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Expression and prognostic significance of YAP, TAZ, TEAD4 and p73 in human laryngeal cancer

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Summary. Objectives. The Hippo signaling pathway plays a critical role in organ size control and tissue homeostasis and its perturbation is associated with tumorigenesis. YAP (Yes associated protein) and TAZ (transcriptional co-activator with PDZ- binding motif) are the major nuclear effectors of the Hippo pathway interacting with TEADs (TEA domain) and p73 transcriptional factors to regulate gene expression. Altered expression of the above proteins promotes tumor initiation, progression and metastasis in a variety of cancer types. This study addresses their expression and prognostic significance in human laryngeal carcinoma. Methods. Protein expression of YAP, TAZ, TEAD4 and p73 was examined by immunohistochemistry in 121 human laryngeal squamous cell carcinomas. Correlations with clinicopathological data and survival were evaluated. Results. All proteins were overexpressed in human laryngeal carcinomas compared to non-neoplastic adjacent epithelium. High expression of YAP, TAZ, TEAD4 and p73 correlated significantly with high grade, advanced stage, supraglottic location of tumor, nodal metastases and recurrence. Furthermore, high expression of all proteins was significantly associated with poor overall and disease- free survival. p73 expression proved to be an independent predictive factor of survival and YAP expression proved to be an independent predictive factor of disease recurrence. Conclusions. Deregulation of the expression of the Hippo pathway proteins is implicated in human laryngeal carcinogenesis and YAP and p73 have prognostic significance in the outcome of the disease.

Key words: Laryngeal cancer, YAP, TAZ, TEAD4, p73

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Introduction

Laryngeal carcinoma is the second most common head and neck cancer. It affects mostly men in the 6th decade of life and the majority of the cases (85-95%) are classified as squamous cell carcinoma. The 5-year survival rate reaches 60% (National Cancer Institute, 2019). The highly invasive and metastatic phenotype of this cancer type is responsible for therapy failure. Exploration of the molecular mechanisms underlying laryngeal carcinogenesis may identify novel biomarkers and develop more effective targeted treatments.

The Hippo pathway is a highly conserved, from Drosophila to mammals, signal transduction pathway that regulates organ size, tissue homeostasis, regeneration and tumorigenesis (Harvey et al., 2013). The core of the Hippo pathway in mammals comprises two pairs of serine-threonine kinases, MST1/2 and LATS1/2, and two transcriptional co-activators YAP (Yes associated protein) and its paralogue TAZ (transcriptional co-activator with PDZ- binding motif) (Piccolo et al., 2014). Because YAP and TAZ lack DNA binding domains they form complexes with various transcriptional factors, including TEAD1-4 (TEA domain family members) (Wu et al., 2008; Zhao et al., 2008) and p73 (Varelas, 2014) among others in order to induce gene expression in the nucleus. When the pathway is active phosphorylation of MST1/2

leads to phosphorylation of LATS1/2 which in turn phosphorylates YAP and TAZ resulting in their cytoplasmic retention and degradation. On the contrary, when the pathway is inactive YAP and TAZ translocate into the nucleus and form complexes with either TEADs that induce the expression of genes regulating tissue growth, repair and differentiation (Moroishi et al., 2015) or p73 that induces apoptosis (Strano et al., 2001). The Hippo pathway is regulated by soluble factors, cellular metabolic status, cell polarity, cell-cell adhesion, mechanotransduction and G-protein-coupled receptor signaling (Meng et al., 2016). Deregulation of Hippo signaling leads to proliferation, migration and survival of cells eventually leading to cancer development (Pan, 2010; Yu et al., 2015). Furthermore the Hippo pathway cross-talks with multiple cancer-associated protein networks including WNT, TGFβ-BMP, Hedgehog (HH), Notch, mTOR, often at the level of YAP and TAZ, an interaction that may lead to tumorigenesis (Ehmer and Sage, 2016).

Elevated expression and nuclear localization of the Hippo pathway components YAP and TAZ have been observed in a wide variety of cancers including laryngeal (Pan et al., 2017; Qiu et al., 2017) as well as breast (Cordenonsi et al., 2011), lung (Zhou et al., 2011), colorectal (Yuen et al., 2013), ovarian (Hall et al., 2010) and prostatic cancer (Zhang et al., 2015). Studies in human tissues have associated high expression of YAP/TAZ with adverse features and poor outcome. In experimental models knockdown of YAP/TAZ impaired tumor growth, while their overexpression led to tumor formation and resistance to treatment (Zanconato et al., 2016). Several studies report TEAD overexpression in different neoplasms and highlighted its prognostic significance (Zhou et al., 2016). p73 overexpression has also been observed in several cancer types including squamous cell carcinomas (DeYoung and Ellisen, 2007). Accumulative evidence therefore highlights the significant role of the Hippo pathway in human cancer and targeting this pathway represents a major opportunity and challenge in the era of targeted cancer therapy (Johnson and Halder, 2014). Considering that there are limited studies of Hippo signaling in laryngeal cancer mainly involving YAP expression and prognostic significance (Pan et al., 2017; Qiu et al., 2017) we aimed to address the expression and prognostic significance of Hippo signaling components in human laryngeal cancer. We therefore evaluated the expression by immunohistochemistry of several key components of the Hippo pathway including YAP, TAZ, TEAD4 and p73 in a series of 121 cases of squamous laryngeal carcinoma and investigated their correlation with clinicopathological parameters and patients' survival.

Material and methods

Patients and samples

We used formalin-fixed paraffin embedded tissue samples obtained from 121 patients with primary invasive squamous cell laryngeal cancer that were diagnosed and treated at the University Hospital of Patras between the years 1994-2010. Clinicopathological data according to the WHO (El-Naggar et al., 2017) and TNM (Sobin et al., 2009) criteria are summarized in Table 1. Immunohistochemistry was performed either on biopsy material conducted for diagnostic purposes in patients with early stage disease (stages I, II) prior to any treatment or on surgical biopsy material from patients with advanced stage disease (stages III, IV) that underwent total laryngectomy. In both cases no treatment (radiotherapy or chemotherapy) was performed prior to the biopsy. Early stage patients were next treated with radiotherapy alone, while advanced stage patients were treated with total laryngectomy (plus neck dissection, radiotherapy or chemotherapy where appropriate). All patients were followed up for at least 5 years. The 5-year overall survival rate was 57.8 % (i.e. 70 patients survived). The study was performed in accordance with the Helsinki Declarations and was approved by the institutional ethical committee.

Antibodies and immunohistochemistry

Immunohistochemistry was performed as previously described (Bravou et al., 2015). In short, 4 μm tissue sections were dewaxed and rehydrated in graded ethanol. For antigen retrieval samples were microwaved in 0,01M citrate buffer (ph=6). Endogenous peroxidase activity was blocked by treatment 3% hydrogen

Table 1. Clinicopathological data of 121 laryngeal SCCs.

Gender n (%)	
Male	119 (98.3)
Female	2 (1.7)
Age, yrs	
Median	65
Range	28-89
Location n (%)	
Glottic	90 (74.4)
Supraglottic	31 (25.6)
Grade n (%)	
I	31 (25.6)
II	74 (61.2)
III	16 (13.2)
Stage n (%)	
Early (Stages I-II)	60 (49.6)
Advanced (Stages III-IV)	61 (50.4)
Neck nodal metastases (N) n (%)	
Absent (N0)	106 (87.6)
Present (N+)	15 (12.4)
Recurrence n(%)	
No	72 (59.5)
Yes	49 (40.5)
Distant metastases	
Absent (M0)	121 (100)
Present (M1)	0 (0)

peroxide, followed by incubation with protein blocking solution (Tris Buffered Saline with 3% of Bovine Serum Albumin, TBS/BSA 3%). Sections were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-nonphosphorylated (active) YAP1 antibody (1A12, Cell Signalling Technology Leiden, The Netherlands 1:300, RRID:AB_2797897), rabbit polyclonal anti-nonphosphorylated (active) TAZ antibody (H-70: sc-48805, Santa Cruz Biotechnology, CA, USA 1:60, RRID:AB_2216639), rabbit polyclonal anti-TEAD4 antibody (PA5-21977 Invitrogen, Rockford, IL, USA 1:75, RRID:AB_11153439) and rabbit monoclonal anti-p73 antibody (EP436Y Abcam, Cambridge, UK 1:100, RRID:AB_776999). Bound primary antibodies were detected with the Envision™ detection kit (DAKO, Hamburg, Germany) and visualized using diaminobenzidine (DAB) as the chromogen. Sections were counterstained with hematoxylin, dehydrated and mounted. Both positive (colorectal carcinomas) and negative controls (by adding the blocking solution TBS/BSA 3% instead of primary antibody) were used.

Immunohistochemical evaluation

All slides were independently evaluated by two expert pathologists blinded to the case (H.P., V.B.). Evaluation of staining was performed in both adjacent normal laryngeal mucosa and carcinoma. Cytoplasmic and nuclear evaluation were evaluated separately. For all proteins immunoreactivity was graded on a scale of 1-3 according to the intensity of staining and percentage of positive cells. Staining intensity was scored as negative (score 0), weak (score 1), moderate (score 2) and strong (score 3). Percentage of positive cells was scored as 0 (staining in less than 1% of tumor cells), 1 (staining in 1-25% of tumor cells), 2 (staining in 26-50% of tumor cells), 3 (staining in 51-75% of tumor cells) and 4 (staining in 76-100% of tumor cells). The two scores were multiplied resulting in values from 0-12 and immunoreactivity score was determined as follows: score 1 (multiplication values 0,1,2) as low expression, score 2 (multiplication values 3,4,6) as medium expression and score 3 (multiplication values 8,9,12) as high expression of the studied proteins.

Statistical analysis

Statistical analysis was performed with the IBM® SPSS® for Windows v.24 (SPSS Inc. Chicago, IL., USA). Correlations of protein expression levels (immunoreactivity scores) with clinicopathological data were analyzed with the chi-square test. Correlations between the expressions of the different proteins were examined with Kendall's tau b coefficient test. Overall survival (OS) was defined as the interval from the date of diagnostic biopsy for early stage patients or total laryngectomy for advanced stage patients to the death from any cause (primary end point), while disease free survival (DFS) was defined as the interval from the date of diagnostic biopsy for early stage patients or total laryngectomy for advanced stage patients to the time of recurrence (secondary end point). Overall and disease-free survival were analyzed using the Kaplan-Meier

Table 2. Expression of YAP in human laryngeal cancer in relation to clinical and pathological parameters.

	YAP Cytoplasmic expression†						p-value ‡	YAP Nuclear expression †						p-value ‡
	1 (Low) n%		2 (Medium) n%		3 (High) n%			1 (Low) n%		2 (Medium) n%		3 (High) n%		
Age														
<median	12	20.0	28	46.7	20	33.3	0.151	25	41.7	16	26.7	19	31.7	0.112
≥median	16	26.2	18	29.5	27	44.3		25	41	8	13.1	28	45.9	
Location														
Glottic	25	27.8	34	37.8	31	34.4	0.081	40	44.4	18	20.0	32	35.6	0.404
Supraglottic	3	9.7	12	38.7	16	51.6		10	32.3	6	19.4	15	48.4	
Grade														
I	12	38.7	10	32.3	9	29.0	0.019	19	61.3	5	16.1	7	22.6	0.001
II	14	18.9	33	44.6	27	36.5		30	40.5	17	23.0	27	36.5	
III	2	12.5	3	18.8	11	68.8		1	6.3	2	12.5	13	81.3	
Stage														
Early	22	36.7	26	43.3	12	20.0	<0.001	36	60.0	11	18.3	13	21.7	<0.001
Advanced	6	9.8	20	32.8	35	57.4		14	23.0	13	21.3	34	55.7	
N														
N0	28	26.4	41	38.7	37	34.9	0.024	46	43.4	22	20.8	38	35.8	0.199
N(+)	0	0.0	5	33.3	10	66.7		4	26.7	2	13.3	9	60.0	
Recurrence														
No	23	31.9	30	41.7	19	26.4	0.001	46	63.9	12	16.7	14	19.4	<0.001
Yes	5	10.2	16	32.7	28	57.1		4	8.2	12	24.5	33	67.3	

† Expression of YAP was scored as described in "Materials and Methods"; ‡Chi-square test: p<0.005 was considered statically significant.

method and differences between subgroups were compared using the Log-Rank test. Cox proportional hazard univariate and multivariate analysis were performed to identify predictors of survival. The significance level was determined as p<0.005.

Results

Increased expression of transcriptional co-activators YAP and TAZ in human laryngeal cancer and association with adverse prognostic factors

Immunoreactivity for non-phosphorylated (active) YAP in adjacent non-neoplastic epithelium was absent or very weak. In carcinomas positive immunohistochemical expression of YAP was found in 119/121 (98.3%) cases. Immunoreactivity for YAP was observed in the cytoplasm and/or the nucleus of cancer cells (Fig. 1). Cytoplasmic expression was found in 118/121(97.5%) cases, whereas nuclear expression was found in 116/121(95.8%) cases. Higher expression of cytoplasmic YAP was associated with advanced stage (p<0.001), poor differentiation (p=0.019), positive lymph nodes (p=0.024) and recurrence (p=0.001). High expression of nuclear YAP was associated with advanced stage (p<0.001), poor differentiation (p=0.001) and recurrence (p<0.001) (Table 2).

Adjacent non-neoplastic laryngeal epithelium demonstrated negative or weak staining for TAZ while positive immunohistochemical expression of TAZ was found in 120/121 (99.1%) cases. Expression of TAZ was cytoplasmic and /or nuclear (Fig. 1). Cytoplasmic expression was found in 120/121(99.1%) cases, whereas nuclear expression was found in 116/121(95.8%) cases. Expression of cytoplasmic TAZ significantly differed among stage and grade groups with advanced stage and high grade tumors showing higher expression of TAZ (p<0.001 and p=0.037 respectively). High cytoplasmic expression of TAZ also correlated significantly with nodal metastases (p=0.013) and recurrence (p=0.013). High nuclear expression of TAZ correlated significantly with advanced stage (p<0.001), high grade (p=0,016) and recurrence (p<0.001) (Table 3).

Increased expression transcriptional factors TEAD4 and p73 in human laryngeal cancer and association with adverse prognostic factors

Epithelial cells of adjacent non-neoplastic tissue showed negative or very weak immunoreactivity for TEAD4 and p73. Positive expression of TEAD4 and p73 in tumor cells was noted in 115/121(95.0%) and 120/121 (99.1%) cases of laryngeal carcinoma respectively. Immunostaining of TEAD4 and p73 was nuclear (Fig. 1). A statistically significant association was found between high TEAD4 expression and supraglottic localization of the tumor (p=0.038), advanced stage (p<0.001), high grade (p=0.010), lymph node metastases (p=0.027) and recurrence (p<0.001). High expression of p73 associated significantly with recurrence (p<0.001) (Table 4).

Table 3. Expression of TAZ in human laryngeal cancer in relation to clinical and pathological parameters.

	TAZ Cytoplasmic expression †						p-value ‡	TAZ Nuclear expression †						p-value ‡
	1 (Low) n%		2 (Medium) n%		3 (High) n%			1 (Low) n%		2 (Medium) n%		3 (High) n%		
Age														
<median	12	20.0	29	48.3	19	31.7	0.581	24	40.0	19	31.7	17	28.3	0.940
≥median	13	21.3	24	39.3	24	39.3		23	37.7	19	31.1	19	31.1	
Location														
Glottic	22	24.4	41	45.6	27	30.0	0.058	37	41.1	30	33.3	23	25.6	0.228
Supraglottic	3	9.7	12	38.7	16	51.6		10	32.3	8	25.8	13	41.9	
Grade														
I	10	32.3	14	45.2	7	22.6	0.037	16	51.6	10	32.3	5	16.1	0.016
II	12	16.2	36	48.6	26	35.1		29	39.2	24	32.4	21	28.4	
III	3	18.8	3	18.8	10	62.5		2	12.5	4	25.0	10	62.5	
Stage														
Early	20	33.3	33	55.0	7	11.7	<0.001	38	63.3	17	28.3	5	8.3	<0.001
Advanced	5	8.2	20	32.8	36	59.0		9	14.8	21	34.4	31	50.8	
N														
N0	25	23.6	48	45.3	33	31.1	0.013	44	41.5	32	30.2	30	28.3	0.276
N(+)	0	0.0	5	33.3	10	66.7		3	20.0	6	40.0	6	40.0	
Recurence														
No	18	25.0	36	50.0	18	25.0	0.013	40	55.6	20	27.8	12	16.7	<0.001
Yes	7	14.3	17	34.7	25	51.0		7	14.3	18	36.7	24	49.0	

†: Expression of TAZ was scored as described in "Materials and Methods"; ‡: Chi-square test: p<0.005 was considered statically significant.

Positive correlation between the expression of YAP, TAZ, TEAD4 and p73 in human laryngeal cancer

There was a significant positive correlation between YAP cytoplasmic expression and its nuclear expression ($p<0.001$, $\tau=0.605$), TAZ cytoplasmic and nuclear expression ($p<0.001$, $\tau=0.742$ and $p<0.001$, $\tau=0.512$ respectively), p73 ($p<0.001$, $\tau=0.365$) and TEAD4 expression ($p<0.001$, $\tau=0.407$). YAP nuclear expression correlated also significantly and positively with TAZ

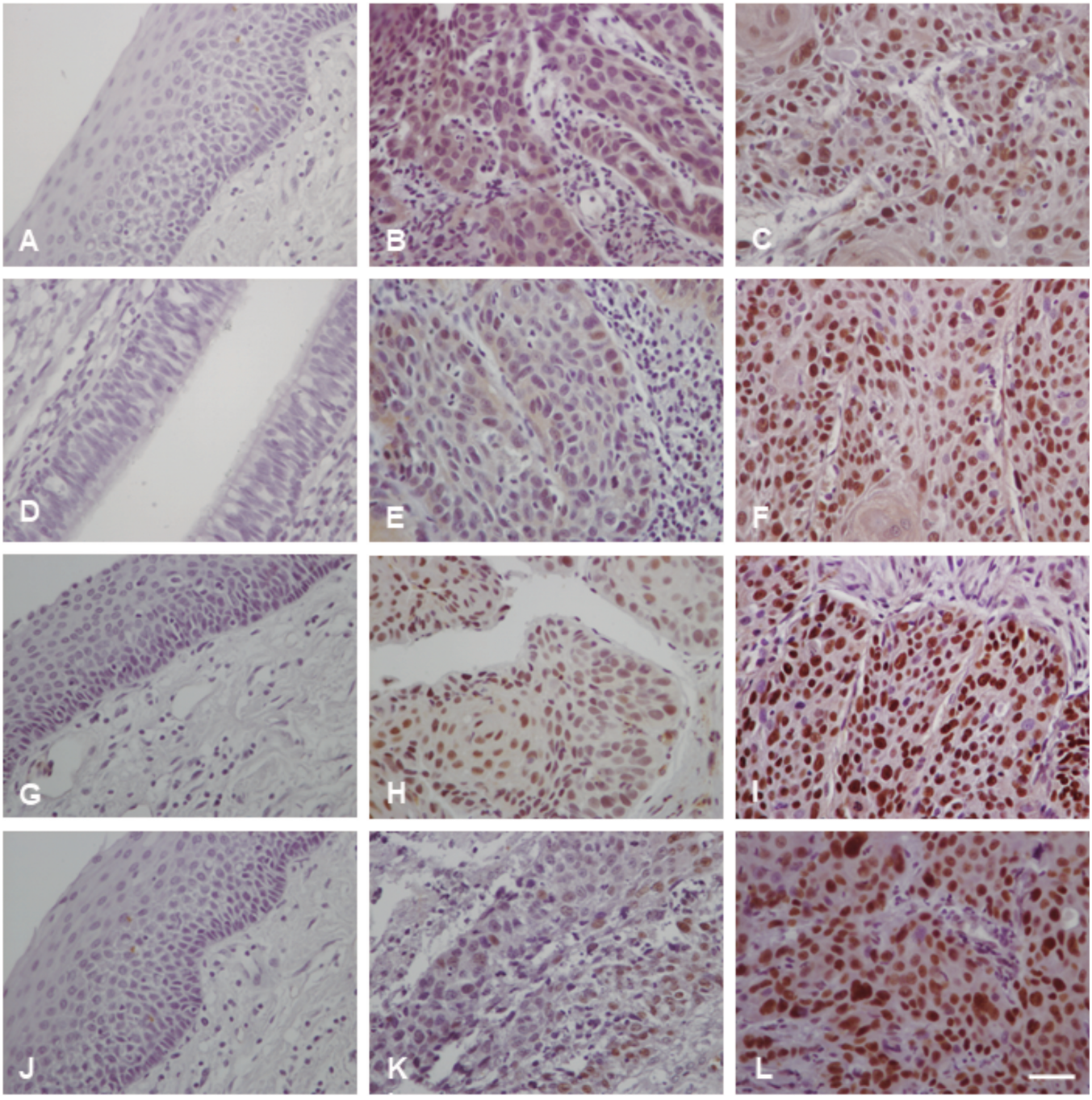

Fig. 1. Immunohistochemical expression of Hippo pathway in human laryngeal cancer. Left column shows negative YAP **(A)**, TAZ **(D)**, TEAD4 **(G)** and p73 **(J)** expression in adjacent non neoplastic epithelium. Representative cases of early stage laryngeal carcinoma (middle column) showing weak expression of YAP **(B)**, TAZ **(E)** TEAD4 **(H)** and p73 **(K)**. Right column shows representative cases of advanced laryngeal cancer showing strong (mainly nuclear) expression of YAP **(C)**, TAZ **(F)**, TEAD4 **(I)** and p73 **(L)**. Scale bar: 50 μm.

cytoplasmic and nuclear expression (p<0.001, τ=0.468 and p<0.001, τ=0.779 respectively), p73 (p<0.001, τ=0.568) and TEAD4 expression (p<0.001, τ=0.627). The cytoplasmic expression of TAZ correlated significantly and positively with its nuclear expression (p<0.001, τ=0.572), p73 (p<0.001, τ=0.374) and TEAD4 expression (P<0.001, τ=0.478), whereas the nuclear expression of TAZ correlated significantly and positively with the expression of p73 (p<0.001, τ=0.511) and TEAD4 (p<0.001, τ=0.562). Finally expression of p73 correlated significantly and positively with the expression of TEAD4 (p<0.001, τ=0.530).

Overexpression of YAP, TAZ, TEAD4 and p73 are associated with poor survival in human laryngeal cancer and the expression of p73 and YAP are independent prognostic factors

Kaplan Meier survival analysis showed a statistically significant association of higher YAP expression with reduced overall (53.3 for low vs 52.1 for medium vs 32.7 for high and Log-rank p<0.001 for cytoplasmic expression; 55.4 for low vs 47.5 for medium vs 32.2 for high and Log-rank p<0.001 for nuclear expression) (Fig. 2) and disease free survival (51.8 for low vs 46.6 for medium vs 29.1 for high and Log-rank p<0.001 for cytoplasmic expression; 57.3 for low vs 38.3 for medium vs 24.0 for high and Log-rank p<0.001 for nuclear expression) (Fig. 3).

There was also a significant association of higher TAZ expression with reduced overall (54.3 for low vs 49.9 for medium vs 33.1 for high and Log-rank p<0.001 for cytoplasmic expression; 56.3 for low vs 45.6 for medium vs 29.1 for high and Log-rank p<0.001 for nuclear expression) (Fig. 2) and disease free survival (47.4 for low vs 46.7 for medium vs 31.0 for high and Log-rank p= 0.001 for cytoplasmic expression; 54.3 for low vs 39.2 for medium vs 24.6 for high and Log-rank p<0.001 for nuclear expression) (Fig. 3).

In addition high nuclear expression of TEAD4 and p73 correlated significantly with poor overall (57.4 for low vs 48.0 for medium vs 32.5 for high expression and Log-rank p<0.001 for TEAD4; 57.0 for low vs 47.0 for medium vs 34.7 for high expression and Log-rank p<0.001 for p73) (Fig. 2) and disease free survival (56.7 for low vs 45.4 for medium vs 25.2 for high and Log-rank p<0.001 for TEAD4 expression; 53.2 for low vs 47.1 for medium vs 27.0 for high and Log-rank p<0.001 for p73 expression) (Fig. 3).

Univariate analysis showed that advanced stage, high grade, supraglottic location of the tumor, nodal metastases, recurrence and high expression of YAP, TAZ, TEAD4 and p73 are significant predictors of overall and disease free survival (Table 5). Furthermore in multivariate analysis advanced stage, recurrence and high expression of p73 were proven to be independent prognostic factors of poor overall survival, whereas the presence of lymph node metastases and medium and high nuclear expression of YAP were proven to be independent prognostic factors for disease free survival (Table 6).

Table 4. Expression of TEAD4 and p73 in human laryngeal cancer in relation to clinical and pathological parameters.

	TEAD4 expression †						p- value ‡	p73 expression †						p-value ‡
	1 (Low) n%		2 (Medium) n%		3 (High) n%			1 (Low) n%		2 (Medium) n%		3 (High) n%		
Age														
<median	18	30.0	22	36.7	20	33.3	0.645	18	30.0	22	36.7	20	33.3	0.297
>=median	14	23.0	23	37.7	24	39.3		11	18.0	25	41.0	25	41.0	
Location														
Glottic	25	27.8	38	42.2	27	30.0	0.038	20	22.2	38	42.2	32	35.6	0.421
Supraglottic	7	22.6	7	22.6	17	54.8		9	29.0	9	29.0	13	41.9	
Grade														
I	14	45.2	9	29.0	8	25.8	0.010	9	29.0	12	38.7	10	32.3	0.251
II	18	24.3	30	40.5	26	35.1		18	24.3	31	41.9	25	33.8	
III	0	0.0	6	37.5	10	62.5		2	12.5	4	25.0	10	62.5	
Stage														
Early	22	36.7	27	45.0	11	18.3	<0.001	18	30.0	24	40.0	18	30.0	0.174
Advanced	10	16.4	18	29.5	33	54.1		11	18.0	23	37.7	27	44.3	
N														
N0	29	27.4	43	40.6	34	32.1	0.027	25	23.6	45	42.5	36	34.0	0.070
N(+)	3	20.0	2	13.3	10	66.7		4	26.7	2	13.3	9	60.0	
Recurrence														
No	29	40.3	30	41.7	13	18.1	<0.001	23	31.9	33	45.8	16	22.2	<0.001
Yes	3	6.1	15	30.6	31	63.3		6	12.2	14	28.6	29	59.2	

†: Expression of TEAD4 and p73 were scored as described in "Materials and Methods"; ‡: Chi-square test: p<0.005 was considered statically significant.

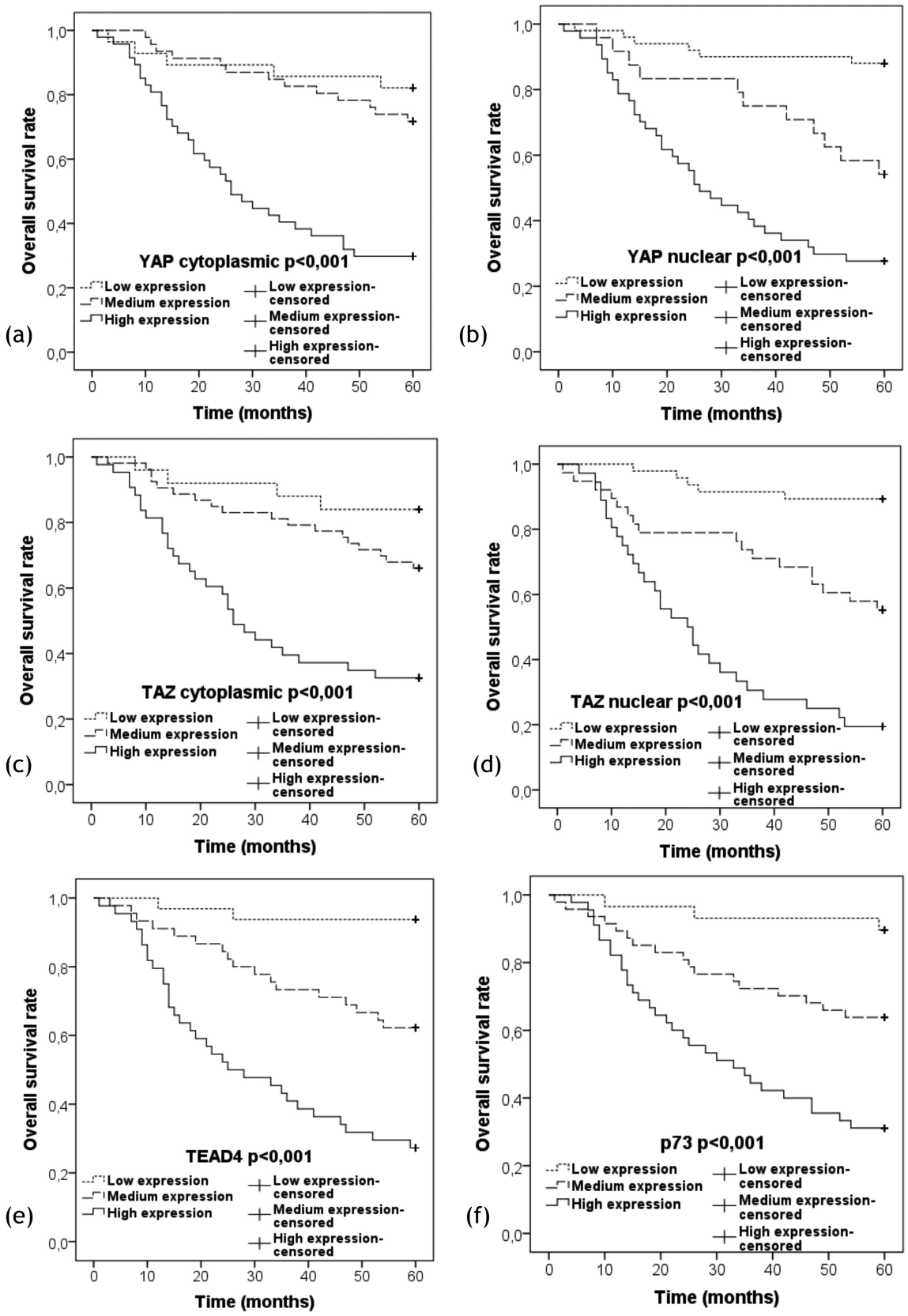

Fig. 2. Kaplan-Meier survival plots. Overall survival estimates according to the expression of YAP **(a, b)**, TAZ **(c, d)**, TEAD4 **(e)** and p73 **(f)**. Log-rank test; p values<0,05 are considered statistically significant.

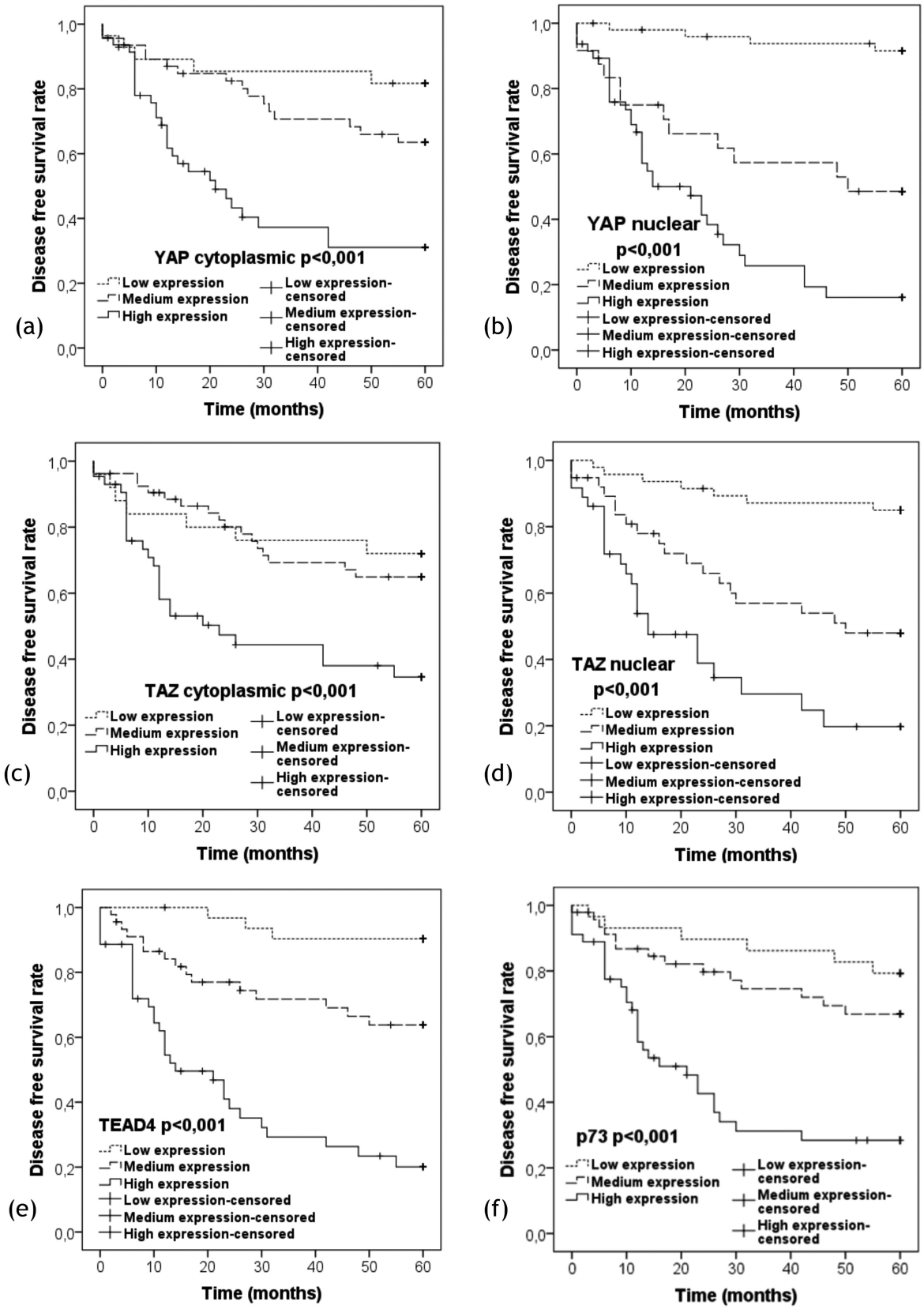

Fig. 3. Kaplan-Meier survival plots. Disease free survival estimates according to the expression of YAP **(a, b)**, TAZ **(c, d)**, TEAD4 **(e)** and p73 **(f)**. Log-rank test; p values<0,05 are considered statistically significant.

Discussion

Understanding the role of Hippo signaling in the molecular biology of laryngeal cancer may prove of clinical importance. In the present study we demonstrate overexpression of the Hippo pathway molecules YAP, TAZ, TEAD4 and p73 in human laryngeal cancer, their association with an aggressive phenotype and reduced survival and we further show that high expression of YAP and p73 are independent prognostic factors of poor survival.

We showed that YAP and TAZ are overexpressed in human laryngeal cancer. YAP and TAZ immunohistochemical expression was both cytoplasmic and nuclear in our samples. The nuclear expression is concomitant with their role in activating transcription

Table 5. Univariate Cox regression analysis for overall and disease free survival. HR: hazards ratio; CI: confidence interval. p values<0.005 are considered statistically significant.

		Univariate analysis					
		Overall survival			Disease Free Survival		
		HR	95%CI	p-value	HR	95%CI	p-value
Age	≥65vs /<65	1.433	0.823-2.494	0.203	0.962	0.549-1.684	0.891
Location	Supraglottic/Glottic	2.041	1.148-3.628	0.015	2.414	1.357-4.294	0.003
Grade				0.001			0.003
	II/I	1.484	0.705-3.127	0.299	1.835	0.843-3.993	0.126
	III/I	4.684	1.961-11.184	0.001	4.801	1.878-12.271	0.001
Stage	Advanced/early	4.738	2.472-9.081	<0.001	2.212	1.234-3.962	0.008
N	Yes/no	4.190	2.215-7.927	<0.001	3.430	1.742-6.751	<0.001
Recurrence	Yes/no	4.956	2.695-9.112	<0.001			
YAP cytoplasmic				<0.001			<0.001
	Med/low	1.605	0.572-4.503	0.368	2.067	0.757-5.646	0.157
	High/low	6.152	2.390-15.835	<0.001	5.644	2.158-14.762	<0.001
YAP nuclear				<0.001			<0.001
	Med/low	4.400	1.626-11.905	0.004	8.745	2.817-27.150	<0.001
	High/low	10.051	4.194-24.088	<0.001	19.665	6.843-56.516	<0.001
TAZ cytoplasmic				<0.001			0.002
	Med/low	2.287	0.774-6.757	0.135	1.204	0.499-2.904	0.680
	High/low	6.610	2.316-18.869	<0.001	3.173	1.364-7.381	0.007
TAZ nuclear				<0.001			<0.001
	Med/low	5.149	1.899-13.964	0.001	4.451	1.855-10.680	0.001
	High/low	14.362	5.512-37.418	<0.001	9.843	4.158-23.303	<0.001
TEAD4				<0.001			<0.001
	Med/low	7.109	1.642-30.778	0.009	4.605	1.332-15.919	0.016
	High/low	19.854	4.742-83.125	<0.001	15.417	4.673-50.860	<0.001
p73				<0.001			<0.001
	Med/low	4.175	1.223-14.251	0.023	1.753	0.673-4.565	0.250
	High/low	10.472	3.191-34.366	<0.001	5.721	2.350-13.929	<0.001

Table 6. Multivariate Cox regression analysis for overall and disease free survival.

		Multivariate analysis							
		Overall Survival					Disease Free Survival		
		HR	95%CI	p-value			HR	95%CI	p-value
Reccurence	Yes/No	2.375	1.234-4.574	0.01	N	Yes/no	2.762	1.392-5.480	0.004
Stage	Advanced/early	3.991	2.014-7.916	<0.001					
p73				0.030	YAP	nuclear			<0.001
	Med/low	3.349	0.949-11.826	0.060		Med/low	8.794	2.832-27.302	<0.001
	High/low	5.294	1.521-18.429	0.009		High/low	18.487	6.412-53.304	<0.001

HR, hazards ratio; CI, confidence interval. p values<0.005 are considered statistically significant. Only parameters that showed a significant difference in univariate analysis were included in the multivariate analysis.

factors and gene expression (Moroishi et al., 2015). It is known that phosphorylation of YAP and TAZ by activated LATS regulates their nuclear-cytoplasmic shuttling by anchoring them to 14-3-3 protein and/or promoting their degradation in the cytoplasm (Zhao et al., 2010; Liu et al., 2010). Another explanation for the cytoplasmic accumulation of YAP/TAZ could be excessive protein synthesis, due to for example genomic amplification, which constantly generates cytoplasmic proteins that then translocate to the nucleus. Other possibilities could be ineffective protein turnover because of altered stability of YAP/TAZ or interaction with Wnt signaling (Steinhardt et al., 2008; Barry et al., 2013). This abundant expression of non-phosphorylated i.e. active form of YAP/TAZ represents the inactivation of the tumor-suppressive Hippo pathway in laryngeal cancer.

Importantly we demonstrated that overexpression of YAP and TAZ in human laryngeal cancer associated with adverse factors, such as advanced stage, high grade and lymph node metastases, suggesting that YAP and TAZ overexpression may contribute to an aggressive tumor phenotype. Further supporting an important role of YAP and TAZ in human laryngeal carcinogenesis we showed that both YAP and TAZ associated with poor overall and disease free survival and YAP nuclear expression proved to be an independent prognostic factor of disease free survival. In agreement with our findings, upregulation of YAP and TAZ has been shown to induce proliferation, resistance to apoptosis, EMT and acquisition of stem cell properties leading to tumor initiation, progression and metastasis (Harvey et al., 2013; Moroishi et al. 2015). YAP and TAZ overexpression has been previously reported in many solid tumors including breast, lung and colorectal cancer and in many cases associated with tumor progression, poor outcome and chemoresistance (Wang et al., 2010; Cordenonsi et al., 2011; Zhou et al., 2011; Yuen et al., 2013; Wang et al., 2013; Piccolo et al., 2014; Bartucci et al., 2015; Lee et al., 2015; Hsu et al., 2016; Zanconato et al., 2016). In the field of head and neck cancer YAP and TAZ correlated with recurrence, resistance to radiotherapy and poor prognosis in oral cancer (Li et al., 2015) while their knockdown impaired tumor growth and metastasis (Hiemer et al., 2015). While little is known regarding TAZ expression in laryngeal cancer, in accordance with our findings, YAP has been reported to be overexpressed in human laryngeal cancer and to correlate with tumor progression parameters and poor survival (Pan et al., 2017; Qiu et al., 2017). Also, knockout of YAP in human laryngeal cancer cells was previously shown to inhibit growth and metastasis suggesting that YAP may serve as a promising therapeutic target (Tang et al., 2019). Based on the above, our findings support the implication of YAP and TAZ in tumor progression of human laryngeal cancer and highlight their prognostic significance in the disease. In this context it would be interesting to study expression of YAP and TAZ in patients with distant metastases. Nevertheless, suppression of YAP/TAZ activity could represent an anticancer therapeutic strategy (Johnson and Halder, 2014).

We also showed overexpression of TEAD4 in our cohort. TEADs are transcription factors activated in response to the Hippo signaling pathway and regulate organ growth and proliferation (Pan, 2010). TEAD genes have been shown to be overexpressed in many cancer types (Zhou et al., 2016) and their oncogenic role is mediated by regulation of multiple genes implicated in cancer development, including CTGF, Cyr61, AXL, Myc and survivin (Holden and Cunningham, 2018). While overexpression of TEADs has been reported in gastric, colorectal, breast (Zhou et al., 2016) and oral cancer (Takeuchi et al., 2017) this is the first report, to the best of our knowledge, implicating TEAD4 overexpression in laryngeal carcinogenesis.

Noteworthy, high expression of TEAD4 in our tumors correlated significantly with supraglottic localization of the tumor, advanced stage, high grade, recurrence and poor survival, suggesting that TEAD4 contributes to an aggressive tumor phenotype and poor prognosis in human laryngeal cancer. In agreement, TEAD4 has been reported to serve as a prognostic indicator in gastric (Lim et al., 2014), breast (Wang et al., 2015), colorectal (Liu et al., 2016) and head and neck squamous cell carcinoma (HNSCC) (Zhang et al., 2018).

Nuclear localization of TEAD4 was observed in our series of laryngeal tumors which is consistent with its role in the regulation of gene transcription. Additionally TEAD4 expression correlated with YAP and TAZ expression, reflecting the functional interaction between these factors in the context of Hippo pathway signaling in human laryngeal cancer. In line with these findings, in order to induce gene expression, TEADs require interaction with transcription co-activators, with YAP/TAZ being the most extensively studied (Yu et al., 2015). Nuclear colocalization of TEAD/YAP has been evidenced from Chip-seq experiments (Zanconato et al., 2015) and a positive correlation between YAP and TEADs expression has been also reported in ovarian cancer (Xia et al., 2014). Furthermore, disruption of TEAD/YAP complex diminishes the oncogenic activity of YAP (Liu-Chittenden et al., 2012) and TEAD knockout suppresses TAZ mediated oncogenic transformation in breast cancer cells (Chan et al., 2009). In this aspect pharmacologic inhibition of the TEAD-YAP/TAZ interaction could be a promising therapeutic strategy for cancer treatment.

Finally, we demonstrated that p73 is overexpressed in our cohort of tumors. The nuclear localization of p73 in our samples is in accordance with its role in the regulation of gene transcription. Moreover high expression of p73 is associated with recurrence and reduced survival suggesting a tumor promoting role in human laryngeal cancer progression. p73 is a transcription factor that belongs to the p53-family which includes p53 itself and also p63. In the context of Hippo signaling, p73 inducts apoptosis in response to DNA damage, chemotherapy and other stimuli suppressing tumor formation (Rufini et al., 2011). However, p73 may

also contribute to tumor progression since elevated expression has been observed in various tumors. Indeed p73 is expressed in either tumor suppressive or oncogenic isoforms (Rufini et al., 2011). Furthermore functional inactivation of p73 can occur due to promoter methylation, increased ratio of oncogenic/tumor suppressive isoforms, interaction with a subset of mutant p53 proteins and inhibition by p63 (DeYoung and Ellisen, 2007). Additionally p63 and p73 can suppress transactivation by p53 and promote tumor formation in the absence of p53 mutation (Rocco and Ellisen, 2006). In this way deregulation of p73 function may be of importance in the pathogenesis of various human tumors.

In support to our findings p73 elevated expression bas been observed in a number of malignant neoplasms including HNSCC (Tannapfel et al., 1999; Uramoto et al., 2004; Concin et al., 2004; Dominguez et al., 2006; Faridoni-Laurens et al., 2008) and studies in other tumors, including breast (Dominguez et al., 2006), colon (Sun, 2002; Dominguez et al., 2006), lung carcinoma (Uramoto et al., 2004) and hepatocellular carcinomas (Tannapfel et al., 1999) showed association of p73 expression with adverse factors and poor prognosis. Making things complicated p73 expression fluctuated in subsets of HNSCC from high to low expression (Ehsanian et al., 2010) and high expression associated with radiosensitivity and better survival in cervical cancer (Liu et al., 2004). In HNSCC despite the observed elevation of p73, p73-dependent apoptosis was inhibited due to the parallel elevation of p63 (Rocco et al., 2006). In our study a significant positive correlation was found between YAP, TAZ, TEAD4 and p73, suggesting a possible synergistic role of the examined proteins in laryngeal cancer. Despite the conflicting data in the literature, based on our findings it could be argued that p73 exerts a tumor promoting role in human laryngeal cancer and represents a poor prognostic indicator of the disease.

To summarize, this study examined the expression of multiple key molecules of the Hippo pathway by immunohistochemistry in human laryngeal cancer. We showed overexpression of YAP, TAZ, TEAD4 and p73 in our samples, we demonstrated a positive correlation between them suggesting a functional cooperation in the context of the Hippo pathway and verified the prognostic significance of YAP and p73 in laryngeal cancer. These findings may be of clinical importance since growing evidence of the importance of Hippo signaling components in cancer could establish their clinical application not only as biomarkers of poor outcome but also as potential therapeutic targets (Moroishi et al., 2015). In this context knockout of YAP in human laryngeal cancer cells has been shown to suppress tumor growth both in vitro and in vivo rendering YAP a promising therapeutic target (Tang et al., 2019). It should be noted that there are limited studies of Hippo signaling in laryngeal cancer mainly involving YAP expression and prognostic significance (Pan et al., 2017; Qiu et al., 2017). In contrast to previous reports our study provides a more comprehensive view of Hippo signaling in laryngeal cancer, since not only YAP but also TAZ and their associated transcriptional factors TEAD4 and p73 were examined in a large series of patients, thus representing a correlative study of multiple partners of the Hippo pathway in the disease. However, although immunohistochemistry is an established tool for biomarker detection we should take into account limitations related to reaction and interpretation bias. For example in our study although proper fixation, antibody selection, negative and positive controls were performed and experimental conditions were optimized, potential differences in the immunoreactivity of small biopsies versus laryngeal surgical specimens should be considered. Also our patient cohort was not homogenous in terms of treatment (radiotherapy alone or total laryngectomy alone or total laryngectomy plus chemotherapy etc.) and this factor may have influenced results regarding patient prognosis. For these reasons, further studies in larger patient series, more homogenous cohorts, as well as in vitro and in vivo functional studies are required to better understand the complex role of the Hippo pathway in human laryngeal cancer. Exploration of the interaction of Hippo pathway components with other tumor pathways would be also of particular interest.

In conclusion, overexpression of YAP, TAZ, TEAD4 and p73 correlates with an aggressive phenotype and reduced survival in laryngeal squamous cell carcinoma highlighting the tumor promoting role and prognostic significance of the Hippo pathway in the disease. These findings render the Hippo pathway a promising therapeutic target in human laryngeal cancer.

Conflict of interest. The authors declare no conflict of interest.

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