Overexpression of *HURP* mRNA in head and neck carcinoma and association with *in vitro* response to vinorelbine

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Abstract. *HURP* gene encodes the hepatoma upregulated protein (HURP), a microtubule associated protein regulating mitotic spindle dynamics, which promotes chromosomal congression and alignment during mitosis, with a potential role in tumorigenesis. In the present study, *HURP* mRNA expression was investigated by reverse transcription-quantitative PCR in oropharyngeal squamous cell carcinoma (OPSCC). Primary OPSCC tumors from 107 patients and 48 adjacent normal tissues, as well as 12 respiratory tract cancer cell lines (9 head and neck squamous cell carcinoma, 2 lung cancer and 1 normal bronchial) were utilised in the present study. mRNA expression levels of *HURP* were higher in malignant OPSCC tissues compared with in normal mucosa ($P<1x10^{-5}$) and significantly associated with sex and smoking status ($P<0.0001$). Vinorelbine *in vitro* toxicity at half-maximal inhibitory concentration (IC_{50}) was measured in the 11 cancer cell lines using an MTT assay. Sensitivity to vinorelbine was significantly correlated with HURP expression ($r=0.636$; $P=0.035$). The data indicated that *HURP* overexpression is frequent in OPSCC tissues and associated with smoking. The correlation between *HURP* mRNA expression and vinorelbine *in vitro* response suggests that HURP is a potential modulator of vinorelbine response; therefore, it should be explored for its possible predictive value for the efficiency of vinorelbine treatment in this type of cancer.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is a collective term to describe malignant tumors of the nasal cavity, nasopharynx, larynx and oral cavity. HNSCC represents the sixth most common malignancy worldwide (1). The main risk factors for developing HNSCC cancer are: a) the synergistic effect of tobacco smoking and alcohol consumption (2,3), b) viral infections such as human papillomavirus (HPV) (4) Epstein–Barr virus (EBV) (5), and c) genetic predisposition (6). Accumulated molecular evidence in recent years has proved that HPV is a key risk factor for increasing incidence of oropharyngeal squamous cell carcinoma (OPSCC) (7-9). Vinorelbine is an antimitotic agent, which belongs to the vinca alkaloids family and is effectively used to treat a variety of cancer cases. Some *in vitro* studies on HNSCC cells have reported an additive effect of vinorelbine to radiation exposure (10,11). In clinical trials, vinorelbine administration has also brought clinical benefits for treating advanced, recurrent/metastatic cases of head and neck squamous cell carcinoma (12-18). This indicates a clinical need to identify the cases that would benefit from vinorelbine. It is essential, therefore, to explore potential biomarkers that have predictive value for vinorelbine based therapy of HNSCC cases alongside the disease prognosis.

Vinorelbine affects MT dynamics and prevents formation of the mitotic spindle, resulting in metaphase arrest deregulation of HURP expression, in relation (19). Therefore, the overexpression of genes involved in spindle assembly and microtubule dynamics could mediate vinorelbine chemo-resistance. HURP protein is encoded by *HURP* or *DLGAP5* (Disks large-associated protein 5) gene, located on chromosome 14q22.3. HURP is a MAP protein associated with kinetochore MTs, that plays a critical role in spindle assembly, chromosome congression, alignment and segregation (20-23) and has a previously reported to be involved in tumorigenesis (24,25). Hence, it can be hypothesised that *HURP* expression may predict response to vinorelbine in cancer cells.

Elevated levels of *HURP* mRNA transcripts have been reported in lung cancer (26) and potentially associated with prognostication (27). The predictive value of HURP expression for radiotherapy has also been reported in prostate cancer cells (28). Increased *HURP* mRNA expression has also been

observed in oral squamous cell carcinoma (OSCC) (29) and anaplastic thyroid carcinoma (ATC) (30).

In this study, we evaluated *HURP* mRNA expression in a large group of oropharyngeal tumors and investigated potential associations with clinicopathological characteristics. In addition, we explored the involvement of *HURP* mRNA expression in the *in vitro* response to vinorelbine, which is a commonly used agent for the management of late stage HNSCCs.

Materials and methods

Patients and specimens. Clinicopathological data (gender, age, and lymph node involvement) were anonymously provided based on the ethical approval already granted (South Sefton Research Ethics Committee; EC.47.01-6 & North West 5 Research Ethics Committee; 09.H1010.5). Primary OPSCC tissues were available from 107 patients. Normal adjacent mucosa was available from 48 of these patients. Snap frozen biopsies were cryo-sectioned using OCT embedding matrix (CellPath Ltd.) to embed frozen blocks for cryosectioning using Cryostat (Thermo Fisher Scientific, Inc.). A 5-mm section fixed in 10% buffered formalin (cat. no. UN 2209, AnalaR) and stained with hematoxylin (cat. no. 6765003, Thermo Fisher Scientific, Inc.) and eosin (cat. no. 6766007, Thermo Fisher Scientific, Inc.) for histopathological examination was followed by two 10 mm sections that were utilised for RNA extraction.

Cell lines and growth conditions. Cancer cell lines utilised in this study were taken from the repositories of the Head and Neck group biobank and the Liverpool Lung Project biobank. All of them are established cell lines representing squamous cell carcinoma of head and neck (UPCI-SCC090) OPSCC, PE/CA-PJ41 (oral squamous epithelium), PE/CA-PJ15 (oral, tongue), UMSCC19 (oropharyngeal, base of tongue), UMSCC12 (laryngeal), UMSCC104 (oral, floor of mouth), UMSCC4 (oropharyngeal base of tongue), HN5 (oral), UMSCC81B (oropharyngeal, tonsil/glottic laryngeal), adenocarcinoma of lung (A549 and CALU6) and a normal non-tumorigenic immortalized bronchial cell line (HBEC-3KT) (31). Cell lines examined in this study were authenticated (GenePrint 10 System; Promega Corporation) and mycoplasma tested (Myco™ plus Mycoplasma PCR Detection kit; iNtRON Biotechnology).

All cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Mixture F-12 (1:1) containing 5% fetal bovine serum (Sigma-Aldrich; Merck KGaA). HBEC-3HT cell line was cultured in Keratinocyte-SFM medium supplemented with 50 mg/ml bovine pituitary extract (BPE) and 5 ng/ml human recombinant epidermal growth factor (rEGF) (Life Technologies; Thermo Fisher Scientific, Inc.). All cell lines were maintained at 37˚C, 5% CO_2.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted utilising the Direct-zol™ RNA MiniPrep kit (Zymo Research Corp.), quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.) and reverse transcribed utilising the High Capacity cDNA Reverse Transcription kit (Life Technologies; Thermo Fisher Scientific, Inc.), according to the manufacturers' instructions. For RT-qPCR, primers and probe for ACTB were designed using the OLIGO 7 software (Molecular Biology Insights) (forward: 5'-GGCACCCAG CACAATGAAG-3', reverse: 5'-CATACTCCTGCTTGCTGA TCCA-3', probe: 5' VIC-CTCCTCCTGAGCGCAAGTACT CCGTG-NFQ 3'), while the predesigned assay for *HURP* mRNA expression (Hs00207323-m1) was purchased from Thermo Fisher Scientific, Inc. RT-qPCR assays were performed in 2 replicates for clinical specimens and 4 replicates for cell lines. mRNA expression levels were calculated as relative quantification (RQ) values, based on the equation $RQ = 2^{-\Delta\Delta Cq}$, where Cq represents quantification cycle values that determined by StepOne software (version 1.2; Thermo Fisher Scientific, Inc.) and normalised to the corresponding Cq value for the endogenous control ACTB, generating ΔCq values (ΔCq = Cq target-Cq ACTB). Sample ΔCq values were further normalised against HBEC-3KT control using the formula: ΔΔCq = (ΔCq sample-ΔCq HBEC-3KT) (32). HPV status of the tumors in the study was previously detected (33,34).

Vinorelbrine exposure. Cell line growth in the presence of varying concentrations of vinorelbine (0-500 nM) was assessed by MTT assay. Exposure lasted for 72 h and measurements were taken from six biological repeats.

HURP mRNA expression differences across categorical clinicopathological parameters were assessed using the Mann-Whitney test. Spearman's correlation was used to test the relationship between *HURP* mRNA expression and response to vinorelbine.

Statistical analysis. All statistical analyses, except the IC_{50} calculation, were performed using IBM® SPSS® statistical software (version 24.0; IBM SPSS). The vinorelbine IC_{50} values were calculated using non-linear regression in Prism 5 (GraphPad Software, Inc.).

Results

Gene expression analysis. RT-qPCR analysis in primary human HNSCC tissues showed that *HURP* transcript levels were significantly higher in tumor tissues compared with those in adjacent normal mucosa (Mann-Whitney test, $P<10^{-5}$, Fig. 1).

HURP transcript levels significantly correlated with patients' gender (Mann-Whitney test, P=0.019) and smoking status (Mann-Whitney test, P=0.041). It has to be noted though that gender was associated with smoking status (χ^2 test, $P<10^{-4}$). No association was observed between *HURP* mRNA expression and other parameters such as age, lymph node involvement, alcohol consumption or HPV status (Table I).

Correlation of HURP mRNA levels with vinorelbine response. All HNSCC and lung cancer cell lines tested here showed significantly higher *HURP* mRNA expression compared to that of immortalized normal HBEC cells that was used as a calibrator (Fig. 2). Notably, the vinorelbine response presented as IC_{50} values (Table II) in the tested cancer cell lines was associated with the level of *HURP* mRNA expression (Spearman's rank test, r=0.636, P=0.035).

Table I. Clinicopathological parameters of the patient cohort in the present study.

Parameter	Patients, n (%) (n=107)	Mean expression	95% CI		P-value[a]
			Lower boundary	Upper boundary	
Sex					0.02
Male	82 (76.7)	99.49	23.55	175.43	
Female	25 (23.3)	18.59	3.96	33.21	
Age, years					0.834
<55	43 (40.2)	158.7	12.41	305.00	
≥55	64 (59.8)	29.31	9.05	49.57	
HPV status					0.981
Positive	40 (37.4)	88.25	20.50	197.00	
Negative	67 (62.6)	67.91	14.00	121.80	
Lymph node involvement					0.14
Yes	71 (66.4)	85.18	11.54	158.82	
No	36 (33.6)	55.48	6.18	117.14	
Tobacco use					0.04
Yes	80 (74.8)	90.03	16.43	163.63	
No	27 (25.2)	42.98	17.06	103.03	
Alcohol consumption					0.161
Yes	90 (84.1)	86.83	23.58	150.09	
No	17 (15.9)	11.65	7.38	15.93	

[a]P-values derived from Mann-Whitney test.

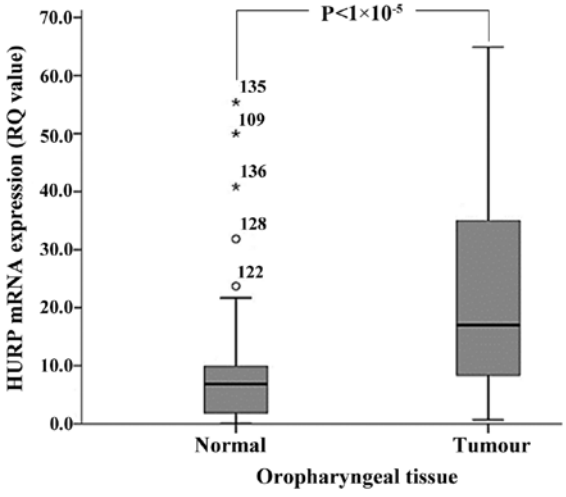

Figure 1. Boxplots demonstrating *HURP* mRNA expression levels (RQ values) in primary OPSCC tumors compared with in normal adjacent tissues. The level of *HURP* transcripts was significantly upregulated in OPSCC tissue compared with in normal tissues. P-values were derived from Mann-Whitney tests. RQ values were calculated using RNA extracted from HBEC-3KT cells as a calibrator. *Extreme values (>3x interquartile range); °Outlier values (>1.5x interquartile range). HURP, hepatoma upregulated protein; OPSCC, oropharyngeal squamous cell carcinoma; RQ, relative quantification.

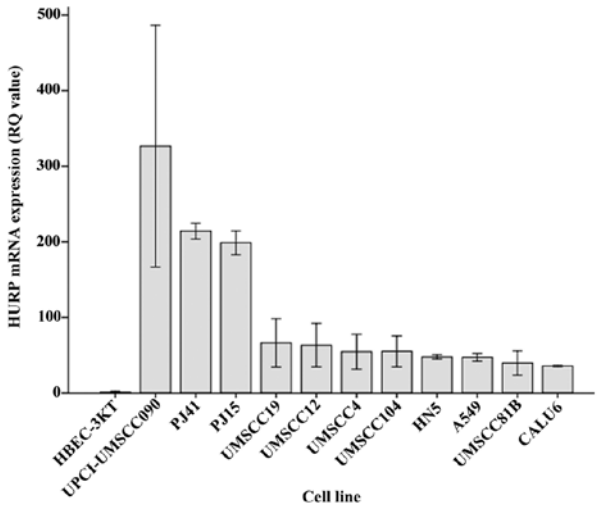

Figure 2. mRNA expression levels of HURP in Respiratory Tract Center cell lines demonstrated that head and neck squamous cell carcinoma cell lines expressed higher levels of HURP transcripts, most notably UPCI-UMSCC090 and PJ41 cells, compared with the normal HBEC cells. The bars depict the mean expression values from four replicates, whereas the error bars represent the 95% CI. HURP, hepatoma upregulated protein; RQ, relative quantification.

Discussion

HURP overexpression has been previously reported in a number of human respiratory malignancies, including non-small cell lung cancer (NSCLC) (26,27,35) and OSCC (29). However, the latter study validated publically available microarray data on an independent set of only 11 patients; therefore, the extent of HURP deregulation in

Table II. Sensitivity of tested cell lines to vinorelbine compared with HURP expression and HPV status.

Cell line	IC_{50}, nM	*HURP* RQ	IC_{50} 95% CI	HPV status
CALU6	0.007	35.70	0.005-0.009	NA
A549	0.032	47.26	0.025-0.042	NA
UMSCC19	0.046	66.17	0.040-0.053	(-)
UMSCC104	0.052	54.96	0.041-0.062	(+)
UMSCC81B	0.055	39.58	0.044-0.071	(-)
UMSCC4	0.074	54.45	0.061-0.0809	(-)
UMSCC12	0.088	63.23	0.074-0.104	(-)
HN5	0.090	47.82	0.073-0.110	(-)
UPCI-SCC090	0.091	326.51	0.075-0.110	(+)
PJ41	0.103	214.21	0.080-0.132	(-)
PJ15	0.062	198.70	0.049-0.080	(-)

HPV, human papilloma virus; HURP, hepatoma upregulated protein; RQ, relative quantification.

HNSCCs is still unclear. In addition, as it is well established that HPV positive oropharyngeal tumors compose a distinct class from a therapeutic perspective (36), it was important to explore the deregulation of HURP expression, in relation to the tumors' HPV status. Our study included 107 oropharyngeal tumors, providing a more robust figure for the reported overexpression. This frequent overexpression of *HURP* indicates a potential role in OPSCC pathogenesis. This is not surprising since HURP regulates mitotic activity of spindle assembly (20-23), therefore alterations in its expression lead to defects in cell division and aneuploidy, contributing eventually to tumorigenesis. This is further supported by the observation that knockdown of *HURP* leads to inhibition of proliferation of human pharyngeal squamous carcinoma cells (37). The tumorigenic activity of HURP has also been reported in breast cancer (24), hepatocellular carcinoma (HCC) (25) and ATC (30,38) indicating its important role in human cancer development.

Higher *HURP* mRNA levels were significantly associated with smoking in our study. This result is consistent with the fact that tobacco smoking is a well-known risk factor of HNSCC cancer alongside alcohol consumption (2,3). Currently the mechanism connecting smoking with HURP deregulation is not known. It still needs to be elucidated whether tobacco use has a causative role in this deregulation or whether it is a secondary effect of tobacco-induced carcinogenesis. The association with gender appears to be coincidental due to the significant correlation between smoking and gender in our dataset, which fits the recognised pattern (males smoke more) in the UK (https://www.cancerresearchuk.org/health-professional/cancer-statistics/risk/tobacco#heading-One).

The lack of correlation between *HURP* mRNA expression and stage and nodal status of the tumors suggests this is an early event in OPSCC tumorigenesis. Furthermore, no correlation with HPV status was observed. However, one has to consider the fact that most of these HPV positive patients were also smokers, which may be a confounding factor in this analysis; therefore, a much larger cohort would be required to enable proper stratification between those parameters.

Vinorelbine has been studied for its clinical efficiency in treating HNSCC (10,11,39,40); however it has been used in late/recurrent stages only (12-18,41). To date, no biomarkers have been suggested as possible companion indicators for vinorelbine treatment in head and neck cancer. However some potential biomarkers have been suggested for predicting VRL efficiency in lung cancer (42,43). Our study is the first to suggest a possible role in predicting response to vinorelbine *in vitro*. A possible explanation behind the correlation we report is possibly related with the ability of HURP to bind directly to MTs and regulate their dynamics (21,22) while vinorelbine blocks mitosis by suppressing MT dynamics (19). Taken together, both lines of evidence may suggest *HURP* overexpression may be a potential predictor for resistance to vinorelbine-involving regimens in HNSCC patients. The next step is to expand this study to samples from relevant clinical trials involving vinorelbine, in order to establish the efficiency of *HURP* expression as a predictor for vinorelbine administration.

In conclusion, our study demonstrates for the first time the significant overexpression of *HURP* mRNA in OPSCC and its association with smoking. It also provides initial *in vitro* evidence for the potential role of HURP in resistance against antineoplastic agent vinorelbine. Future studies are required to uncover its potency to serve as a therapeutic stratification marker in regimens including this antimitotic agent, incorporating the fact that VRL is always used in combination with another chemotherapeutic agent.

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Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ASKAK made substantial contributions to the conception and design of the study, the acquisition, analysis and interpretation of data, generated the datasets and drafted the work. PP sufficiently participated in the acquisition, analysis and interpretation of data, generated the datasets and revised the manuscript. AS collected clinical samples of primary oropharyngeal squamous cell carcinoma tissues and normal adjacent mucosa, performed their histological examination, contributed to the acquisition, analysis and interpretation of data, and revised the manuscript. AAS participated in the work sufficiently, contributed to the acquisition, analysis and interpretation of data, generated the datasets and revised the manuscript, gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JMR contributed to the acquisition, analysis and interpretation of data, and revised the manuscript. RJS collected clinical samples of primary oropharyngeal squamous cell carcinoma tissues and normal adjacent mucosa, performed their histological examination, contributed to the acquisition, analysis and interpretation of data, and revised the manuscript. TL contributed to the acquisition of data, performed data analysis and interpretation, and revised the manuscript. All authors read and approved the final version of the manuscript to be published.

Ethics approval and consent to participate

Clinicopathological data (sex, age and lymph node involvement) were anonymously provided based on the ethical approval already granted (South Sefton Research Ethics Committee, EC.47.01-6 and North West 5 Research Ethics Committee, 09.H1010.5).

Patient consent for publication

All clinical samples were collected after informed consent under previously granted ethical approval (South Sefton Research Ethics Committee, EC.47.01-6; North West 5 Research Ethics Committee, EC.09.H1010.5) from cases treated at the Liverpool Head and Neck Oncology Service, Liverpool, UK.

Competing interests

The authors declare that they have no competing interests.

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