Original Research Article

P4HA1: A single-gene surrogate of hypoxia signatures in oral squamous cell carcinoma patients

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ABSTRACT

Background and purpose: Hypoxia gene expression signatures are of high prognostic value for head and neck cancer patients. Recently, the prognostic information of a multiple-gene hypoxia signature was found to be provided by the mRNA level of P4HA1 alone (Tawk et al., 2016). Therefore, we studied the prognostic value of P4HA1 in an independent cohort of oral squamous cell carcinoma (OSCC) patients.
Material and methods: Frozen tumor samples of 118 adult OSCC patients were analysed for P4HA1 mRNA level by quantitative real-time TaqMan™ PCR analysis. Kaplan-Meier analysis and Cox's regression analysis were performed to characterize the prognostic impact of P4HA1 mRNA level in OSCC patients.
Results: The analyzed patient cohort was divided into four subgroups according to the quartiles of the P4HA1 mRNA levels. The highest intratumoral P4HA1 mRNA level was significantly correlated with a poor overall survival (RR = 2.2; P = 0.04) and an increased risk of locoregional recurrence (RR = 4.8; P = 0.02). In patients who received radiotherapy (n = 82) highest intratumoral P4HA1 mRNA level was significantly correlated with a poor overall survival (RR = 3.4; P = 0.01) and an increased risk of locoregional recurrence (RR = 10.3; P = 0.005). Moreover, significant correlations between the P4HA1 mRNA level and the mRNA level of several EMT and stem cell markers were found.
Conclusions: A high P4HA1 mRNA level, as a single-gene surrogate of hypoxia, is an independent prognostic marker for the overall survival and locoregional recurrence of OSCC patients.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the ten most common cancers worldwide with an incidence of 780.000 per year, whereby most of the HNSCC are oral squamous cell carcinomas (OSCC) [1,2]. The 5-year overall survival time has stagnated since years at about 40–50% [3]. In OSCC, tumor hypoxia is a characteristic feature as shown by the overexpression of the transcription factor hypoxia-inducible factor 1 (HIF-1) [4,5].

Efforts to characterize the extent of hypoxia within a tumor tissue and the adverse prognostic consequences in head and neck cancer have led to the evaluation of hypoxia gene signatures. Recently, the clinical relevance of three common hypoxia signature studies was established [6–8]. However, prolyl 4-hydroxylase (P4HA1), which is the only gene common to all identified hypoxia signatures, was found to provide a similar prognostic information regarding overall survival of head and neck cancer patients as the full signatures [9]. In human fibroblasts the transcriptional activity of HIF1 on P4HA1 had an impact on matrix stiffness, extracellular matrix production and cell-matrix interaction that affects cancer cell adhesion and invasion [3,10]. P4HA1 expression has consistently been described as stably increased under hypoxia on the mRNA as well as on the protein level [11,12].

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The prolyl 4-hydroxylase (*P4HA1*) gene is coding for a protein involved in the hydroxylation of proline residues in posttranslational collagen synthesis. The human prolyl 4-hydroxylases are tetrameric isoenzymes composed of two β subunits (encoded by *P4HB*) and two (catalytic) α subunits. There are three different α subunits encoded by *P4HA1*, *P4HA2* and *P4HA3* [13]. The knockout of P4HA1 in mice is lethal and is associated with overall developmental delay, due to the lack of collagen IV in the extracellular matrix and a disrupted basement membrane [14]. Recently, *P4HA1* was found to be overexpressed in gliomas and the expression correlated with tumor microvessel density. That fact demonstrated that *P4HA1* influences the neovascularization in gliomas, whereas a knockdown of *P4HA1* decreases the levels of collagen IV and disrupts the vascular basement membrane [15]. The authors believe *P4HA1* may have a role in the transdifferentiation process of glioma stem cells into endothelial cells [15].

To evaluate the prognostic potential of *P4HA1* in an independent data set, we studied its mRNA level in the tumor tissue of 118 OSCC patients and determined its association with overall survival and locoregional control as well as with mRNA levels of selected epithelial mesenchymal transition (EMT) and stem cell markers.

Material and methods

Tissue samples and histopathological data

We examined frozen primary tumor samples of 118 OSCC patients. All patients had been treated with surgery at the Department of Oral and Maxillofacial Plastic Surgery, Martin Luther University Halle Wittenberg, Germany. The tissue samples were cut by a cryocut microtome and the first and the last histologic sections were stained with hematoxylin and eosin. Experienced pathologists (UB, DB) verified the sections. We defined samples as tumor tissue when >70% of the first and the last histologic sections were tumor tissue. All patients gave written informed consent. The study was carried out in compliance with the Helsinki Declaration, and it was approved by the Ethics Committee of the Medical Faculty of Martin Luther University Halle-Wittenberg.

51 patients were alive after a median observation time of 31 months (mean 33 months), whereas 67 patients died after a median time of 13 months (mean 16 months) after diagnosis. The histopathological and clinical data have been summarized in Tables 1 and 2.

Cell culture

To study the hypoxic P4HA1 mRNA level in relevant *in vitro* models, we analyzed the human cell lines CAL-33 (derived from a primary tumor of the tongue; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), SAS (derived from a primary tumor of the tongue; Deutsches Krebsforschungszentrum, Heidelberg, Germany) and XF354 (derived from a primary SCC of the floor of the mouth; Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cells were cultured as monolayers in RPMI 1640 medium (Lonza, Walkersville, MD, USA) containing 10% fetal calf serum, 1% sodium pyruvate, 180 U/ml penicillin and 180 μg/ml streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere supplemented with 5% CO_2.

Cells were cultured in RPMI medium containing glutamine and 10% fetal calf serum overnight under normoxic (21% oxygen) or hypoxic conditions (<1% oxygen) which was achieved using a gas generator system as described previously [16]. Cells were then harvested by treatment with trypsin and RNA was isolated.

RNA-Isolation

Snap-frozen tumor samples were cut into 20 μm tissue sections and RNA was isolated by Trizol reagent according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany). The RNA of treated cell lines was isolated equally. DNA contaminations were removed by DNAse I digestion (Qiagen, Hilden, Germany). The RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific, Karlsruhe, Germany).

Quantitative RT-PCR

6 μg of total RNA was used for cDNA synthesis (tissue samples) and 1 μg for cell line samples according to standard protocols (Fermentas, St. Leon-Rot, Germany) as previously described [17]. The cDNA was amplified by automated real-time quantitative TaqMan™ assays for *P4HA1* and *RPII* as a housekeeping gene using kits from Thermo Fisher Scientific (Darmstadt, Germany). *P4HA1* transcript amounts were normalized to *RPII* transcript amounts using the ΔΔCt method [18].

Moreover, the same cDNA was used to analyze the normalized mRNA levels of the EMT-markers *ZEB2, Twist, TGFβ, MMP7, CTGF*, the stem cell markers *Oct3/4, Snai1, Snai2, LGR4, LGR5FL*, the structure proteins *CDH1, vimentin, KRT13* the hypoxic markers *CA9, VEGF, Glut1, HIF1α* and mRNA of *MDM2, survivin, HER2, EGFR, PDL-1* and *Osteopontin* using TaqMan™ assays (Thermo Fisher Scientific (Darmstadt, Germany) or as previously described [19,20]. The primers for the analysis of *CTGF* were: fw: 5′gag cag ctg caa gta cca gt, rw: 5′gtc ttc cag tcg gta agc cg. The mRNA-level of *P4HA1* was correlated with those markers via Spearman's rank correlation (see Table 3).

Statistical analysis

Cox's regression hazard model and Kaplan-Meier analysis were used to estimate a correlation of *P4HA1* mRNA with overall survival of OSCC patients. Cox's regression hazard model for analysis of overall survival and locoregional control was adjusted for the prognostic effect of covariates (T-stage and N-stage and grading), and the relative risk (RR) was calculated. Survival times were calculated from the day of tumor diagnosis. The end point for the overall survival analysis was the time of death of the patient. The end point for the locoregional control analysis was the first recurrence. The interrelationships between the different mRNA levels were tested with the Spearman's rank correlation (r_s, correlation coefficient). The correlation of the P4HA1 mRNA level, T-stage, N-stage, grading and gender of the patients were tested with the Kruskal Wallis test. A probability (*P*) of <0.05 was defined as significant. Statistical analyses were carried out using SPSS software version 20.0 (SPSS Inc., Chicago, USA).

Results

Correlation of the P4HA1 mRNA level with the survival of OSCC patients

The *P4HA1* mRNA level of 118 OSCC samples was normalized to the *RPII* mRNA level. The OSCC cohort was than divided into quartiles (low *P4HA1* mRNA level, <75.7; moderate *P4HA1* mRNA level, 75.71–131.9; high *P4HA1* mRNA level, 131.91–174.1 and very high *P4HA1* mRNA level, >174.1). The transcript ratios of 118 OSCC samples ranged from 13 to 565. (mean 162; median 127.5).

The 3-year overall survival rate was 70% for patients with a low, 45% with a moderate, 45% with a high and 33% with a very high intratumoral *P4HA1* mRNA level. OSCC patients with a very high

Table 1
Clinicopathological data of OSCC patients.

Category	Number of cases	P4HA1 mRNA level			
		Low	Moderate	High	Very high
Total	118	30	29	29	30
Gender					
Men	94	19 (63%)	23 (79%)	24 (83%)	28 (93%)
Women	24	11 (37%)	6 (21%)	5 (17%)	2 (7%)
Age (years)					
<60	67	17 (57%)	18 (62%)	17 (57%)	15 (50%)
>60	51	13 (43%)	11 (38%)	12 (41%)	15 (50%)
T-stage					
I	19	6 (20%)	5 (17%)	4 (14%)	4 (13%)
II	35	8 (27%)	10 (35%)	12 (41%)	5 (17%)
III	21	7 (23%)	7 (24%)	3 (10%)	4 (13%)
IV	43	9 (30%)	7 (24%)	10 (35%)	17 (57%)
N-stage					
N0	44	11 (37%)	14 (48%)	*10 (35%)*	9 (30%)
N1-3	74	19 (63%)	15 (52%)	*19 (65%)*	21 (70%)
Grading					
1	12	5 (17%)	2 (7%)	*2 (7%)*	3 (10%)
2	82	19 (63%)	20 (69%)	*24 (83%)*	19 (63%)
3	22	5 (17%)	7 (24%)	3 (10%)	7 (23%)
X	2	1 (3%)	0	0	1 (3%)
Radiation therapy	106				
Yes	82	16 (64%)	21 (81%)	25 (86%)	20 (77%)
No	24	9 (36%)	5 (19%)	4 (14%)	6 (23%)
Median dose (Gy)	54				
Range (Gy)	14–72				
Recurrence until 3 years after diagnosis					
Yes		3 (10%)	7 (24%)	12 (41%)	10 (33%)
No		27 (90%)	22 (76%)	17 (59%)	20 (67%)

Table 2
Survival analysis of OSCC patients.

Category	No. of pts	Overall survival (OS)		Locoregional control (LRC)	
		Univariate analyses	*Multivariate analyses*	*Univariate analyses*	*Multivariate analyses*
		RR (95% CI) p-value	RR (95% CI) p-value	RR (95% CI) p-value	RR (95% CI) p-value
T-stage					
I + II	54	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)
II + IV	64	**2.5 (1.5–4.2) *p* = 0.001**	**2.4 (1.3–4.4) *p* = 0.003**	1.5 (0.8–3.1) *p* = 0.24	1.9 (0.8–4.1) *p* = 0.13
N-stage					
N0	44	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)
N1-3	74	**1.9 (1.1–3.3) *p* = 0.014**	1.3 (0.7–2.4) *p* = 0.37	1.3 (0.6–2.7) *p* = 0.43	1.0 (0.4–2.2) *p* = 0.99
Grading (b)					
1	12	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)
2	82	0.7 (0.3–1.5) *p* = 0.36	0.6 (0.3–1.1) *p* = 0.13	1.0 (0.3–3.2) *p* = 0.93	0.5 (0.2–1.9) *p* = 0.33
3	22	0.7 (0.3–1.6) *p* = 0.37	0.5 (0.2–1.4) *p* = 0.1.9	1.3 (0.3–4.9) *p* = 0.73	0.7 (0.2–2.9) *p* = 0.61
P4HA1 mRNA level					
Low	30	1.0 (reference)	1.0 (reference)	1.0 (reference)	1.0 (reference)
Moderate	29	1.5 (0.7–3.3) *p* = 0.27	1.8 (0.9–4.0) *p* = 0.12	2.6 (0.7–10.0) *p* = 0.17	2.6 (0.7–10.3) *p* = 0.16
High	29	1.6 (0.7–3.3) *p* = 0.24	2.0 (0.9–4.3) *p* = 0.07	**4.9 (1.4–17.4) *p* = 0.014**	**5.8 (1.5–21.3) *p* = 0.008**
Very high	30	1.9 (0.9–4.0) *p* = 0.08	**2.2 (1.0–4.8) *p* = 0.039**	**4.4 (1.2–16.0) *p* = 0.025**	**4.8 (1.2–18.7) *p* = 0.023**

b- information not available for 2 patients.

intratumoral *P4HA1* mRNA level died in median 20 months earlier compared to patients with lower intratumoral *P4HA1* expression. Multivariate Cox's regression hazard analysis revealed an increased risk of earlier death (RR = 2.2; *P* = 0.039) (see Table 2, see Fig. 1) for patients with a very high intratumoral *P4HA1* mRNA level compared to patients with a low intratumoral *P4HA1* mRNA level (see Table 2, see Fig. 1, Supplemental Fig. 1).

Cox's regression analysis also identified a higher intratumoral *P4HA1* mRNA level as associated with a higher risk of locoregional recurrence. The risk of recurrence was calculated as RR = 2.6 (*P* = 0.16) for a moderate, RR = 5.8 (*P* = 0.008) for a high and RR = 4.8 (*P* = 0.023) for a very high intratumoral P4HA1 mRNA expression compared to the control group (low level). (see Fig. 2, Table 2, Supplemental Fig. 3).

Moreover, multivariate Cox's regression hazard analysis of those OSCC patients of the same cohort received a radiotherapy after surgery (*n* = 82; see Table 1) showed an increased risk of earlier death (RR = 3.4; *P* = 0.009) (see Supplemental Fig. 2) for patients with a very high intratumoral *P4HA1* mRNA level compared to patients with a low intratumoral *P4HA1* mRNA level.

Table 3
Bivariate correlations between *P4HA1* level in tumor tissues and clinicopathological parameters of OSCC patients (Kruskal Wallis test) or mRNA level of different biomarkers (Spearman's Rho test) (r_s-correlation coefficient).

	r_s	*P*-value	n
Clinicopathological parameters			
T-stage		0.081	118
Gender		**0.005**	118
N-stage		0.599	118
Grading		0.486	118
EMT-markers			
ZEB 2	0.250	**0.009**	108
Twist	0.406	**0.008**	108
TGFβ	0.548	**<0.001**	108
MMP7	0.216	0.070	71
CTGF	0.341	**0.004**	69
Stem cell marker			
Oct3/4	0.253	**0.033**	71
Snai1	0.360	**0.002**	71
Snai2	0.292	**0.014**	71
LGR4	0.171	0.077	108
LGR5FL	0.308	**0.010**	69
Structure proteins			
CDH	0.011	0.912	108
Vimentin	0.356	**0.002**	71
KRT13	−0.251	**0.039**	71
Hypoxic markers			
CA9	0.063	0.604	71
VEGFa	0.118	0.329	71
Glut1	0.084	0.488	71
HIF1alpha	−0.037	0.765	69
Others			
MDM2	0.391	**0.001**	71
Survivin	0.322	**0.006**	71
HER2	−0.420	**<0.001**	69
EGFR	−0.112	0.36	69
PDL-1	0.427	**<0.001**	105
Osteopontin	0.461	**<0.001**	69

The patients received a radiotherapy after surgery had a risk to have a recurrence calculated as RR = 2.8 (*P* = 0.22) for a moderate, RR = 5.4 (*P* = 0.032) for a high and RR = 10.3 (*P* = 0.005) for a very high intratumoral *P4HA1* mRNA expression compared to the control group (low level) (Supplemental Fig. 2).

Correlation of P4HA1 mRNA level with tumor–specific parameters and other molecular markers of OSCC patients

The *P4HA1* mRNA level narrowly missed the significance threshold when correlated with the T-stage (*P* = 0.081) but correlated with the gender of the patients (*P* = 0.005), whereas no correlation was found between the *P4HA1* mRNA level and the grade of the tumor, or the nodal status using Kruskal-Wallis test (see Table 1).

Using Spearman's rank correlation as a bivariate correlation we found significant associations between the intratumoral *P4HA1* mRNA level and the intratumoral mRNA level of EMT markers like *ZEB2, TWIST, TGFβ* or *CTGF* or stem cell markers like *Oct3/4, Snai1* and *2* and *LGR5FL*. However, no correlations to the intratumoral mRNA level of hypoxic marker like *CA9, VEGFA* and *Glut1* were found (see Table 3). Moreover, Spearman's rank analyses showed significant correlations between the intratumoral *P4HA1* mRNA level and the mRNA level of *vimentin, KRT13, MDM2, survivin, HER2, PDL-1* and *osteopontin* (see Table 3).

mRNA level of P4HA1 in OSCC cell lines cultivated under different oxygen conditions

To validate the hypoxic increase of the *P4HA1* mRNA level *in vitro*, we determined the level of *P4HA1* in three different OSCC -cell lines cultivated 24 h under (I) normoxic (21% oxygen) and (II) hypoxic conditions (0.1% oxygen). As expected, the mRNA level of *P4HA1* was massively increased in those cell lines cultivated under
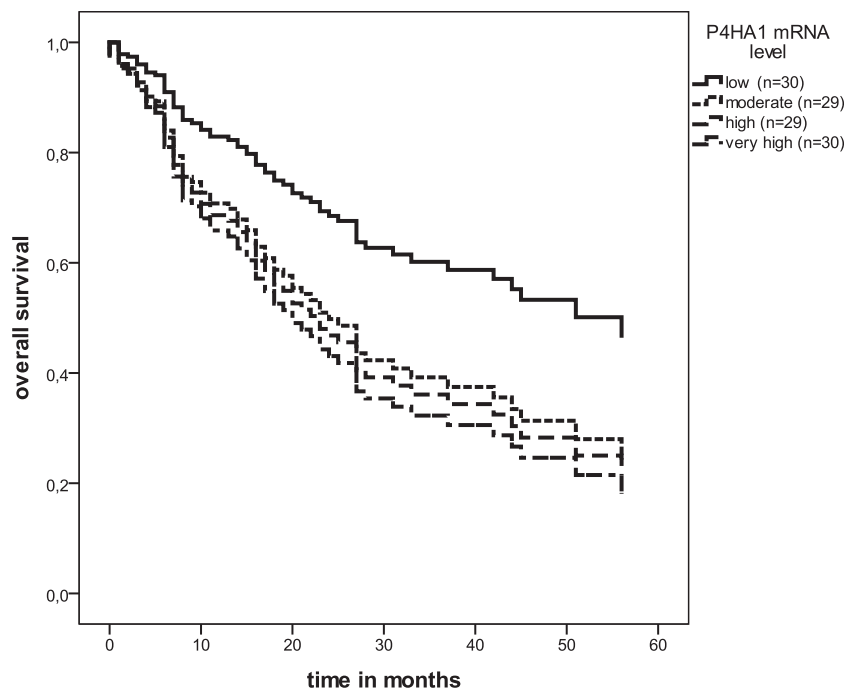

Fig. 1. Multivariate Cox's hazard regression model: association of *P4HA1* mRNA expression level and survival of OSCC patients. The model was adjusted to patients tumor stage, lymph status (N-stage) and grading of the tumor. The OSCC cohort was divided into four groups (quartiles) according to the intratumoral *P4HA1* mRNA level (low, moderate, high, very high). The patients risk of death was calculated as RR = 1.8 (*P* = 0.12) for a moderate, RR = 2.0 (*P* = 0.075) for a high and RR = 2.2 (*P* = 0.039) for a very high intratumoral *P4HA1* mRNA expression compared to the control group (low level).
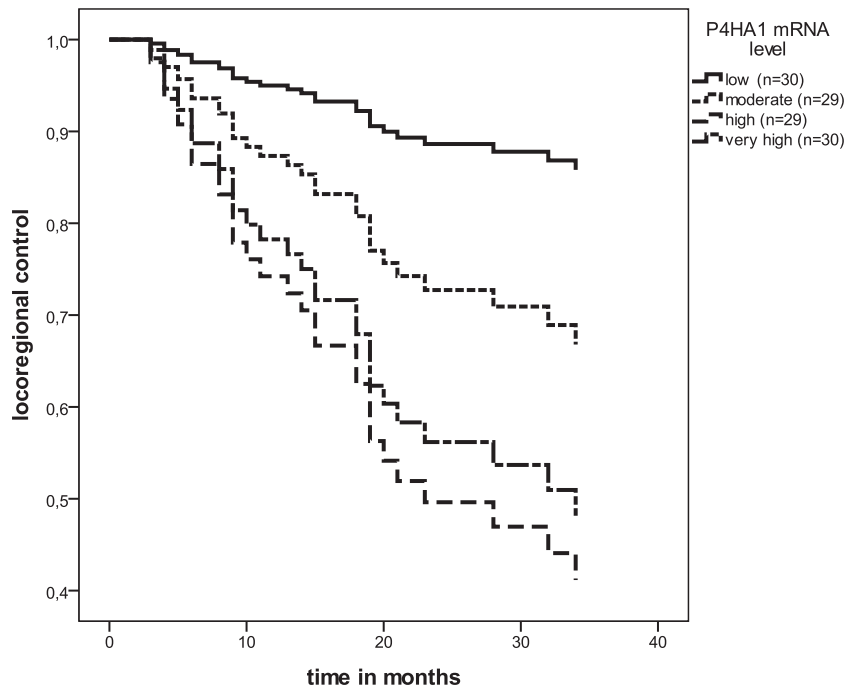
Fig. 2. Multivariate Cox's hazard regression model: association of *P4HA1* mRNA expression level and locoregional recurrence of OSCC patients. The model was adjusted to patients tumor stage, lymph status (N-stage) and grading of the tumor. The OSCC cohort was divided into two groups according to the median intratumoral *P4HA1* mRNA levels. The risk of recurrence was calculated as RR = 2.6 (P = 0.16) for a moderate, RR = 5.8 (P = 0.008) for a high and RR = 4.8 (P = 0.023) for a very high intratumoral *P4HA1* mRNA expression compared to the control group (low level).

hypoxic conditions. In the cell lines CAL-33, XF354 and SAS the *P4HA1* mRNA level was increased due to oxygen deprivation by a factor of 10.9, 9.8 and 4.8, respectively (see Fig. 3).

Discussion

A negative prognostic impact of tumor hypoxia has been described for different tumor entities [21,22], including tumors of the head and neck region. Furthermore, we demonstrated a negative prognostic impact of a higher intratumoral *P4HA1* mRNA level for the overall survival and locoregional control in a cohort of 118 OSCC patients (see Tables 1 and 2; see Figs. 1 and 2). In bivariate correlation analyses the *P4HA1* mRNA level of OSCC samples correlated significantly with the mRNA level of EMT or stem cell markers like *ZEB2, Twist, TGFβ, Oct3/4, Snai1, Snai2* and *LGR5FL*. Our findings indicate an association of P4HA1 with different EMT and stem cell markers. That is remarkable, because other authors believe that *P4HA1* may have a role in the transdifferentiation process of e.g. glioma stem cells into endothelial cells [15].

However, no correlation of the *P4HA1* mRNA level with the mRNA level of hypoxic induced markers like *CA9, VEGFA* and *Glut1 was found* (see Table 3). Hence, we analyzed the mRNA level of the hypoxia-associated marker *P4HA1* in three OSCC cell lines and compared its expression level under hypoxic and normoxic culture conditions (see Fig. 3). This *in vitro* analysis showed an increased mRNA level of *P4HA1* under hypoxic conditions (see Fig. 3). Moreover, we found a hypoxia specific increase of the mRNA level of *CA9, VEGFA* and *Glut1* (data not shown). These data demonstrate that correlations of biomarkers in *in vivo* samples do not necessarily reflect the *in vitro* situation, as it was found for the mRNA level of *P4HA1* and *CA9, VEGFA, Glut1* (*HIF1*-target genes) in the three analysed OSCC cell lines.

In the literature, *P4HA1* has previously been shown to be transcriptionally regulated by HIF1. [10,23,24]. Prolyl 4-hydroxylases are associated with hypoxic processes during osteoarthritis [25].
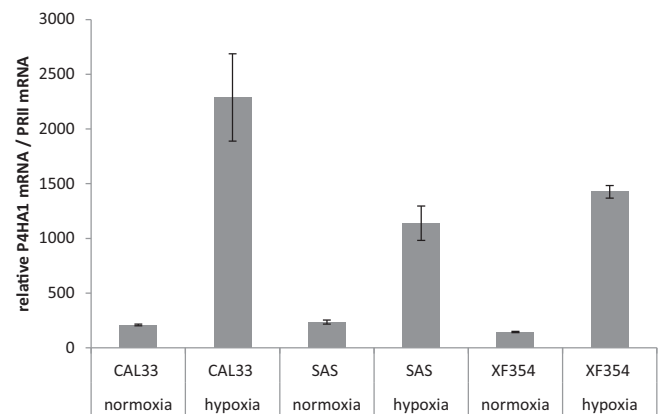

Fig. 3. *P4HA1* mRNA expression level of the OSCC cell lines CAL-33, XF354 and SAS. Cells were cultivated for 24 h under culture conditions of 21% oxygen (normoxia) or 0.1% oxygen (hypoxia). The mRNA level in the cell lines CAL-33, XF354 and SAS were upregulated, when cells were cultivated under hypoxic conditions by the factor of 10.8, 9.8 and 4.8, respectively.

Moreover, the collagen prolyl hydroxylase *P4HA1* was found to be associated with lymphatic and lung metastasis in breast cancer patients [26]. In addition, *P4HA1* is transcriptionally regulated by E2F, which was described for an E2F knock-out mouse model in which knock-out of E2F decreased the level of P4HA1 and led to remodelling of the extracellular matrix and supported the process of metastasis. [27]. In our analysis, *P4HA1* mRNA was not significantly increased in tumor samples of patients with nodal metastasis. The observation that *P4HA1* mRNA and protein levels are stably upregulated via hypoxia suggests *P4HA1* as a clinically useful and prognostic relevant marker to identify hypoxic tumors [12]. A meta-analysis of genome–wide RNA sequencing data identified this hypoxic marker as a unique prognostic marker for hypoxic tumors [9]. Tawk et al. analysed three different hypoxia gene

signatures studies in a set of 302 head and neck tumor patients [6–9]. Our data confirmed the prognostic power of *P4HA1* in an independent cohort of 118 OSCC patients.

The possible cause for that finding might be the transcriptional impact of HIF1 on the level of hypoxic *P4HA1* [10,24]. However, due to the short half-life time of HIF1, indirect measurements of its transcriptional activity such as *P4HA1* may be the ideal surrogate indicators of hypoxic gene expression. Tumor hypoxia is associated with HIF1 activation, metastasis, and resistance to chemotherapy and radiotherapy, as well as poor patient survival [28]. Rankin and Giaccia concluded that HIF1 promotes each step during metastasis including the remodelling of the ECM, which implies the activity of *P4HA1* [28]. HIF1 promotes signalling in the primary tumor which contributes to the expression of secreted factors that are involved in formation of the premetastatic niche [28] which then not only modifies the ECM but also affects the activity of the immune system [3]. These findings are supported by the correlation of the *P4HA1* mRNA level with the mRNA level of stem cell markers or genes associated with EMT, like *ZEB2, Twist, LGR4, LGR5* and *TGFβ* (see Table 3). These results are in accordance with the regulation of multiple steps within the metastatic cascade by HIF1. Although, *ZEB2, Twist, LGR4, LGR5* and *TGFβ* are not HIF1-regulated they are also strongly associated with epithelial mesenchymal transition (EMT), a mechanism essential for metastasis [29–33]. In addition the correlation of the *P4HA1* mRNA level with the transcript levels of *HER2* and *PDL1* could help to stratify OSCC patients for future targeting and/or immunotherapies.

Conclusion

In this study, we showed for the first time that *P4HA1* is an independent prognostic factor for overall survival of OSCC patients and is associated with a higher risk of locoregional recurrence. The data confirm the potential use of *P4HA1* as a single-gene surrogate of multiple-gene hypoxia signatures in head-and-neck cancer, whereby different tumor-specific and hypoxic independent processes like EMT may influence the expression of *P4HA1*, too.

Conflict of interest statement

On behalf of all co-authors, the corresponding author declare, that there is no conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ctro.2017.05.002.

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