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The prognostic value of glycerol-3-phosphate dehydrogenase 1-like expression in head and neck squamous cell carcinoma

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Aims: In this study, we sought to determine the prognostic significance of glycerol-3-phosphate dehydrogenase 1-like (GPD1L) expression in head and neck squamous cell carcinoma (HNSCC).
Methods and results: The mRNA levels of *GPD1L* were measured in 70 paired HNSCC and corresponding adjacent normal tissues using real-time PCR. GPD1L protein levels were evaluated in HNSCC from 135 patients using immunohistochemical staining. Correlations were analysed between GPD1L levels and local recurrence rate, regional recurrence rate, second primary malignancy rate), disease-free survival (DFS) and disease-specific survival (DSS). The results of real-time PCR showed that, compared with the paired normal tissues, mRNA levels of *GPD1L* were decreased significantly in HNSCC ($P < 0.001$). Patients whose tumours showed high GPD1L protein expression had a significantly better prognosis than those whose tumours showed low expression (61.3% versus 21.4%, $P < 0.001$ for DFS; 68% versus 39.3%, $P = 0.001$ for DSS). High GPD1L expression was associated with a lower local recurrence rate than low GPD1L expression ($P = 0.049$). Multivariate survival analysis also showed that GPD1L expression was an independent prognostic factor ($P = 0.001$).
Conclusions: Our results indicate that the GPD1L expression is a strong predictor for local recurrence and survival in HNSCC.

Keywords: biomarker, glycerol-3-phosphate dehydrogenase 1-like, head and neck, prognosis, squamous cell carcinoma

Introduction

Worldwide, approximately 635 000 new cases of head and neck cancer are diagnosed annually; more than 12% of these cases are in China.[1] Despite numerous advances in diagnosis and treatment over the last three decades, mortality rates have essentially remained unchanged.[2]

In a previous study we showed that the gene encoding the glycerol-3-phosphate dehydrogenase 1-like protein (*GPD1L)* is down-regulated significantly in head and neck squamous cell carcinoma (HNSCC) samples tested by cDNA microarray.[3]*GPD1L*, which is located at 3p22.3, has 84% homology with the gene encoding glycerol-3-phosphate dehydrogenase 1 (*GPD1*).[4] It was discovered in 2002 when the National Institutes of Health Mammalian Gene Collection (MGC) Program, which aims to identify and sequence a cDNA clone containing a complete open reading frame for each human and mouse gene, was completed.[5] However, the function of *GPD1L* was unclear at that time. In 2007, London *et al.* identified that *GPD1L* may affect trafficking of the cardiac Na+ channel to the cell surface. A *GPD1L* mutation decreases surface membrane expression of the sodium channel

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SCN5A, reduces inward Na+ current and causes Brugada syndrome.[6] Since then, the majority of research has focused upon its role in arrhythmias. Recently, Kelly *et al.*[7] found that GPD1L is a novel regulator of hypoxia-inducible factor 1-alpha (HIF-1α) stability, a direct target of miR-210, and can regulate HIF1α expression negatively by the suppression of prolyl hydroxylase (PHD) activity. Based on these interesting findings, we speculate that the gene may be involved directly or indirectly in energy metabolism, angiogenesis, cell proliferation and survival, among other factors. In this study, we investigated the expression of *GPD1L* in HNSCC tissues and its correlation with clinical parameters and prognosis.

Methods

PATIENT SAMPLES

This study was approved by the Institutional Review Board of the Stomatological Hospital of Peking University. Samples were collected from a cohort of 70 patients who were diagnosed with primary HNSCC between November 2012 and June 2013. Fresh samples were collected after surgery and frozen quickly in liquid nitrogen until total RNA was extracted. In parallel, a separate cohort of 135 patients was assembled from a large pool of patients in the database with a histological diagnosis of HNSCC; all the patients had undergone radical surgery in the department of oral and maxillofacial surgery at the Stomatological Hospital of Peking University between June 1999 and September 2007. Patient inclusion criteria included: (i) a primary tumour without evidence of distant metastasis (clinical stages I–IV; UICC/AJCC, 7the dn, 2009); (ii) no previous treatment; and (iii) complete medical information and follow-up data. The identifier data were terminally coded to maintain patient anonymity.

TREATMENT PROTOCOLS AND DETAILS

Surgery

Initially, all the patients were treated surgically. The surgical procedure was selected by surgeons according to tumour site and local practice. All primary tumours were completely excised, and margins were negative. Neck lymph node dissection was performed in 121 (89.6%) of the 135 patients, including: unilateral supraomohyoidal neck dissection (SOND) in 54 cases; unilateral radical or modified radical neck dissection (RND) in 45 cases; bilateral SOND in 12 cases; SOND on one side and RND on the other side in nine cases; and bilateral RND in one case. Tissue reconstruction, using free-flap transfer, was performed in 78 cases, including a radical forearm flap in 45 cases, a fibular flap in 25 cases, a lateral arm flap in four cases, an anterolateral thigh flap in two cases and a fibular flap plus radical forearm flap in two cases.

Radiotherapy

For the patients with pN+ neck, postoperative radiotherapy (RT) to the neck was advised, and patients underwent radiotherapy within 2–6 weeks of completing surgery. A conventional radiotherapy regimen of five fractions per week from Monday to Friday with 200 cGy per day was administered. The total dose for each area was as follows: primary tumour area and neck with positive nodes >6000 cGy; and neck with negative nodes >5000 cGy.

FOLLOW-UP PROTOCOL

Postoperative patients were advised to return for regular visits every 2 months in the first year; every 3 months in the second year; 6 months in the third, fourth and fifth years; and once every 6 months to 1 year thereafter. Telephone interviews were completed every 6 months for survivors. The above policy for follow-ups has been routine practice in our hospitals.

RNA PREPARATION AND REAL-TIME PCR

Total RNA was extracted from all of the HNSCC and corresponding adjacent normal tissues using TRIzol reagent (Invitrogen, Life Technologies, Gaithersburg, MD, USA) following the manufacturer's protocol. Total RNA was reverse-transcribed using TakaRa reverse transcriptase reagents (TakaRa, Mountain View, CA, USA) following the manufacturer's protocol.

Quantitative real-time reverse transcription PCR was performed with the Thermal Cycler Dice real-time system TP800 (TakaRa) according to the standard protocol of the SYBR Premix ExTaq perfect real-time system (TakaRa). The following primers for *GPD1L* and *β-actin* were used as references for normalization: *GPD1L* sense 5′-ACGGTGGTTGATGATGC AGACACT, antisense 5′-CGGATGACGGCCGCT TTG GT; and *β-actin* sense 5′-ATCGTCCACCGCAAATGCTT CTA, antisense 5′-AGCCATGCCAATCTCATCTTGTT. *GPD1L* and *β-actin* products are 122 and 118 bp, respectively. Thermal cycling conditions were 95°C for 1 min, 95°C for 15 s and 40 cycles at 60°C for 1 min.

The fold change of *GPD1L* expression in HNSCC samples relative to adjacent normal samples was quantified using $2^{-\Delta\Delta C_t}$ according to our previous study.[8]

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IMMUNOHISTOCHEMISTRY

Tissue sections of 3-μm thickness were cut from formalin-fixed, paraffin-embedded tissue samples. The avidin–biotin complex (ABC) technique was performed, following the manufacturer's instructions for the Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanol, treated with citrate buffer for antigen retrieval and quenched in hydrogen peroxide. Tissue sections were blocked with 2.5% normal serum and incubated overnight at 4°C using a rabbit polyclonal antibody against the GPD1L protein (1:50 dilution; HPA035284; Atlas Inc., Stockholm, Sweden), followed by incubation with secondary antibody and then the ABC reagent. Diaminobenzidine was used as a chromogen, and the sections were counterstained with Mayer haematoxylin (Sigma-Aldrich Corporation, St Louis, MO, USA). Immunohistochemical staining of normal human salivary gland tissue specimens was used for positive controls; negative controls were processed using the same procedure, except that 10% non-immune mouse serum (Santa Cruz, Inc., Santa Cruz, CA, USA) was substituted for the primary antibody.

The percentage of positive area (P) of GPD1L expression was assessed by manual counting, aided by analysis using Image-pro Plus version 6.0 (IPP 6.0; Media Cybernetics, Inc., Bethesda, MD, USA). The scores for intensity of staining (I) were determined as 1, negative or light yellow colour (weak staining); 2, brown colour (moderate staining); and 3, dark brown colour (strong staining). The total scores (S) were designated as $P \times I \times 100$ for each section. Because positive staining was distributed evenly in the tumour sections, GPD1L expression was determined by counting 1000 cells in 10 random large graticules that were visible under the microscope. All the images that were analysed using IPP 6.0 were verified by two pathologists who were blinded to the results of the previous assessments. When a disagreement arose, consensus was reached by discussion. A cut-off value was set as the median of the labelling index: a value ≥100 was considered to indicate high expression and a value <100 low expression.

STATISTICAL ANALYSIS

The patient characteristics were expressed as percentages or means. The baseline data of the two groups were compared using a chi-squared test, except that age was compared using an independent-sample *t*-test. Disease-specific survival (DSS) was calculated as the period from the first day after treatment until death from any cause or until the date of the last follow-up, at which point the data were censored. Disease-free survival (DFS) was defined as the time from the first day after treatment to death from any cause or to disease progression. The log-rank test was used to analyse univariate associations between GPD1L expression levels and DFS and DSS. All potential prognostic factors with P-values < 0.05 from the univariate analysis were incorporated into multivariate analyses. Hazard ratios with corresponding 95% confidence intervals (CIs) and P-values are reported. Cox proportional hazard models were utilized for univariate and multivariate analyses of molecular biomarkers and other baseline factors with DSS. All calculations and analyses were performed using the SPSS 17.0 Statistical Package (SPSS Inc., Chicago, IL, USA) for Windows and were two-tailed where appropriate.

Results

PATIENT CHARACTERISTICS

The primary sites of tumour for the 70 patients from whom fresh specimens were collected were the oral cavity ($n = 64$) and oropharynx ($n = 6$). There were 49 males and 21 females; 31 patients were aged ≥60 years, and 39 <60 years. In total, seven patients (10%) were at TNM stage I, 10 patients (14.3%) at stage II, 15 patients (21.4%) at stage III and 38 patients (54.3%) at stage IV. For pathological grade, 13 tumours (18.6%) were grade I, 49 (70%) were grade II and eight (11.4%) were grade III.

The demographic data of the 135 successive patients who underwent surgery with or without postoperative radiotherapy are summarized in Table 1. In these patients' data, the cut-off date of follow-up was 1 April 2013 for survivors. The median follow-up for surviving patients was 73 months [interquartile range (IQR), 69–103].

GPD1L MRNA EXPRESSION IS DOWN-REGULATED IN HNSCC TISSUES

The results of the real-time PCR showed that *GPD1L* expression was decreased significantly in HNSCC tissues compared with adjacent normal tissues. Using a cut-off value of 0.5, the expression ratio was <0.5 in 74.3% of patients (52 of 70). For the mRNA levels of *GPD1L*, a significant difference was found between HNSCC and adjacent normal tissues ($P < 0.001$,

Table 1. The baseline demographics and GPD1L protein expression of the 135 patients in this study

	Patients (*n* = 135)		GPD1L expression (*n* = 131)*		
Variable	No.	%	Low expression	High expression	*P*
Age, years: mean ± SD	59.0 ± 12.3		60.2 ± 12.2	57.9 ± 12.5	0.288
Gender					
Male	82	60.7	31	49	0.280
Female	53	39.3	25	26	
Sites					
Tongue	53	39.3	20	32	0.950
Buccal	22	16.3	8	12	
Mandibular gingiva	19	14.0	8	11	
Floor of the mouth	16	11.8	8	8	
Maxillary gingiva	14	10.4	7	6	
Oropharynx	7	5.2	3	4	
Hard palate	4	3.0	2	2	
T stage					
T1	34	25.2	13	17	>0.999
T2	59	43.7	25	34	
T3	12	8.9	5	7	
T4	30	22.2	13	17	
N status					
N−	61	45.2	25	35	>0.999
N+	60	44.4	24	35	
Unknown	14	10.4	7	5	
Pathologic grade					
I	55	40.7	21	31	0.097
II	68	50.4	33	34	
III	12	8.9	2	10	
Growth pattern					
Exophytic	44	32.6	17	24	>0.999
Ulcerative	38	28.1	16	21	
Infiltrative	53	39.3	23	30	
Smoking history					
Smoker	69	51.1	27	42	0.479
Nonsmoker	66	48.9	29	33	
Alcohol history					
Drinker	49	63.7	19	30	0.584
Nondrinker	86	36.3	37	45	

*GPD1L expression (*n* = 131).
Four cases were excluded from final evaluation due to the lack of tumour cells in the tissue sections.

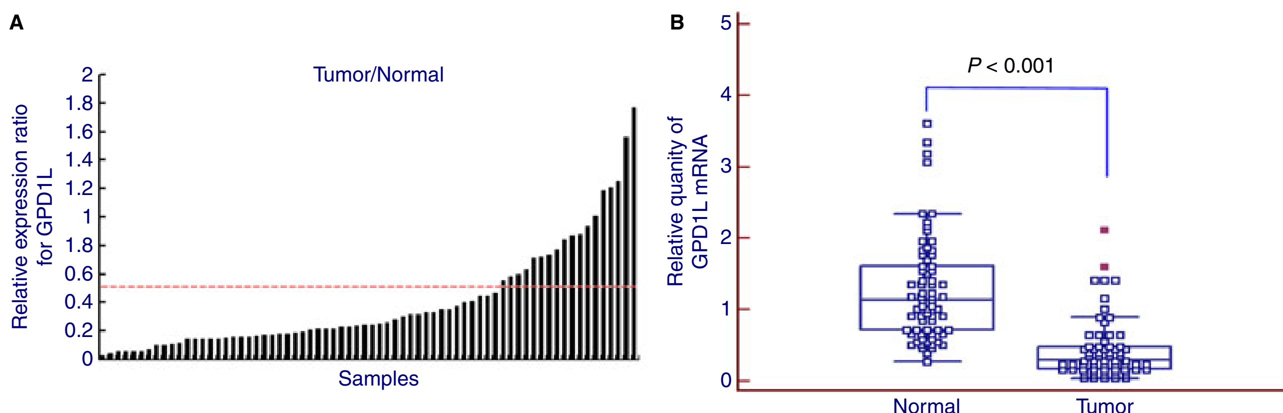

Figure 1. Analysis of the expression of *GPD1L* by real-time PCR using 70 pairs of samples. **A**, Fold change in relative mRNA expression of *GPD1L* in samples from tumour and adjacent normal tissues (cut-off value of fold change: 0.5). **B**, Comparison of the relative expression of *GPD1L* between HNSCC and adjacent normal tissues. Boxes: distribution of expression values from the 25th to 75th percentiles for *GPD1L*; horizontal lines in boxes: median values; whiskers: 5th and 95th percentiles; dots: outliers.

Figure 1). The results confirmed that *GPD1L* was a down-regulated gene in HNSCC. No association was found between *GPD1L* mRNA expression and any clinical and demographic data.

GPD1L PROTEIN EXPRESSION

GPD1L protein expression was assessed in 135 cases by immunohistochemistry; four cases were excluded from final evaluation due to a lack of tumour cells in the tissue sections. Obvious cytoplasmic and plasma membrane staining of normal epithelial and cancer cells was defined as positive immunostaining. Ultimately, 56 cases were evaluated as showing low expression of the GPD1L protein, and 75 cases as showing high expression (Figure 2). As determined by chi-squared test, no association was found between GPD1L protein expression and any baseline demographics.

GPD1L PROTEIN EXPRESSION IS CORRELATED SIGNIFICANTLY WITH THE PROGNOSIS OF PATIENTS WITH HNSCC

During the follow-up period, 66 (48.9%) of the 135 patients had died. Six patients died as a result of causes unrelated to cancer: four of cardiac failure and brain stroke, one from an uncontrolled lung infection, and one of uncertain cause. The sites of recurrence of enrolled patients are shown in Table 2. Thirty-seven (27.4%) patients developed local recurrence. In total, 16 (11.9%) patients developed nodal recurrence alone without associated local recurrence. In addition, 27 (20%) patients developed second primary carcinoma, including 14 in the head and neck region, four in the colorectum, three in the breast, two in the lung and bronchus, and one case each in the oesophagus, liver, prostate and kidney. The DFS rate was 44.4% (60 of 135) and the DSS rate was 55.6% (75 of 135), respectively.

Through analysis of GPD1L protein expression and prognosis, we found that the DFS rates were significantly different between low- and high-GPD1L protein expression subgroups (21.4% and 61.3%, respectively, $P < 0.001$, Figure 3A). A similar result was observed with the DSS rates for patients with tumours showing low and high GPD1L protein expression (39.3% and 68%, respectively, $P = 0.001$, Figure 3B).

GPD1L PROTEIN EXPRESSION IS CORRELATED MORE CLOSELY WITH LOCAL RECURRENCE THAN WITH SECOND PRIMARY MALIGNANCY OR REGIONAL RECURRENCE

The patients with low GPD1L protein expression had an increased risk of local recurrence compared to those with high GPD1L protein expression (35.7% versus 20%, $P = 0.049$). However, no significant association was found between GPD1L protein expression and second primary malignancy rate (low versus high expression: 28.6% versus 14.7%, $P = 0.079$) or regional recurrence rate (low versus high expression: 14.3% versus 10.7%, $P = 0.595$).

GPD1L PROTEIN EXPRESSION IS AN INDEPENDENT RISK FACTOR FOR DSS

To assess the prognostic value of GPD1L protein expression on a continuous scale, univariate survival

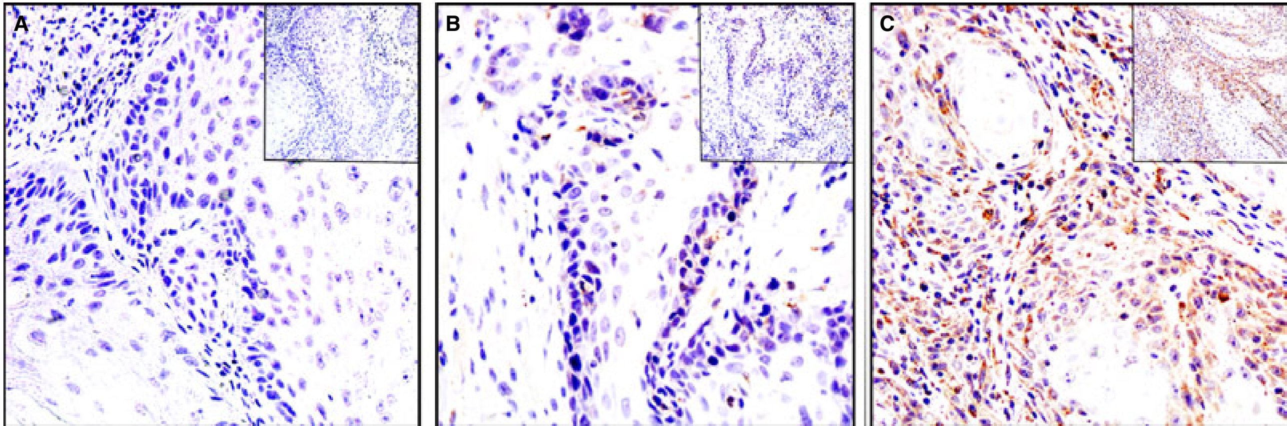

Figure 2. Typical cases showing a close association between GPD1L protein expression and prognosis. **A**, Negative control; **B**, A poor prognosis case with low GPD1L protein expression; **C**, A good prognosis case with high GPD1L protein expression.

Table 2. The correlation between GPD1L protein expression and the sites of first recurrence and treatment results of patients in this study

Sites of the first recurrence	Patients (n = 135)	Treatment after recurrence	GPD1L expression (n = 131)*		P
			Low expression (%)	High expression (%)	
Local ± node					
Presence	37	25 op; 6 op+RT; 6 RT	20 (57.1)	15 (42.9)	0.049
Never	96	–	36 (37.5)	60 (62.5)	
Node only					
Presence	16	9 op; 5 op+RT; 2 RT	8 (50)	8 (50)	0.595
Never	119	–	48 (41.7)	67 (58.3)	
SPM					
Presence	27	12 op; 7 CTRT; 8 CT	16 (59.3)	11 (40.7)	0.079
Never	108	–	40 (38.5)	64 (61.5)	
Distant	Missing	–	–	–	–

SPM, Second primary malignancy; op, operation; RT, radiotherapy; CT, chemotherapy; CTRT, concurrent chemoradiotherapy.
Underlined values represent a statistically significant difference
*GPD1L expression (n = 131): four cases were excluded from final evaluation due to the lack of tumour cells in the tissue sections.

analysis was also performed using the Cox proportional hazards model. The results showed that GPD1L expression ($P = 0.001$), T stage ($P = 0.005$) and lymph node status ($P = 0.021$) were associated with DSS. In a multivariate analysis that included the above three factors, GPD1L protein expression ($P = 0.001$), T stage ($P = 0.013$) and lymph node status ($P = 0.046$) were found to be independent prognostic factors (Table 3).

Discussion

HNSCC are characterized by marked heterogeneity in their biological behaviour, and tumours of the same stage often respond differently to the same treatment.[9] Identification of novel biomarkers that reflect the physiological state and change of cells during disease progression may permit individualized therapy and prediction of prognosis for patients with cancer.[10]

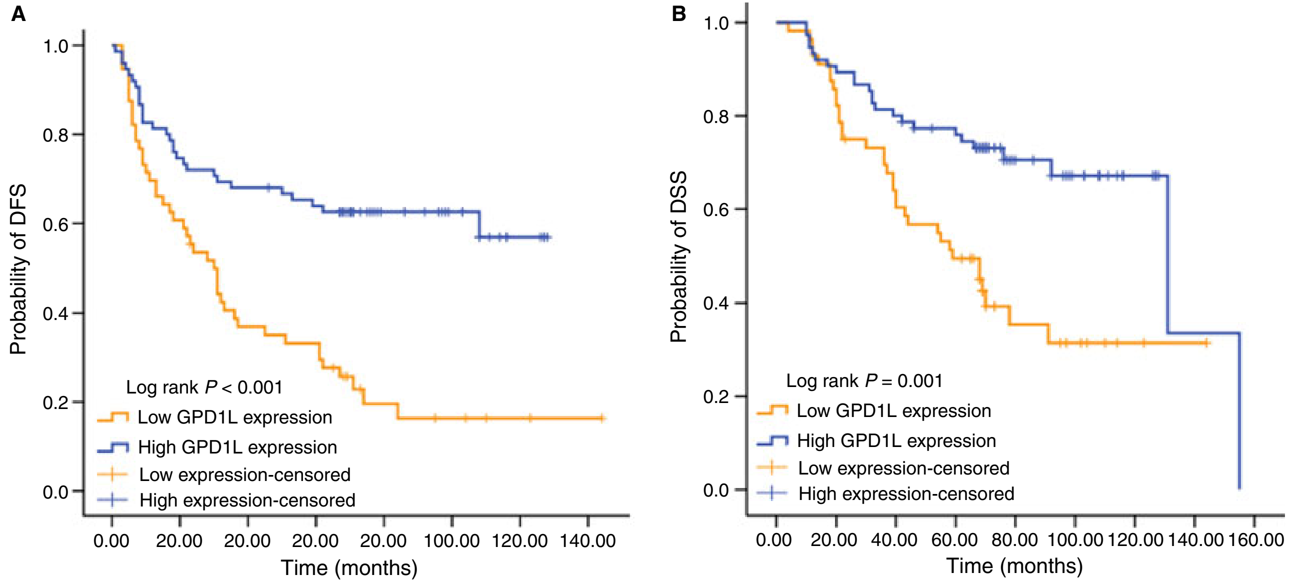

Figure 3. Kaplan–Meier survival curves demonstrating correlations between GPD1L protein expression and prognosis for patients with HNSCC. **A**, GPD1L protein expression and disease-free survival (DFS); **B**, GPD1L protein expression and disease-specific survival (DSS).

Using tissue microarrays, we have previously identified several differentially expressed genes in HNSCC tissues compared with normal tissues. GPD1L is one of the down-regulated genes, and its function in tumours is not clear.[3] Recently, Descotes *et al.*[11] identified potential prognostic biomarkers for node-nega-

Table 3. Cox proportional hazards regression models in estimating DSS

Variable	Hazard ratio	95% Confidence interval	*P*
Univariate analysis			
Age	1.006	0.984–1.028	0.606
T stage (T1, T2, T3, T4)	1.402	1.108–1.772	0.005
Pathologic grade (I, II, III)	1.045	0.700–1.561	0.829
Smoking history (smoker versus nonsmoker)	1.100	0.656–1.844	0.717
Alcohol history (drinker versus nondrinker)	1.517	0.903–2.547	0.115
Site (oral versus oropharynx)			0.634
Gender (male versus female)	1.134	0.673–1.910	0.637
Lymph node status (N+ versus N−)	1.938	1.103–3.403	0.021
Growth pattern (exophytic, ulcerative, infiltrative)	1.169	0.866–1.578	0.307
GPD1L protein expression (low versus high)	0.405	0.238–0.689	0.001
Multivariate survival analysis			
T stage (T1, T2, T3, T4)	1.393	1.071–1.811	0.013
Lymph node status (N+ versus N−)	1.794	1.010–3.185	0.046
GPD1L protein expression (low versus high)	0.386	0.220–0.678	0.001

Underlined values represent a statistically significant difference

tive breast tumours by proteomic analysis; their study found that GPD1, the homologous molecule of GPD1L, was differentially expressed, allowing prediction of tumour relapse. In our study, decreased expression of *GPD1L* was quantitatively detected at the mRNA level in HNSCC tissues compared with adjacent normal tissues, suggesting that *GPD1L* may suppress initiation and development of HNSCC. Moreover, low expression of the GPD1L protein was correlated with a high local recurrence rate and thereby led to a poorer prognosis for patients with HNSCC. GPD1L protein expression, as detected by immunohistochemistry, was an independent predictor of adverse DFS and DSS. The present study has, for the first time, detected *GPD1L* expression in both mRNA and protein levels in HNSCC by comparing cancer with adjacent normal tissues, and revealed the correlation between its expression levels and patients' clinical parameters.

In conclusion, the results suggest that *GPD1L* is down-regulated and possibly acts as a tumour suppressor in HNSCC. A further prospective study is needed to determine the value of *GPD1L* as a candidate biomarker in HNSCC. Furthermore, experiments to determine the effect of the expression of *GPD1L* on cellular biological behaviour *in vitro* or *in vivo* are necessary to understand its role in cancer, and specifically in HNSCC. This potential biomarker may in future serve as a novel factor for recurrence surveillance and prognostic evaluation.

Acknowledgements

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