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Angiopoietin-like 4 induces head and neck squamous cell carcinoma cell migration through the NRP1/ABL1/PXN pathway

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Abstract

Objectives: The molecular mechanisms whereby angiopoietin-like 4 (ANGPTL4), a pluripotent protein implicated in cancer development, contributes to head and neck squamous cell carcinoma (HNSCC) growth and dissemination are unclear.

Materials and Methods: We investigated ANGPTL4 expression in human normal oral keratinocytes (NOKs), dysplastic oral keratinocytes (DOKs), oral leukoplakia cells (LEUK1), and HNSCC cell lines, as well as in tissue biopsies from patients with oral dysplasia, and primary and metastatic HNSCC. We further examined the contribution of ANGPTL4 cancer progression in an HNSCC orthotopic floor-of mouth tumor model and the signaling pathways linking ANGPTL4 to cancer cell migration.

Results: ANGPTL4 expression was upregulated in premalignant DOKs and HNSCC cell lines compared to NOKs and was increased in tissue biopsies from patients with oral dysplasia, as well as in primary and metastatic HNSCC. We also observed that downregulation of ANGPTL4

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expression inhibited primary and metastatic cancer growth in an HNSCC orthotopic tumor model. Interestingly, ANGPTL4 binding to the neuropilin1 (NRP1) receptor led to phosphorylation of the focal adhesion protein, paxillin (PXN), and tumor cell migration; this was dependent on the tyrosine kinase ABL1. Treatment with the ABL1 inhibitor, dasatinib and small interfering RNA silencing of NRP1 or ABL1 expression blocked PXN phosphorylation and tumor cell migration.

Conclusion: Our findings suggest an early, sustained, and angiogenesis-independent autocrine role for ANGPTL4 in HNSCC progression and expose ANGPTL4/NRP1/ABL1/PXN as an early molecular marker and vulnerable target for the prevention of HNSCC growth and metastasis.

Keywords

angiopoietin-like 4; head and neck squamous cell carcinoma; oral dysplasia; cell migration; neuropilin; ABL Proto-Oncogene 1; paxillin

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a group of cancers arising from the mucosal lining of the oral cavity, sinonasal cavity, pharynx, and larynx [1]. HNSCC remains one of the ten most prevalent cancers worldwide, with 600,000 new diagnoses each year [1]. Advanced therapeutic options, including surgery, radiation, chemotherapy, and molecular-based therapies, are available to treat HNSCC patients and preserve their quality of life. However, the 5-year overall survival of these patients remains poor (i.e., 50%) and has not significantly changed over the last two decades [1]. One of the challenges to improving this dismal mortality rate has been the advanced stage of the cancer at the time of diagnosis. More than 60% of HNSCC patients present with involvement of the surrounding tissues and/or lymph nodes (stage III) or distal metastases (stage IV). HNSCC tumors with local or distal metastasis translates to a high risk of locoregional recurrence despite treatment. This underscores the need for novel biomarkers and more effective therapeutic targets for HNSCC.

Genetic/epigenetic changes and signaling dysregulation have been associated with HNSCC tumorigenesis [2]. Interestingly, compelling data support an important role of angiopoietin-like 4 (ANGPTL4) in HNSCC cell migration and lymph node metastasis [3–11]. ANGPTL4 is a circulating protein with sequence homology to Angiopoietin (ANGPT)1 and ANGPT2. ANGPTL4 has pleotropic effects in disparate pathological disorders, including ischemic ocular disease, cardiac and lung disease, cancer, joint disease, diabetes, atherosclerosis, and nephrotic syndrome [12–27]. Secreted ANGPTL4 is cleaved by furin-like proprotein convertases into two major domains that accomplish unrelated functions. The ANGPTL4 N-terminal domain (nANGPTL4) is an adipokine that inhibits lipoprotein lipase (LPL) [28], the enzyme responsible for the hydrolysis of circulating triglycerides (TGs), thereby affecting metabolic functions including nutrient partitioning and insulin sensitivity. Recent data has demonstrated the therapeutic potential of targeting ANGPTL4 for cardiovascular and metabolic diseases [29, 30].

Conversely, the ANGPTL4 C-terminal domain (cANGPTL4) is involved in angiogenesis, vascular hyperpermeability, anoikis resistance, and tumor growth and metastasis [19, 23]. A

role for ANGPTL4 as a diagnostic and/or prognostic marker of cancer progression has been proposed for colon cancer [31], gastric cancer [32], head and neck squamous cell carcinoma [3–11], breast cancer [33–35], ovarian cancer [36], cervical cancer [37], prostate cancer [38], hepatocellular carcinoma [39], Kaposi's sarcoma [40, 41], uveal melanoma [42], and renal cell carcinoma [43, 44]. Overall, ANGPTL4 upregulation is correlated with poor prognosis and diminished cancer-free survival rates [10, 35–37]. In HNSCC, ANGPTL4 promotes cell migration and dissemination [3–5, 7, 9–11]. However, the molecular mechanism(s) whereby ANGPTL4 mediates these effects are not known. Using cell-based and animal models and corroboration in human tissue, we set out here to identify the signaling networks that link ANGPTL4 to tumor cell migration in HNSCC.

MATERIALS AND METHODS

Cell culture and reagents

Spontaneously immortalized human-derived normal oral keratinocytes (NOK) [45] and human oral leukoplakia cancer cell line (LEUK1), derived from a dysplastic leukoplakia adjacent to an early invasive tongue OSCC (T1N0M0) [46], were grown in keratinocyte serum free medium with growth factor supplement (Gibco) and 1% antibiotic/antimycotic. Human-derived dysplastic oral keratinocytes derived from dorsal tongue showing epithelial dysplasia [47] were grown in high glucose DMEM with 10% FBS, 0.05% Hydrocortisone, 1% Penicillin-Streptomycin. Human-derived HNSCC cell lines HN13 (tongue - T2N2M0), HN6 (base of tongue - T3N2M0), HN4 (base of tongue - T4N1M0), HN12 (lymph node - T4N1M0), and CAL27 (tongue) [48] were cultured as DOK but without 0.05% Hydrocortisone. LEUK1 were provided by Dr. Hening Ren (University of Maryland, Baltimore). The other cell lines were provided by Dr. Silvio Gutkind (University of California, San Diego). Cells were tested and authenticated at the UMB Translational Laboratory Shared Service. Recombinant human full-length ANGPTL4 (ANGPTL4), C-terminal ANGPTL4 (cANGPTL4) and N-terminal (nANGPTL4) proteins were purchased from R&D Systems. Dasatinib was obtained from Tocris Bio-Techne Corporation.

Western Blot analysis, immunofluorescence, immunoprecipitation, and ELISA

Westen Blot analysis, immunofluorescence, and immunoprecipitation were performed as in [40]. Antibodies were ANGPTL4 (Proteintech), NRP1 (Abcam), NRP2 (Santa Cruz Biotechnology), phospho-PXN (Cell Signaling Technology), PXN (Cell Signaling Technology), ABL1 (Santa Cruz Biotechnology), actin (Proteintech), GAPDH (Cell signaling Technology), anti-mouse HRP (Bio-Rad), anti-rabbit HRP (Bio-Rad), Alexa Fluor 488 conjugated goat anti-rabbit (Abcam) and FITC-conjugated phalloidin (Sigma-Aldrich). Secreted ANGPTL4 was measured using ANGPTL4 DuoSet ELISA kit (R&D Systems).

Immunohistochemistry

Fifteen representative biopsied samples of human normal oral epithelium, oral dysplasia and HNSCC were selected from archived tissue banks of the UMB Department of Oncology and Diagnostic Sciences of the School of Dentistry and the Greenebaum Comprehensive Cancer Center Pathology Biorepository Shared Service, following UMB Institutional Review Board guidelines involving anonymous tissue. Immunohistochemical staining was performed

using anti-ANGPTL4 (Proteintech), anti-NRP1 (Abcam) and anti-phospho-PXN (Abcam) antibodies. A board-certified oral and maxillofacial pathologist diagnosed the dysplasia cases (WHO three-tier grading), and a board-certified anatomic pathologist diagnosed the HNSCC cases.

Migration assays

For wound closure assays, cell monolayers were scratched using BioTek's Autoscratch wound-making tool. Gap closures were quantified using BioTek Cytation 5. Gen5 3.11 software and gap closures were calculated as ([Initial Area-Final Area]/Initial Area) x100. For transwell assays, cells were seeded and exposed to conditions. Migrated cells were stained with crystal violet. For microspike analysis, cells were stained with FITC-phalloidin (0.3μM). Cell nuclei were counterstained with Hoechst 33342. Images were captured by Gen5 imaging system (60 x) and quantified with FiloQuant v1.0 (plugin in ImageJ).

siRNA and cDNA expression

siRNAs were expressed in cells using HiPerfect (Qiagen) or Nucleofector™ kit (Amaxa Biosystems). Scrambled, ANGPTL4, NRP1 and ABL1 siRNAs were obtained from Qiagen. Stably expression of ANGPTL4 was achieved by transfection of pcDNA3.1-ANGPTL4-mycHis [40].

CRISPR-induced ANGPTL4 knockdown expression

CAL27 ANGPTL4 KO was generated in the UMB Translational Laboratory Shared Service CRISPR Core. HN13 ANGPTL4 KO was generated using ANGTPL4 CRISPR/Cas9 KO plasmid (Santa Cruz Biotechnology).

Orthotopic floor-of mouth mouse model

Animal procedures were approved by the Institutional Animal Care and Use Committee and carried by the Translational Laboratory Shared Service. CAL27 and CAL27 ANGPTL4 KO were transduced with a lentiviral vector expressing YFP/luciferase, rendering CAL27-LUC and CAL27-LUC ANGPTL4 KO, respectively. NRG mice were injected in the floor of the mouth with 1×10^5 CAL27-LUC or 1×10^5 CAL27-LUC ANGPTL4 KO mixed with NIH 3T3 cells (2:1 ratio) cells with 33% Matrigel. Tumor growth was observed daily for seventeen days. Bioluminescence imaging was performed with a CCD camera. At the end of the experiment, mice were euthanized, and lungs were harvested for analysis.

GEPIA analysis

The expression of ANGPTL4, NRP1, and PXN in HNSCC was determined by Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/), which utilizes RNA sequencing data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) consortium projects. Five hundred and twenty-eight HNSCC and forty-four normal samples from the TCGA and the GTEx databases were included. Sample inclusion criteria followed the ones of these databases. The survival curves of HNSCC patients based on gene expression levels of ANGPTL4, NRP1and PAX were generated according to [49].

Statistics

Statistical analyses were performed with the Prism 9.0 biostatistics program (GraphPad Software). Data were expressed as mean value ± standard error of the mean. Unpaired Student's t-test and one-way ANOVA with Bonferroni posttest (or Tukey test where indicated) were used for statistical differences between two or multiple heterogeneous groups. Statistically significant results are labeled as *p<0.05, **p<0.01, ***p<0.001 or ****P < 0.0001.

RESULTS

ANGPTL4 is upregulated in oral dysplasia and HNSCC and it is relevant in HNSCC development.

To study the contribution of ANGPTL4 to HNSCC tumorigenesis, we first investigated the levels of ANGPTL4 expression at different stages of HNSCC progression. We obtained cellular extracts and conditioned media from human normal oral keratinocytes (NOK) [45], human leukoplakia-derived oral keratinocytes (LEUK1) [47], human dysplastic oral keratinocytes [47], and (tongue) squamous cell carcinoma cell lines (i.e., HN13, HN6, HN4, HN12, and CAL27), previously reported as cellular models of HNSCC development [48]. While we did not observe detectable levels of ANGPTL4 in extracts from NOK or LEUK1, we found high levels of intracellular ANGPTL4 in premalignant DOK, and HN13, HN6 and CAL27 (Fig. 1A). ANGPTL4 was not detected in HN4 and HN12, derived from tumors of the same patient in the base of tongue and lymph node, respectively. Upregulation of cellular ANGPTL4 correlated with an increase in ANGPTL4 in conditioned media, analyzed by Western blotting (Fig. 1B) and ELISA (Fig. 1C).

To validate the relevance of ANGPTL4 in HNSCC tumorigenesis in vivo, ANGPTL4 was knocked-out in CAL27 using CRISPR-mediated gene editing and both cell lines (CAL27 and CAL27 ANGPTL4 KO) were transduced to express YFP/luciferase (LUC). CAL27 LUC and CAL27 LUC ANGPTL4 KO were injected in the floor of the mouth of NRG mice, and oral and distal tumor growth was quantified by bioluminescence over time. Inhibition of ANGPTL4 expression led to decreased oral tumor growth (Fig. 1D–E) and metastatic growth (Fig. 1F) in this HNSCC orthotopic tumor model.

Using paraffin-embedded biopsied samples of human normal oral epithelium, oral dysplasia and HNSCC, we analyzed ANGPTL4 expression using a specific anti-ANGPTL4 antibody. Immunohistochemical staining of these tissues showed no detectable ANGPTL4 expression in normal palatal mucosa (Fig. 1G), consistent with our observations in NOK. However, diffuse, and strong cytoplasmic ANGPTL4 expression was observed in mild-moderate epithelial dysplasia of the lateroventral tongue (Fig. 1H), moderate epithelial dysplasia of the palatal gingiva (Fig. 1I), tongue squamous cell carcinoma (Fig. 1J–K), hypopharynx squamous cell carcinoma (Fig. 1L), and HNSCC metastasis in tonsil (Fig. 1M). Gene expression profiling interactive analysis (GEPIA) [49] of 528 HNSCC and 44 normal samples from the TCGA and GTEx databases demonstrated an elevated relative expression of ANGPTL4 in HNSCC tumors compared to normal tissue (Fig. 1N). Collectively, these results demonstrate a correlation between the levels of ANGPTL4 and HNSCC cancer

progression, with increased ANGPTL4 expression detected at early stages – and remaining high in later stages – of HNSCC tumorigenesis, suggesting that ANGPTL4 may play an early and sustained role in HNSCC development.

ANGPTL4 regulates cell migration in dysplastic oral keratinocytes and HNSCC cell lines.

We next set out to investigate whether ANGPTL4 could regulate the motility of ANGPTL4-expressing DOKs and HNSCCs. For this, we used RNA interference (RNAi) to knock down ANGPTL4 expression in DOK and CAL27 (Fig. 2A); scrambled siRNA was used as a control. Knock down of ANGPTL4 expression caused a decrease in the migratory potential of DOK and CAL27 (Fig. 2B). These results paralleled our observations using CRISPR-mediated knockout of *ANGPTL4* in HN13 and CAL27 (Fig. 2C–D). We next took advantage of the observation that increased ANGPTL4 expression was not detected in NOK, HN4 and HN12, and ectopically overexpressed ANGPTL4 in each of these cell lines (NOK-ANGPTL4 C1 and NOK-ANGPTL4 C2, HN4-ANGPTL4, and HN12-ANGPTL4, respectively; Fig. 2E–G), using stable transfection of pCDNA3.1-ANGPTL4-mycHis [40]. Cell lines ectopically overexpressing ANGPTL4 acquired cell migratory capabilities compared to their parental cell lines. Collectively, these results suggest that autocrine expression of ANGPTL4 contributes to the promotion of tumor cell migration in HNSCC.

ANGPTL4 binds the neuropilin1 receptor in dysplastic oral keratinocytes and HNSCC lines, leading to tumor cell migration.

We recently reported that binding of ANGPTL4 to the receptors neuropilin (NRP)1 and NRP2 on vascular endothelial cells promotes break down of vascular cell tight junctions promoting vessel hyperpermeability [19]. These two plasma membrane glycoproteins act as coreceptors for other ligands, including VEGFs and Semaphorin3s, and have been previously reported to regulate critical proliferative and immunological processes in cancer [50, 51]. We therefore examined whether NRP1 or NRP2 influenced tumor cell migration in response to ANGPTL4. Although levels varied, all HNSCC cell lines expressed both NRP1 and NRP2 (Fig. 3A). Interestingly, co-immunoprecipitation experiments showed robust binding of endogenous ANGPTL4 and NRP1 in DOKs as well as HN13 and CAL27 (Fig. 3B). However, we could not consistently reproduce the binding of ANGPTL4 to NRP2 in these cell lines despite its expression in oral premalignant and HNSCC cells. To determine whether NRP1 contributed to HNSCC cell migration, we knocked down this receptor using RNAi and observed a decrease in the migratory potential of HN13 (Fig. 3C–E) and CAL27 (Fig. 3F–H) compared to scrambled RNAi control. Collectively, these results suggest that ANGPTL4 and NRP1 interact in premalignant dysplastic keratinocytes and HNSCC cell lines to promote tumor cell migration in response to ANGPTL4 in HNSCC.

ANGPTL4 regulates the focal adhesion protein, paxillin through an NRP1/ABL1 dependent pathway.

Cell motility depends on the reorganization of the actin cytoskeleton and the assembly and disassembly of focal adhesions (FAs), both regulated by the interplay of multiple structural and signaling proteins. A central component of FAs is paxillin (PXN), a multidomain scaffold protein that acts as protein recruitment point at the extending leading edge of

the migrating cell [52]. Interestingly, when we exposed NOKs to ANGPTL4, we observed a rapid phosphorylation of PXN-Tyr^{118} (Fig. 4A). PXN phosphorylation was decreased in CAL27 ANGPTL4 KO compared to parental CAL27 (Fig. 4A). ANGPTL4-induced phosphorylation was not significantly enhanced by pre-coating plates with the α5β1 ligand, fibronectin, although it was maintained for a longer time (Fig. 4B). However, PXN phosphorylation did not correlate with changes in phosphorylation of focal adhesion kinase (FAK) [53] (data not shown), suggesting a different signaling pathway for PXN activation.

In this regard, it has previously been reported that the tyrosine kinase ABL1 plays an important role in the NRP1-dependent promotion of cell migration in retinal angiogenesis [54]. We therefore examined whether ANGPTL4/NRP1-induced PXN phosphorylation could be mediated by ABL1. siRNA-induced knockdown of either NRP1 (Fig. 4C) or ABL1 (Fig. 4D) expression in NOKs led to inhibition of PXN-Tyr^{118} phosphorylation. Similar results were obtained when NOKs were pretreated with dasatinib, a pharmacologic BCR-ABL1 inhibitor, before exposure to ANGPTL4 (Fig. 4E). We next investigated whether NRP1/ABL1 contributed to ANGPTL4-induced phosphorylation of PXN in HNSCC. Inhibition of the NRP1/ABL1 pathway, either by knocking down expression of NRP1 (Fig. 4F), or ABL1 (Fig. 4G) or using dasatinib to inhibit ABL1 (Fig. 4H), led to the downregulation of PXN phosphorylation. Accordingly, co-immunoprecipitation studies showed interaction between NRP1 and PXN in DOKs, HN13 and CAL27 (Fig. 4I). Collectively, these results demonstrate that ANGPTL4/NRP1/ABL1 promotes PXN phosphorylation in HNSCC.

ANGPTL4 regulates cell migration through an NRP1/ABL1/PXN dependent pathway.

To determine whether the ANGPTL4/NRP1/ABL1/PXN signaling pathway regulates tumor cell motility in HNSCC, we assessed trans-well cell migration in DOK and CAL27 in which NRP1 or ABL1 expression was inhibited by expression of their specific siRNAs. We observed a decrease in the migratory capacity of DOK upon expression of NRP1 siRNA or ABL1 siRNA, with respect to cells expressing scrambled siRNA (Fig 5A); this effect correlated with a decrease in the phosphorylation levels of PXN (Fig. 5B). These results were corroborated in CAL27 (Fig. 5C–D). Both recombinant full length ANGPTL4 and recombinant C-terminal ANGPTL4, but not recombinant N-terminal ANGPTL4, were able to induce cell migration (Fig. 5E) and PXN phosphorylation (Fig. 5F). Treatment of DOKs with either protein also resulted in the formation of membrane microspikes/filopodia, a phenotypic change characteristic of migrating cells; this was inhibited by expression of NRP1 siRNA or ABL1 siRNA (Fig. 5G–H). Collectively, these results suggest that ANGPTL4/NRP1/ABL1/PXN regulate tumor cell migration in HNSCC.

The ANGPTL4/NRP1/PXN axis may have clinical significance in HNSCC dissemination.

To assess the clinical implications of the activation of ANGPTL4/NRP1/ABL1/PXN in patients with HNSCC, we performed immunohistochemical analyses of ANGPTL4, NRP1 and phosphorylated PXN in HNSCC biopsies. We observed robust expression of all three proteins in the HNSCC tumors, within the same area (Fig. 6A). To further interrogate this pathway in HNSCC patients, we next evaluated the expression profiles of NRP1 and PXN using GEPIA analysis of 528 HNSCC and 44 normal samples from the TCGA

and the GTEx databases. Expression of NRP1 (Fig. 6B) and PXN (Fig. 6C) were both significantly higher ($P < 0.01$) in human HNSCC compared to normal tissue. Interestingly, ANGPTL4/NRP1/PXN signature expression was associated with poor disease-free survival (Fig. 6D; log-rank $p = 0.043$) and poor overall survival (Fig. 6E; log-rank $p = 0.046$) in HNSCC. Collectively, these studies suggest that the ANGPTL4/NRP1/ABL1/PXN cascade may represent an important molecular target for the prevention and/or treatment of HNSCC progression and dissemination.

DISCUSSION

Over the last ten years there has been a growing appreciation of the role of ANGPTL4 in cancer. It was recently reported that ANGPTL4 single nucleotide polymorphisms can influence susceptibility to cancer development and invasiveness [60]. Increased expression of this pluripotent factor has been identified in the proteomic signature of many solid tumors and a variety of tumorigenic events [23]. However, the signaling mechanisms associated with ANGPTL4-induced tumorigenic functions remain unclear. Our results extend our understanding of the contribution of ANGPTL4 to HNSCC progression and dissemination. We report here increased ANGPTL4 expression in human dysplastic oral keratinocytes and several cultured HNSCC cell lines. Increased expression of ANGPTL4 was observed both at early stages of HNSCC tumorigenesis in primary tumors, as well as in metastatic HNSCC tissue, suggesting an early and sustained role for ANGPTL4 in HNSCC.

Motivated by these observations, we explored the molecular pathways whereby ANGPTL4 contributes to HNSCC tumorigenesis. We demonstrate an important role for ANGPTL4 in regulating HNSCC tumor cell motility through NRP1/ABL1/PXN. Overexpression of NRPs has been reported in a wide range of malignancies and is associated with a poor prognosis. Our observations further suggest that dysregulation of this ANGPTL4/NRP1-mediated pathway may affect the behavior of HNSCC cells through an autocrine manner. Besides NRPs, ANGPTL4 has been shown to bind to other membrane proteins in cancer cells including integrin $\alpha5\beta1$, leading to cell proliferation, anoikis resistance, and changes in energy metabolism [23]. Conversely, secreted ANGPTL4 binds NRP1 in endothelial cells leading to paracrine effects, including the activation of proangiogenic and pro-permeability signaling events that may contribute to tumor metastasis. NRP1 is upregulated in dysplastic epithelium and oral SCC, as well [55–57]. Upregulation of both ANGPTL4 and NRP1 may be advantageous for tumor cells to escape and metastasize into proximal or distal tissues, an unfortunately frequent occurrence in patients with HNSCC.

Of note, tumors expressing an ANGPTL4 T266M variant, which has decreased $\alpha5\beta1$ integrin binding and signaling, are characterized by impaired tumor cell proliferation, anoikis resistance, and migratory and metastatic capabilities compared to tumor cells expressing wild-type ANGPTL4 [60]. $\alpha5\beta1$ integrin interacts and cooperates with NRP1 in many signaling mechanisms. Whether integrins participate in ANGPTL4/NRP1 signaling and whether ANGPTL4 activate these alternative pathways to induce a migratory phenotype warrant further investigation.

Similar to NRP1, it has been previously suggested that expression of NRP2 may serve as an important biomarker for HNSCC [58]. NRP2 promotes HNSCC tumor progression, affecting tumor cell proliferation, migration, and invasion and downregulating the Wnt/β-catenin pathway [58]. Although we also observed expression of NRP2 in all cultured NOK, DOK and HNSCC cell lines, we did not observe evidence for direct binding of this isoform to ANGPTL4, which we previously reported in vascular endothelial cells [19]. While we cannot rule out a role for NRP2 in the promotion of HNSCC tumorigenesis by ANGPTL4, these studies suggest that binding affinities for neuropilin ligands may be influenced by other factors (e.g., tumor microenvironment). Whether ANGPTL4 exerts its tumorigenic effects, in part, through NPR2 in HNSCC is currently under investigation.

We also did not observe increased expression of ANGPTL4 in two HNSCC cell lines, HN4 and HN12. These two tumor cell lines were derived from the same patient: one from the base of tongue (HN4) and the other from a metastasis isolated from a lymph node (HN12). The absence of increased ANGPTL4 expression in these two cell lines suggests a role for additional (ANGPTL4-independent) pathways in the promotion of cancer cell progression in this particular patient. These observations highlight the potential of combining molecular biomarkers (e.g., ANGPTL4 over-expression) with gene-product targeted therapies (e.g., those targeting the ANGPTL4/NRP/ABL1/PXN pathway) when developing novel cancer therapies. Nevertheless, our results build upon extensive studies reported over the past decade which collectively support a key role for ANGPTL4 in the progression of HNSCC and other human malignancies.

Multiple stimuli have been reported to upregulate the expression of ANGPTL4, including the PPAR receptors, TGF-β and hypoxia-inducible factor 1 (HIF-1) [23]. We speculate that ANGPTL4 may act in cooperation with other HNSCC growth and angiogenic factors whose expression is dysregulated by similar transcriptional signals. For example, both *ANGPTL4* and *VEGF* are HIF-1-responsive genes that demonstrate increased expression in similar tissues and conditions, including HNSCC development, and have been reported to synergize in pathogenic responses [12, 42, 59]. VEGF and ANGPTL4 share the ability to interact with NRP1 [59], which acts as a point of convergence for several additional ligands relevant in HNSCC, including FGF, PDGF, and TGFβ. Furthermore, NRP1 suppresses anti-tumor immunity by reinforcing intratumoral regulatory T cell (T_{reg}) cell function and by interfering with antitumor $CD8^+$ T-cell immunity [50]. Therapies targeting NRP1 may therefore simultaneously inhibit tumor-induced angiogenesis and tumor cell migration, while also strengthening tumor immunosurveillance. Our investigations further identity ABL1 as an ANGPTL4/NRP1 effector. ABL1 inhibitors are already FDA-approved for the treatment of BCR-ABL1-related cancers such as chronic myeloid leukemia. Collectively, our studies identify novel opportunities for the development of therapies targeting the ANGPTL4-induded NRP1/ABL1/PXN signaling axis for the treatment of patients with HNSCC. These therapies would complement current treatment regimens and may help prolong the survival of patients with this aggressive cancer.

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ABBREVIATIONS

ABL1	ABL1 proto-oncogene 1, non-receptor tyrosine kinase
ANGPT	Angiopoietin
ANGPTL4	Angiopoietin like -4
DOK	Dysplastic oral keratinocytes
DMEM	Dulbecco’s modified Eagle’s media
FAs	Focal adhesions
FAK	focal adhesion kinase
FBS	Fetal bovine serum
GEPIA	Gene expression profiling interactive analysis
HNSCC	Head and neck cancer squamous cell carcinoma
LEUK	Leukoplakia-derived oral keratinocytes
LPL	Lipoprotein lipase
NOK	Normal oral keratinocytes
NRP1	Neuropilin1
PXN	Paxillin
RNAi	RNA interference
SNPs	Single nucleotide polymorphisms
Treg	Regulatory T cell
TG	Triacylglycerol
TCGA	The cancer genome atlas program
VEGF	Vascular endothelial growth factor

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HIGHLIGHTS

- ANGPTL4 is overexpressed in early and late stages of head and neck carcinogenesis.
- ANGPTL4 is important in both HNSCC growth and metastasis in vivo.
- ANGPTL4 regulates cell migration through an NRP1-dependent pathway.
- ANGPTL4/NRP1/ABL1/PXN is a molecular marker in HNSCC development.

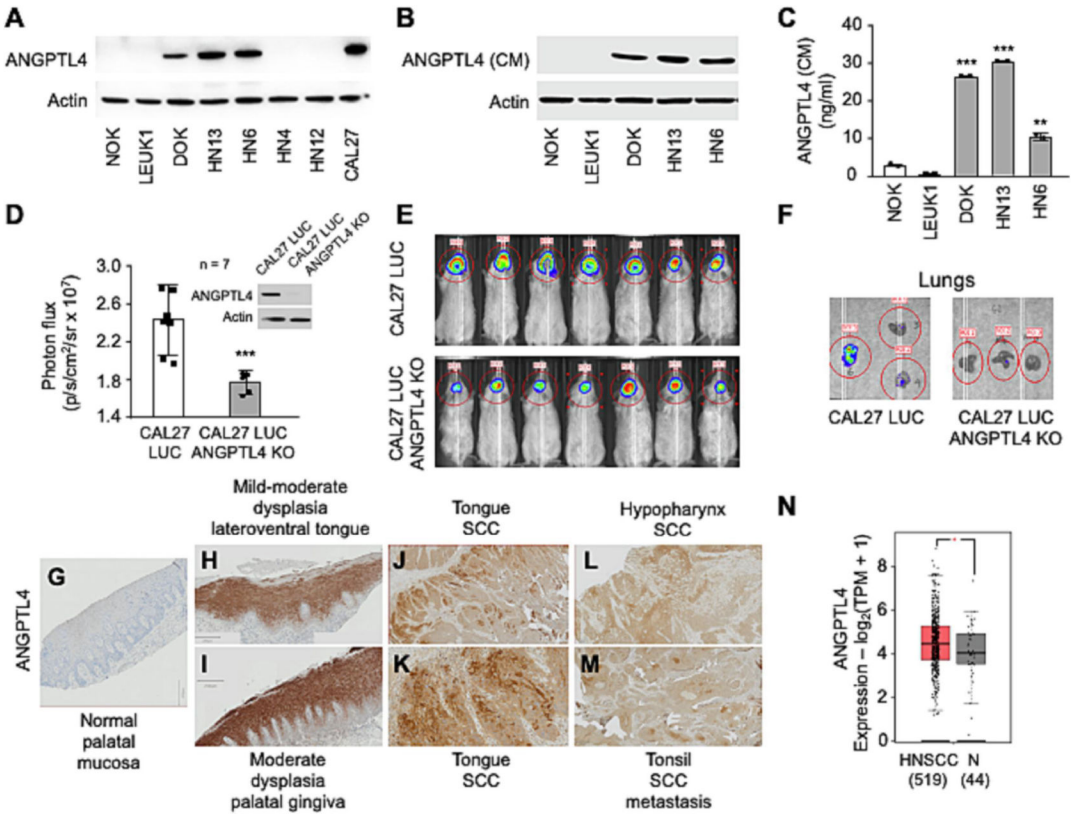

Figure 1. ANGPTL4 is upregulated in premalignant oral dysplasia and HNSCC, and it is relevant in HNSCC development.

A) Western blot of ANGPTL4 expression in total cell lysates of NOK, premalignant LEUK and DOK, and malignant HN13, HN6, HN4, HN12, and CAL27. B-C) Analysis of secreted ANGPTL4 in the conditioned media of NOK, LEUK, DOK, HN13, and HN6 using Western blot (B) or ELISA (C). HN4, HN12, and CAL27 secretions not included here. D-E) Photon flux analysis (end of experiment, mice number = 7) (D) and bioluminescence images of tumors (E) from NGR mice injected in the floor of mouth with CAL27 LUC or CAL27 LUC ANGPTL4 KO. F) Bioluminescence images of lung metastasis in NGR mice injected in the floor of mouth with CAL27 LUC or CAL27 LUC ANGPTL4 KO. G-M) IHC staining of ANGPTL4 in representative samples of human normal, dysplastic, and HNSCC tissues. Negative staining in the normal palatal mucosa (20x) (G), positive staining in mild-moderate dysplasia of lateroventral tongue (20x) (H) and moderate dysplasia of palatal gingiva (20x) (I), and positive staining in tongue SCC (20x) (J) and (100x) (K), hypopharynx SCC (20x) (L), and metastatic squamous cell carcinoma in tonsil (20x) (M). N) Comparison of the mRNA levels of ANGPTL4 in HNSCC tissues (n = 519) and normal tissues (n =44) from the TCGA and GTEx databases, respectively, using GEPIA 2.0. TPM: transcripts per kilobase million. Student's t test, *P < 0.05; **P < 0.01; ***P < 0.001.

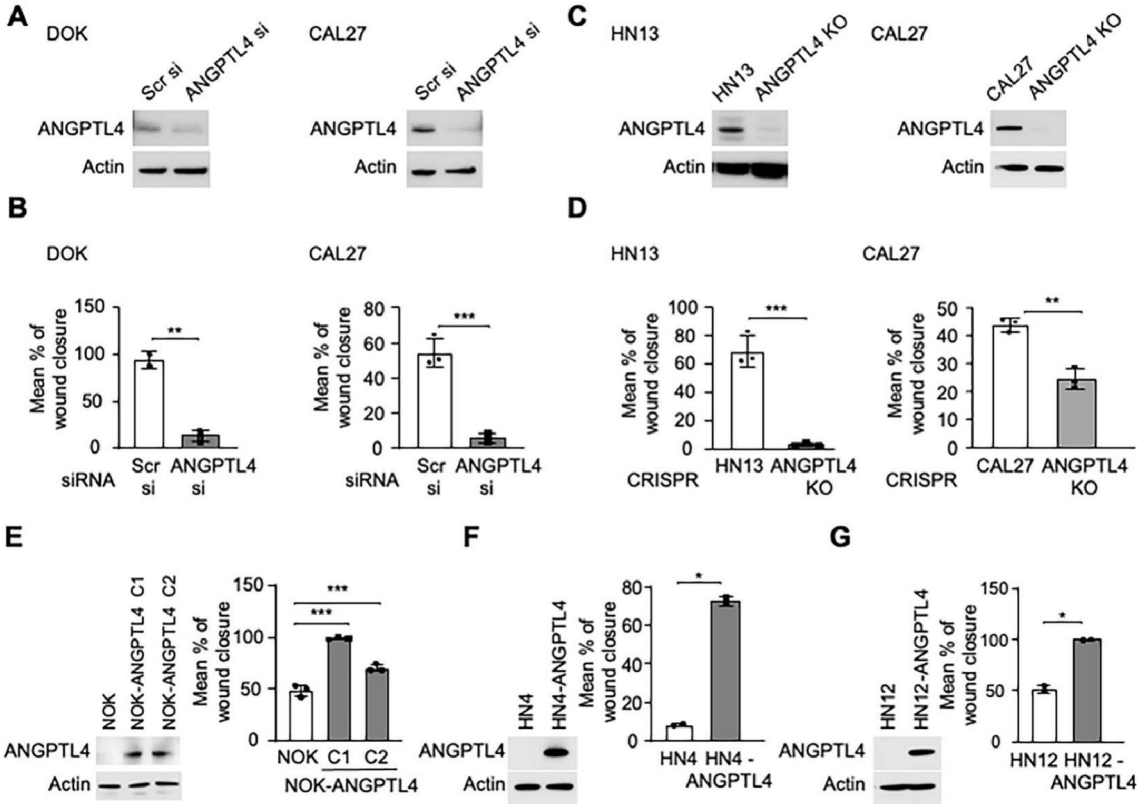

Figure 2. ANGPTL4 is necessary and sufficient for the induction of cell migration of oral premalignant and HNSCC cells.

A-B) Western blot of ANGPTL4 expression (A) and wound closure migration quantification (B) upon transfection of DOK or CAL27 with 50nM Scrambled siRNA (Scr si) or 50nM ANGPTL4 siRNA (ANGPTL4 si). C-D) Western blot of ANGPTL4 expression (C) and wound closure migration quantification (D) upon CRIPSR-mediated ANGPTL4 knock-out (ANGPTL4 KO) in HN13 or CAL27. E-G) Western Blot analysis of ANGPTL4 expression and wound closure migration quantification upon stable transfection of pCDNA3.1-ANGPTL4-mycHis in NOK (NOK-ANGPTL4C1 and NOK-ANGPTL4C2) (E), HN4 (HN4-ANGPTL4) (F) and HN12 (HN12-ANGPTL4) (G). Student's t test, *P < 0.05; **P < 0.01; ***P < 0.001.

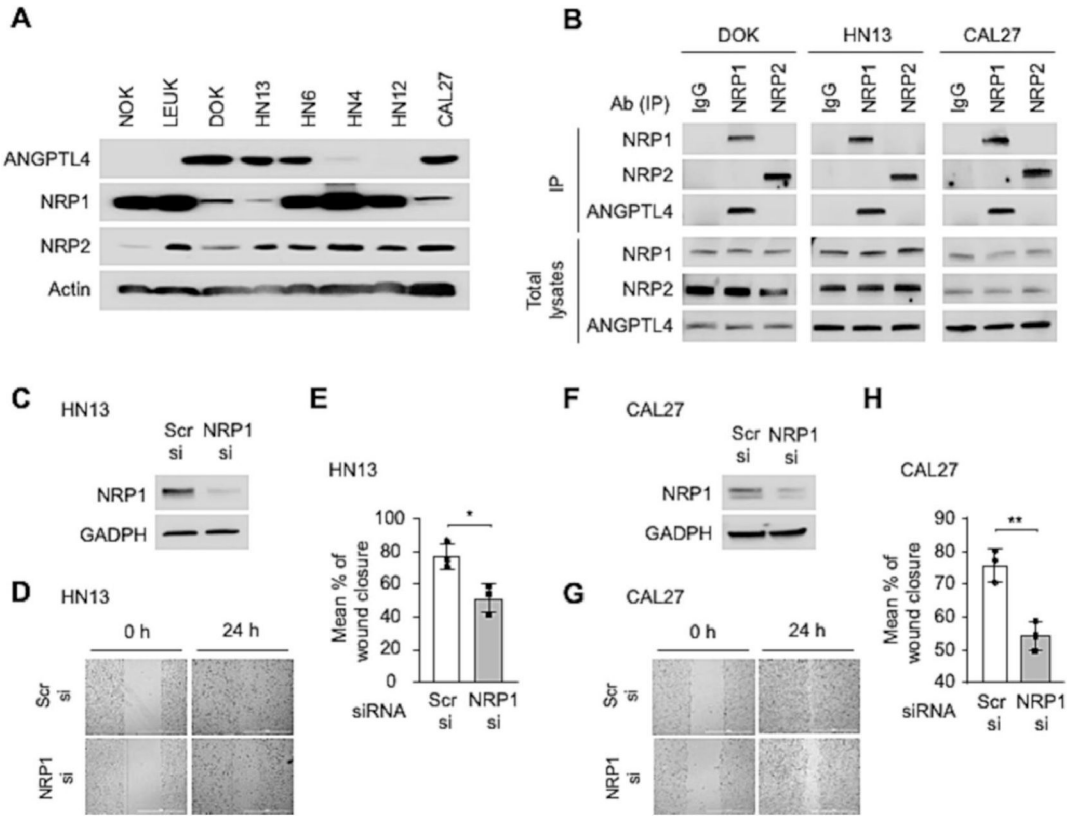

Figure 3. ANGPTL4 binds NRP1 in dysplastic oral keratinocytes and HNSCC lines.

(A) Western blot analysis of NRP1 and NRP2 expression in total cell lysates of NOK, LEUK, DOK, HN13, HN6, HN4, HN12, and CAL27. (B) Immunoprecipitation of NRP1 or NRP2 with endogenous ANGPTL4 in DOK, HN13, and CAL27. C-E) Western blot of ANGPTL4 expression (C) and wound closure migration assay (D) and its quantification (E), upon transfection of HN13 with 50nM Scrambled siRNA (Scr si) or 50nM NRP1 siRNA (NRP1 si). F-H) Western blot of ANGPTL4 expression (F) and wound closure migration assay (G) and its quantification (H), upon transfection of CAL27 with 50nM Scrambled siRNA (Scr si) or 50nM NRP1 siRNA (NRP1 si). Student's t test, *P < 0.05; **P < 0.01; ***P < 0.001.

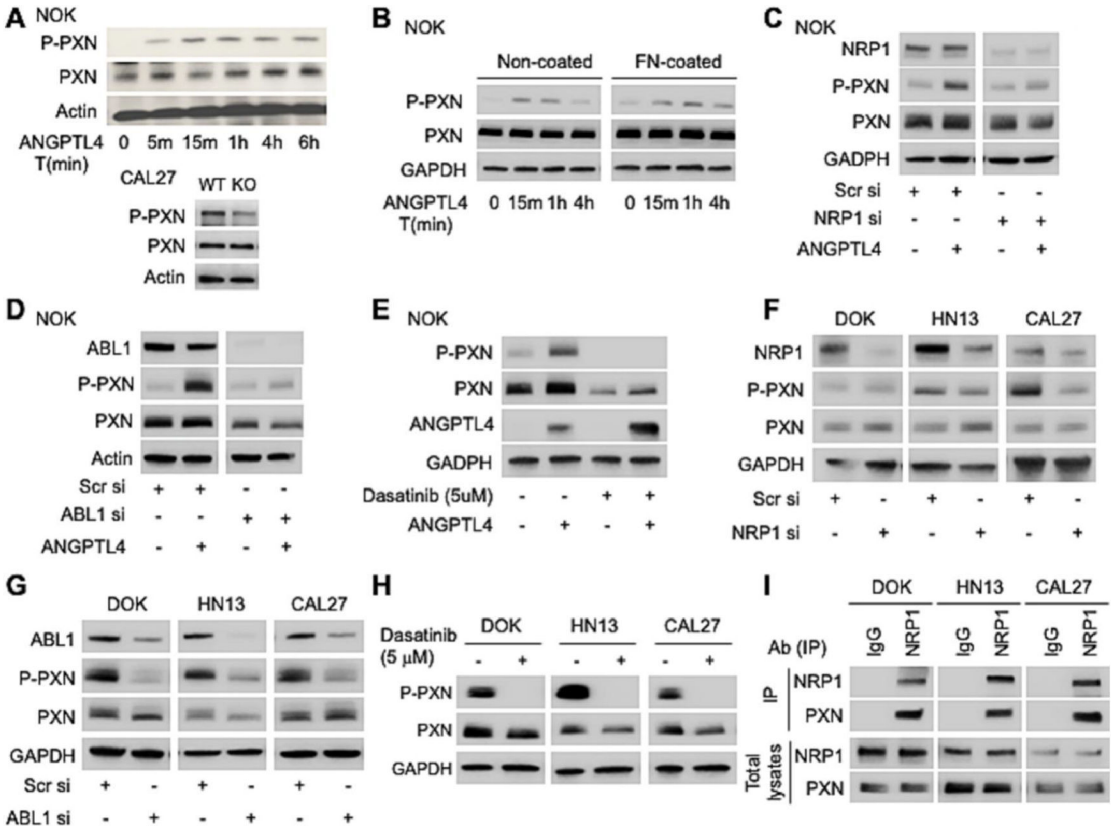

Figure 4. ANGPTL4/NRP1 activates PXN phosphorylation through ABL1.

A) Western blot analysis of P-PXN-Tyr^{118} levels upon treatment of NOK with (5 μg/mL) ANGPTL4 over time, and P-PXN-Tyr^{118} endogenous levels in CAL27 (WT) and CAL27 ANGPTL4 KO (KO). B) Western blot analysis of P-PXN-Tyr^{118} levels at different times upon treatment of NOK with (5 μg/mL) ANGPTL4, in non-coated plates or plates coated with (10 μg/mL) fibronectin (FN). CD) Western blot analysis of P-PXN-Tyr^{118} upon transfection of NOK with 50nM Scrambled siRNA (Scr si) (C-D) or 50nM NRP1 siRNA (C) or 50nM ABL1 siRNA (D), followed by treatment with (5 μg/mL) ANGPTL4. E) Western blot analysis of P-PXN-Tyr^{118} upon pretreatment of NOK with 5μM Dasatinib, followed by treatment with (5 μg/mL) ANGPTL4. F-G) Western blot analysis of P-PXN-Tyr^{118} upon transfection of DOK, HN13 or CAL27 with 50nM Scrambled siRNA (Scr si) (F-G) or 50nM NRP1 siRNA (F) or 50nM ABL1 siRNA (G), followed by treatment with (5 μg/mL) ANGPTL4. H) Western blot analysis of P-PXN-Tyr^{118} upon pretreatment of DOK, HN13 or CAL27 with 5μM Dasatinib, followed by treatment with (5 μg/mL) ANGPTL4. I). Co-immunoprecipitation of NRP1 with endogenous PXN in DOK, HN13 and CAL27.

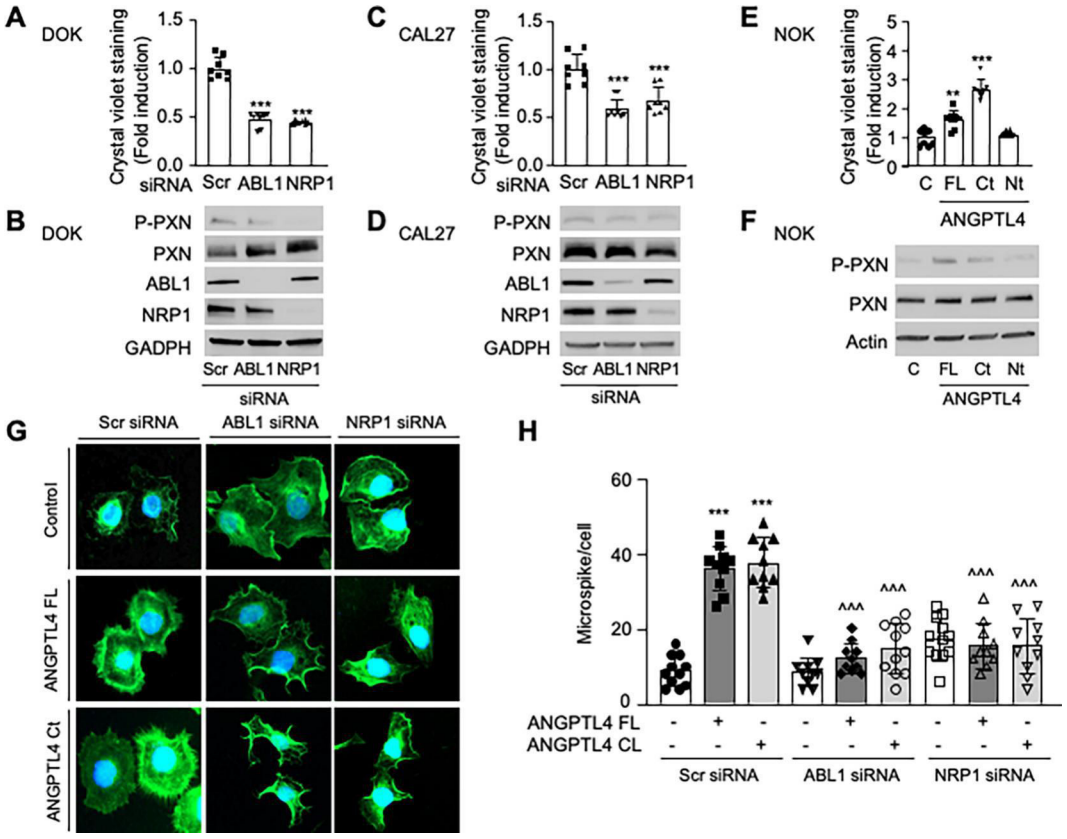

Figure 5. ANGPTL4 regulates cell migration through an NRP1/ABL1 dependent pathway.

A-B) Transwell migration assay (A) and Western blot analysis of P-PXN-Tyr^{118} (B) upon transfection of DOK with 50nM Scrambled, NRP1 or ABL1 siRNA. C-D) Transwell migration assay (C) and Western blot analysis of P-PXN-Tyr^{118} (D) upon transfection of CAL27 with 50nM Scrambled, NRP1 or ABL1 siRNA. E-F) Transwell migration assay (E) and Western blot analysis of P-PXN-Tyr^{118} (F) upon treatment of NOK with (5 μg/mL) full length ANGPTL4 (ANGPTL4 FL), C-terminal ANGPTL4 (ANGPTL4 Ct) or N-terminal ANGPTL4 (ANGPTL4 Nt). G-H) Visualization (G) and quantification (H) of phalloidin-stained membrane microspikes in NOK upon transfection of 50nM Scrambled, ABL1, or NRP1 siRNA, followed by treatment of cells with (5 μg/mL) full length ANGPTL4 (ANGPTL4 FL) or (5 μg/mL) C-terminal ANGPTL4 (ANGPTL4 Ct). Cell nuclei are stained with Hoechst 33342. Tukey test is used for post hoc comparison in ANOVA, *P < 0.05; **P < 0.01; ***P < 0.001.

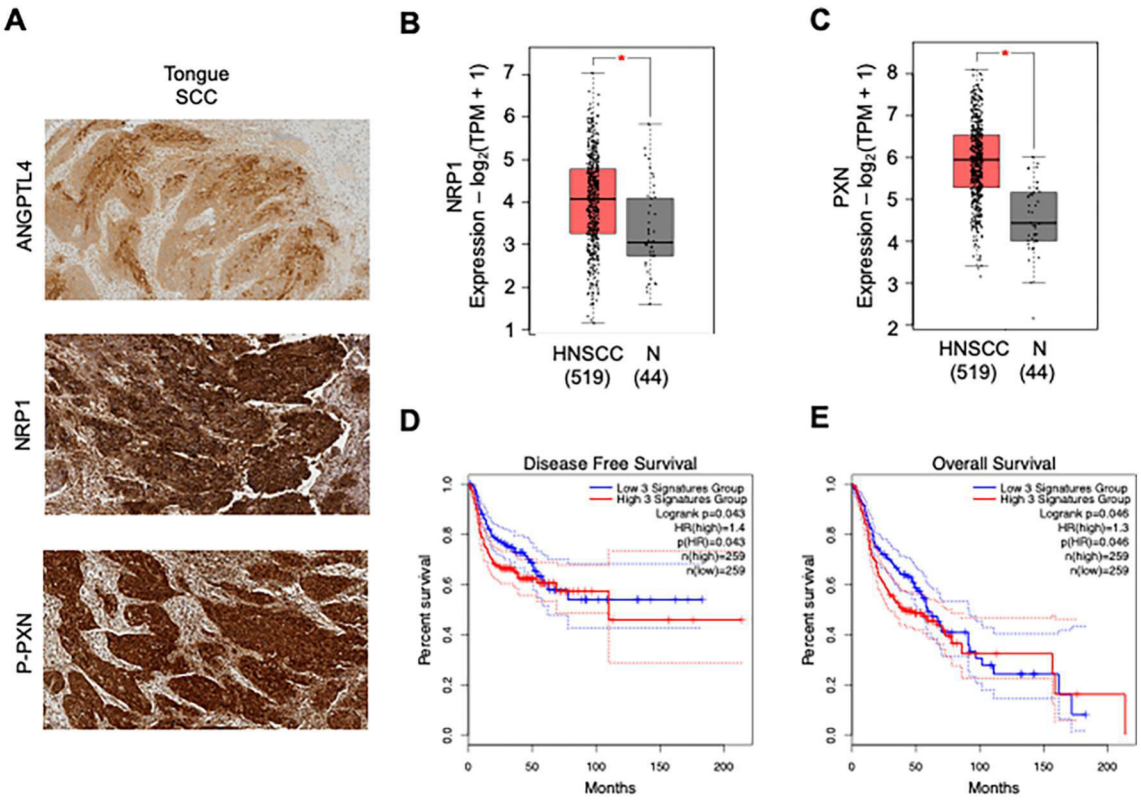

Figure 6. ANGPTL4, NRP1 and PXN are molecular targets for the treatment of HNSCC.

A. Immunohistochemical staining of ANGPTL4, NRP1 and P-PXN proteins in the same region of tongue SCC (20 x). B-C) Comparisons of the mRNA expression of NRP1 (B) and PXN (C) between HNSCC samples (n=519) from TCGA database and normal samples (n=44) from GTEx database. TPM: transcripts per kilobase million. * $p < 0.05$. D-E) Association of ANGPTL4, NRP1, and PXN signature expression with poor disease-free survival (log-rank p= 0.043) (D) and poor overall survival (log-rank p= 0.046) (E) in HNSCC, using GEPIA 2.0 analysis.