells (Supplementary Fig. 2d–f). However, the difference was not statistically significant ($P > 0.05$).

**LYVE-1 knockdown significantly reduced metastasis in vivo**

Next, we addressed the pivotal question of whether LYVE-1 is a pro-metastatic in vivo by xenotransplantation of siLYVE-1-inhibited OSCC cells into zebrafish larvae. Indeed, zebrafish is emerging as an attractive addition to animal models in cancer research, which provided important data regarding metastatic events in vivo and personalised cancer approaches$^{21}$. The siLYVE-1 and siControl HSC-3 cells, labelled with CellTrace™ Far Red fluorochrome, were microinjected into the perivitelline space. On the fourth day post-injection, larvae were analysed by microscopic imaging to discern fish with a tumour metastasised outside the yolk sac region. Intriguingly, the siControl group had significantly more fish with metastasis to cloaca and tail regions compared with the siLYVE-1 group, where tumour remains largely
Fig. 3 (See legend on next page.)
confined within the yolk sac area (61.1 vs. 24.1%, respectively; \(P < 0.05\)) (Fig. 4g, h).

The main cause of cancer-related mortality is metastasis, a process mediated by the access of cancer cells to blood and lymphatic vasculatures\(^2\). Indeed, the identification of tumour cell-derived VM, as a novel model of neovascularization, has opened a new perspective in cancer research\(^2\). Increasing evidence shows that VM is strongly associated with poor survival in cancer patients, and hence represents an attractive therapeutic target\(^2\). In OSCC tumour tissues, we observed a phenomenon that could be similar to the VM. About one-third of randomly selected OSCC patients had vessel-like structures with surrounding CK\(^+\)/LYVE-1\(^-\) cells. These structures were expressing LYVE-1 but, unlike normal LEC, they were entirely negative for D2-40, implying a key role of LYVE-1 in cancer cell plasticity and metastatic potential. Unlike sarcomas, carcinomas including HNSCC utilise lymphatic vessels as the preferential route of metastasis by initially spreading to the regional cervical lymph nodes\(^9,10\). Therefore, the presence of cervical lymph node metastases is considered one of the most important prognostic indicators in OSCC patients\(^25\). In this regard, the localisation of lymphatic vessels in the tumour tissue matters. Intratumoural LYVE-1\(^+\) lymphatic vessels, unlike the peritumoral ones, were indeed associated with higher relapse rate and poor disease-specific survival in HNSCC patients\(^26\). To our knowledge, the present study is the first to report intratumoural CK\(^+/\)LYVE-1\(^-\) mosaic vessel-like structures in OSCC tumours. In support of our findings, it was recently shown that aggressive breast cancer cells can form intratumoural LM channels to access lymphatic vasculature\(^27\). These channels were concomitantly positive for tumour and lymphatic vessel markers including cytokeratin and LYVE-1, respectively\(^27\). Furthermore, we found that multiple keratinocytes and cancer cell lines differentially express LYVE-1 in vitro—a trait not widely discussed before. Interestingly, such expression levels and localisation of LYVE-1 were also retained in the 3D organotypic myoma model. The functional effects of LYVE-1 in cancer cells pose an interesting avenue for studies on cancer cell plasticity and metastatic potential.

The VM structures are traditionally identified in tumour tissues as periodic acid-Schiff positive (PAS\(^+\)) vessel-like spaces, with the absence of any specific endothelial cell markers\(^28\). However, this method has been criticised for its limited specificity. In fact, PAS staining detects extracellular matrix components, and hence PAS\(^+\) regions may also represent non-functional structures irrelevant to VM\(^29,30\). On the other hand, the mosaic
vessel-like pattern, where tumour and endothelial cell markers are simultaneously expressed, has gained attention not only for identifying VM but also as a prognostic marker. This mosaic pattern was long thought to be formed by a coalescence of tumour and endothelial cells during carcinogenesis. However, recent studies revealed that aggressive tumour cells can express genes associated with non-cancerous cell phenotype, such as endothelial cell precursors.

Phenotype plasticity allows cancer cells to reversibly transform phenotypes and acquire functional adaptation to thrive in a harsh tumour microenvironment. For instance, glioblastoma cancer cells were able to differentiate into functional CD31+ endothelial cells, which...
created VM structures in a xenograft mouse model. Likewise, breast cancer stem-like cells differentiated into endothelial cells and formed vessel-like structures on Matrigel.

Consistently with these reports, we show that LYVE-1 is expressed by OSCC cell lines, but only HSC-3 cells, and not the less aggressive SCC-25 cells, were able to form LYVE-1+ vessel-like network on Matrigel. The in vivo LM phenomenon was also evident in an orthotopic mouse model of OSCC. Of note, such LM vessel-like structures were strongly positive for CD44—a biomarker implicated in the plasticity of aggressive tumour cells and VM formation. These findings support the notion that the mimicry phenomenon is associated with more aggressively growing tumours. A function for LYVE-1 as a lymphangiogenic factor is fully consistent with its evident expression at the tubular extensions and intercellular junctions on Matrigel. These in vitro and in vivo findings suggest that intratumoural CK+/LYVE-1+ lumens in the clinical samples are likely LM structures rather than basement membrane sleeves of pruned vessels, as could be encountered when identifying mimicry structures.

The LYVE-1 is an essential mediator of tumour lymphangiogenesis and correlates with lymph node metastasis in HNSCC patients. Indeed, LYVE-1-driven effects are mediated by binding with its ligand—hyaluronan (HA). Importantly, HA is synthesised by both tumour and stromal cells in OSCC, and it has been shown to regulate various cancer processes from initiation to metastasis. Thus, it is logical to assume that LYVE-1+ OSCC cells may utilise HA/LYVE-1 interaction to facilitate oral carcinogenesis. This assumption is supported by an interesting study showing that aggressive breast cancer cells can harness their HA content to bind LYVE-1 and facilitate adhesion, hence invasion and metastasis. Notably, the high-metastatic cells used in our study, HSC-3, were proven to be highly sensitive and responsive to...
HA-signalling in vitro. Here, we have shown that HSC-3 exhibit marked suppression of tube formation, migration and invasion potential in 3D in vitro models following the siRNA-mediated knockdown of LYVE-1. More importantly, inhibition of LYVE-1 in HSC-3 cells significantly reduced their metastatic potential in zebrafish larvae. In agreement with our report, it was recently shown that anti-LYVE-1 monoclonal antibody inhibited primary tumour formation and metastasis to axillary lymph nodes in xenograft models of breast cancer.

Finally, these pieces of evidence may bear considerable therapeutic implications. Despite the major advances in the development of new angiogenic inhibitors, the clinical success of these drugs remains, however, considerably limited. As a result, to date there are no FDA-approved therapeutics that target tumour-related lymphangiogenesis. The de novo formation of tumour-derived mimicry channels was suggested as a plausible factor that could reduce the effects of anti-angiogenic therapy. Thus, a better understanding of LYVE-1 function in tumour cells and its role in LM formation could benefit the development of targeted therapeutics for blocking tumour lymphangiogenesis in OSCC. Clearly, more detailed studies, including larger sample size and complete deletion models of LYVE-1, are necessary in order for such phenomenon to be better defined in the future.

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