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Human Papillomavirus-related tumours of the oropharynx display a lower tumour hypoxia signature

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SUMMARY

Objectives: Human Papillomavirus (HPV)-related oropharyngeal squamous cell carcinoma (OSCC) patients have improved prognosis compared to other head and neck (HNSCC) cancers. Since poor prognosis is associated with tumour hypoxia, we studied whether the hypoxic response is different in HPV-related cells and tumours.

Material and methods: HPV-positive and -negative cells were incubated in hypoxia and analyzed by qRTPCR, western blotting and cell proliferation assays. Tumours formed by xenografting these cells in nude mice were studied by IHC. HNSCC patient samples were analyzed by unsupervised clustering of hypoxia-related gene expression, quantitative real-time PCR (qRTPCR) and immunohistochemical (IHC) detection of neo-blood vessels.

Results and conclusion: HPV-positive and -negative cells responded differently to hypoxia, in terms of gene expression (HIF-1α, PHD-3, GLUT-1 and VEGF-A) and cell survival. Tumour xenografts formed by HPV-positive cells had fewer hypoxic areas than those formed by HPV-negative cells. HPV related tumours were less hypoxic, expressed lower levels of hypoxia-responsive genes, and had a higher density of neo-blood vessels. HPV-related OSCC display lower tumour hypoxia, which could be linked to the distinct intrinsic abilities of HPV-positive tumour cells to adapt to hypoxia and to their better prognosis.

Introduction

Human Papillomavirus (HPV)-related tumours of the oropharynx are a distinct clinical subgroup of head and neck squamous cell carcinoma (HNSCC) with clinical, pathological and molecular features that are different from their HPV-negative counterpart [1–3]. Patients with HPV-related OSCC also have a prolonged disease-free survival and overall survival (for a comprehensive review, see [1] and references therein). These tumours are therefore thought to be more sensitive to both chemo- and radiation-therapies [4–7].

Several molecular and cellular features of HPV-positive HNSCC have been proposed to account for their prognosis. This includes a lower tumour hypoxia, as suggested from a retrospective analysis of the outcome of patients who were included in the DAHANCA 5 trial [8]. This could be consistent with prolonged survival, since hypoxia is a well-known poor prognostic factor in many cancers [9], and is involved in radioresistance in HNSCC [10]. However, the hypoxia status of HPV-related compared to HPV-unrelated HNSCC is not clearly established, and the published results are inconsistent and discrepant [11–15].

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We report that selected HPV-positive and HPV-negative cells respond differently to hypoxic growth conditions in cell culture and have different hypoxia statuses in vivo. These properties reflect findings in HPV-related and -negative human tumours. Our results show that HPV-related OSCC have an altered adaptation to hypoxia, which could influence the evolution of these tumours and should be taken into account when considering reasons for their better prognosis and treatment options.

Material and methods

Cell culture and reagents

The human head and neck squamous cell carcinoma (hHNSCC) SQ20B cell line was a kind gift from Rodrigez-Lafrasse [16]. The SQ20B cell line originates from a HPV-negative laryngeal SCC. The hHNSCC Cal33 cell line was purchased from the ATCC. It originates from a HPV-negative SCC of the tongue. The hHNSCC SCC90 cell line [17] was a kind gift from Prof. Susan Gollin (University of Pittsburgh). SCC90 originate from a HPV16-positive oropharyngeal tumour, and expresses wild-type *TP53* [17]. The 147T cell line was a kind gift from Snijders [18]. It was established from an HPV-positive SCC of the oral cavity. Cells were maintained at 37 °C in normoxic (20% O_2) or hypoxic (94% N_2, 5% CO_2, 1% O_2) conditions in Dulbecco's modified Eagle's medium (DMEM, PAN Biotech GmbH, Dominique Dutscher, Brumath, France) supplemented with 10% fetal bovine serum.

Western blot

3×10^5 SQ20B, Cal33, 147T and SCC90 cells were seeded and were harvested at given time points in Laemli buffer (Tris–HCl 6.25 mM, DTE 1.5 mg/ml, 1% SDS, pH6.8). 10 μg proteins were analyzed by SDS–PAGE according to standard methods. Proteins were detected with monoclonal mouse anti-HIF-1α, mouse monoclonal anti-β tubulin, ECL sheep anti-mouse IgG, HRP-conjugated antibody (1:10,000; GE Healthcare, Saclay, France). See Suppl. Material and methods for details.

Cell viability assay

SQ20B, Cal 33, SCC90 et 147T were seeded in 96 well plates (3×10^3, 3×10^3, 24×10^3 and 24×10^3 cells per well, respectively, to take into account different doubling times; 8 wells per time point), and, after 24 h at 20% oxygen, plates were incubated in either hypoxia (3% oxygen for 24 h followed by 1%) or normoxia (20% oxygen). Cell proliferation was measured at various times on separate plates using a tetrazolium salt-based method, according to the manufacturer's instructions [Alexis Cell counting Kit-8 (WST-8)].

Animal models and tumour xenografts immunohistochemistry (IHC)

SCC90 and SQ20 heterotypics xenografs were performed by injecting cell suspensions subcutaneously into the flank of mice as detailed in Suppl. Material and methods.

HIF-1α and CAIX IHC stainings of xenografts sections were carried out as described in Suppl. Material and methods.

Patients and tumour samples

The Affymetrix GeneChip analysis was performed on cohort of 98 patients. See Suppl. Table 1 for detailed demographics, and see Suppl. Material and methods for detailed description of the cohort. The gene expression assays and IHC analyses were performed on a consecutive series of 88 patients. See Table 1 and Suppl. Material and methods for a detailed description.

Table 1
Detailed demographics and clinical features of a cohort of 88 squamous cell carcinoma of the oropharynx. This cohort is constituted of 34 HPV-positive and 54 HPV-negative OSCC.

	HPV-positive patients $N = 34$	HPV-negative patients $N = 54$
Gender		
Male	22 (65%)	46 (85%)
Female	12 (35%)	8 (15%)
Age		
<57 years old	15 (44%)	24 (44%)
⩾57 years old	19 (56%)	30 (56%)
Tobacco smoking		
No	9 (26%)	4 (7%)
Yes	23 (68%)	39 (72%)
NA	2 (6%)	11 (20%)
Alcohol drinking		
No	9 (26%)	5 (9%)
Yes	23 (68%)	39 (72%)
NA	2 (6%)	10 (19%)
Pathological lymph node staging (pN)		
N0	5 (14.5%)	9 (17%)
N1	7 (20.5%)	6 (11%)
N2	20 (59%)	31 (57%)
N3	2 (6%)	5 (9%)
NA	/	3 (6%)
Pathological tumour size staging (pT)		
T1	4 (12%)	3 (6%)
T2	16 (47%)	28 (51%)
T3	13 (38%)	19 (35%)
T4	1 (3%)	3 (6%)
NA	/	1 (2%)
Tumour stage		
Stage I	0 (0%)	2 (4%)
Stage II	1 (3%)	6 (11%)
Stage III	11 (32%)	11 (20%)
Stage IV	22 (65%)	33 (61%)
NA	/	2 (4%)
Tumour histology		
Well differentiated	0 (0%)	10 (18%)
Moderately differentiated	18 (53%)	32 (59%)
Poorly differentiated	15 (44%)	9 (17%)
Non differentiated	1 (3%))	0 (0%)
NA	/	3 (6%)
Treatment		
Surgery	1 (3%)	7 (13%)
Surgery + radiotherapy	19 (56%)	37 (68.5%)
Surgery + chemoradiotherapy	14 (41%)	10 (18.5%)

Gene expression assays

Gene expression analysis was performed as described in [19–21]. Specific primer pairs are listed in Suppl. Material and methods.

Human tumour IHC

The expression of CAIX and CD105 were evaluated by IHC. A detailed protocol is available in Suppl. Material and methods.

Unsupervised clustering and statistical analysis

Statistical methods are extensively described in Suppl. Material and methods.