Hippo-YAP signaling activation and cross talk with PI3K in oral cancer: A retrospective cohort study

Running title: Hippo-YAP and PI3K in oral cancer

Juan P. Rodrigo [1, 2, 3, 4] †

Tania Rodríguez-Santamarta [1, 5] †

Daniela Corte [6]

Vanessa García-de-la-Fuente [1]

Nerea Rodríguez-Torres [5]

Paloma Lequerica-Fernández [1, 7]

Corina Lorz [4, 8, 9]

Juana M García-Pedrero [1, 2, 4]

Juan C. de Vicente [1, 3, 5]

[1] Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo. Oviedo, Spain.

[2] Department of Otolaryngology, Hospital Universitario Central de Asturias (HUCA). Oviedo, Spain.

[3] Department of Surgery, University of Oviedo, Oviedo, Spain

[4] Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Instituto de Salud Carlos III Madrid, Spain

[5] Department of Oral and Maxillofacial Surgery, Hospital Universitario Central de Asturias (HUCA). Oviedo, Spain.

[6] Tumor Biobank Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), Hospital Universitario Central de Asturias (HUCA), Oviedo, Spain.

[7] Department of Biochemistry, Hospital Universitario Central de Asturias (HUCA), Oviedo, Spain.

[8] Molecular Oncology Unit, CIEMAT, Madrid, Spain.

[9] Research Institute 12 de Octubre i+12, University Hospital 12 de Octubre, Madrid Spain

† These authors contributed equally to this work

*** Corresponding author:** Juan Carlos de Vicente. Department of Oral and Maxillofacial Surgery, Hospital Universitario Central de Asturias, School of Medicine. c/ Catedrático José Serrano s/n. 33006. Oviedo. Spain. Tel.: +34-85-103638. E-mail: jvicente@uniovi.es ORCID: 0000-0002-0991-9465

*** Co-corresponding author:** Juana María García Pedrero, Hospital Universitario Central de Asturias, Instituto de Investigación Sanitaria del Principado de Asturias, Edificio FINBA Lab ORL – IUOPA, Avda. Roma s/n 33011 Oviedo, Spain. Tel: +34 985 107937 E-mail: juanagp.finba@gmail.com ORCID: 0000-0002-5891-9488

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Abstract

Objectives: This study aimed to investigate the clinical and prognostic relevance of the Hippo-YAP transactivators YAP1 and TAZ in oral squamous cell carcinoma, and their possible relationship with PI3K/mTOR pathway activation.

Materials and Methods: Immunohistochemical analysis of YAP1, TAZ, PIK3CA (p110α), p-AKT (Ser473) and p-S6 (Ser235) was performed in paraffin-embedded tissue specimens from 165 OSCC patients. Correlations between protein expression and clinical data were further assessed.

Results: YAP1 expression was detected in both cytoplasm and nucleus of tumor cells, whereas TAZ expression was only found in the nucleus. Nuclear YAP1 was significantly associated with tumor size (*p*=.03), neck lymph node metastasis (*p*=.02), TNM stage (*p*=.02), and poor differentiation (*p*=.04). Nuclear TAZ was associated with tobacco (*p*=.03) and alcohol consumption (*p*=.04), and poor tumor differentiation (p=.04). There was a positive significant correlation between nuclear and cytoplasmic YAP1, nuclear TAZ, p110α expression, and mTORC1 activation pS6 (S235). Combined expression of nuclear and cytoplasmic YAP1 was prognostic in both univariate and multivariate analyses. Active nuclear YAP1 was significantly and independently associated with poor disease-specific (*p*=.005, HR = 2.520; 95%CI = 1.319 – 4.816) and overall survival (*p*=.015, HR = 2.126; 95%CI = 1.155 – 3.916).

Conclusion: Nuclear YAP1 is an independent predictor of poor survival in oral squamous cell carcinoma.

Keywords

Hippo-YAP pathway, YAP1, TAZ, PIK3CA, oral squamous cell carcinoma, prognosis.

1 INTRODUCTION

Oral squamous cell carcinoma (OSCC) arises from the mucosal epithelium of the oral cavity. It affects more than 350,000 people each year worldwide (Siegel et al., 2021). The major risk factors for OSCC development include smoking, alcohol abuse, inherited genetic traits such as Fanconi anemia, and infection with oncogenic viruses (Marur & Forastiere, 2016). Tumor size, regional and distant metastasis, depth of invasion, and positive surgical margins remain the most important prognostic factors for OSCC. However, other factors such as stromal patterns, especially desmoplastic reactions, have recently gained attention as indicators of malignant potential in cancer (Amano et al., 2022). Five-year survival rates are approximately 50–60% (Bray et al., 2018), figures that have not improved in the past three decades due to locoregional tumor recurrence, distant metastasis, and treatment resistance (Huang et al., 2019). The first-line therapy for OSCC is surgery, typically combined with adjuvant radiotherapy or even chemotherapy in advanced disease. Unsatisfactory treatment outcomes have led to the search for personalized approaches focused on the establishment of genetic biomarkers for progression prediction and to identify tumors that may respond poorly to therapy (Makarov & Gorlin, 2019). Whole-exome sequencing analyses of head and neck squamous cell carcinoma (HNSCC) showed that this disease is extremely heterogeneous, and there is no single genetic alteration or unique dysregulated molecular pathway responsible for its development or progression (Giudice & Squarize, 2013). Mounting evidence has established that the Hippo pathway is a major regulator in organ size control, stem cell homeostasis, and tumorigenesis in mammals (Zhao et al., 2011). Besides, it has also been involved in the tumor metastatic cascade (Dupont et al., 2011). Amplification of the 11q22 chromosomal region, which includes the Yes-associated protein 1 (YAP1) gene, the main downstream effector of the Hippo pathway, is detected in 5-15% of OSCC (Ono et al., 2019). TAZ, also known as WWTR1 (WW-domain containing transcriptional regulator 1), is a paralog of YAP1. Both share 46% amino acid sequence identity (Liu et al., 2021) and have overlapping, but not completely redundant, transcriptional targets (Plouffe et al., 2018). The Hippo pathway consists of a core kinase cascade in which MST1/2 (mammalian STE20-like protein kinase) and the cofactor SAV1 (human Salvador homology 1) phosphorylate the kinases LATS1/2 (large tumor suppressor 1/2) and the adaptor protein MOB1 (MOB kinase activator) (Yu et al., 2015). The LATS/MOB complex later phosphorylates the downstream effectors YAP1 and TAZ. When the Hippo pathway is activated, YAP1/TAZ becomes phosphorylated and remains inactively located in the cytoplasm through binding 14-3-3 proteins, and are lately degraded in the proteasome after ubiquitination (Wang et al., 2021). In contrast, inactivated Hippo pathway results in an

unphosphorylated YAP1/TAZ that is translocated to the cell nucleus where it functions as co-transcriptional coactivator. YAP1/TAZ forms complexes with a range of transcription factors, most notably the TEAD family and Smads (Zhao et al., 2011; Vassilev et al., 2001), inducing the expression of genes that contribute to the establishment of a pro-tumorigenic phenotype (Santos-de-Frutos et al. 2019). Dysregulated YAP1/TAZ activity has been implicated in a variety of cancers, including HNSCCs (Shin & Kim, 2020). In OSCC, in a similar fashion to other tumors, YAP1 oncogene has been reported to be critical for tumor initiation, sustaining tumor growth (Chen et al., 2017), inhibition of apoptosis, and induction of epithelial-mesenchymal transition (EMT) (Ge et al., 2011; Zhao et al., 2007). Furthermore, high YAP1 activity has been associated with poor prognosis (Hiemer et al., 2015).

PI3K-AKT-mTOR pathway relies on three main driving molecules: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), protein kinase B (AKT), and mammalian target of rapamycin (mTOR) (Starzyńska et al., 2020). The p110α catalytic subunit of PI3K is encoded by *PIK3CA* gene located at 3q26.32 locus. Genomic alterations of *PIK3CA* gene, including mutation, amplification and/or overexpression, are detected in approximately 55% of HNSCC patients (García-Escudero et al., 2018). *PIK3CA* activating mutations have been identified up to 20% of cases, and gene amplification ranges between 9% and 50% of OSCC patients (Starzyńska et al., 2020; Cancer Genome Atlas Network, 2015). *PIK3CA* gene overexpression has been found to be a poor prognosis factor in HNSCC, and associated with YAP activation (García-Escudero et al., 2018). On the other hand, YAP1 and TAZ have been proposed as poor prognosis markers and, at least in OSCC, YAP1 has a more prominent transcriptional role than TAZ (Santos-de-Frutos et al., 2019).

In this study, we sought to investigate the clinical significance of YAP1 and TAZ protein expression pattern in a large cohort of 165 OSCC patients and their possible relationship with p110α expression and PI3K/mTOR pathway activation.

2 MATERIALS AND METHODS

2.1 Patients and tissue specimens

Surgical tissue specimens from 165 patients with histologically confirmed OSCC who underwent surgical treatment with curative purposes at the Hospital Universitario Central de Asturias between 1st March 2000 and 31st December 2010 were retrospectively collected, in accordance with approved institutional review board guidelines. All experimental procedures were

conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Hospital Universitario Central de Asturias and by the Regional Ethics Committee from Principado de Asturias (date of approval 14 May 2019; approval number 136/19, for project PI19/01255). Due to the retrospective nature of the study the need for written informed consent from patients was waived. Inclusion criteria in the study were: (i) diagnosis of OSCC, and (ii) radical resection of the primary tumor with simultaneous neck lymph node dissection. Disease stage was defined in line with the 8th edition of the AJCC Cancer Staging Manual (Lydiatt et al., 2017).

Clinicopathological data were collected from medical records, as summarized in Table 1. Tissue specimens were provided by the Principado de Asturias BioBank (PT17/0015/0023), integrated in the Spanish National Biobanks Network, and representative tissue areas from the 165 OSCC tumors were obtained from archival paraffin-embedded blocks to construct tissue microarrays (TMAs), as previously described (de Vicente et al., 2017). Three morphological representative areas were selected from each tumor paraffin block, avoiding necrotic areas. Tumor grading was classified according to the World Health Organization (WHO) 2017 criteria (El-Naggar et al., 2017). Surgical specimens were processed routinely, included in paraffin and finally stained with haematoxylin and eosin. The number of lymph nodes removed during the different types of neck dissection ranged between 12 and 47, with an average of 20.5 lymph nodes. All metastatic nodes were examined in search of extranodal extension (ENE), which is histopathologically defined as an extension of metastatic carcinoma from within a lymph node, through the node capsule, and into the surrounding connective tissue, regardless of the stromal reaction (Arun et al., 2021).

2.2 Immunohistochemistry (IHC)

The TMAs were cut into 3-µm sections and dried on Flex IHC microscope slides (DakoCytomation, Glostrup, Denmark). The sections were deparaffinized with standard xylene and hydrated through graded alcohols into water. Antigen retrieval was performed by heating the sections with Envision Flex Target Retrieval solution, either high pH (Dako) or low pH (for YAP1). Staining was done at room temperature on an automatic staining workstation (Dako Autostainer Plus, Dako) with rabbit polyclonal YAP1 antibody (Invitrogen # PA1-46189) at 1:500 dilution, mouse monoclonal anti-TAZ antibody (CL0371, Abcam # ab242313) at 1:100 dilution, rabbit monoclonal PI3 Kinase p110α antibody (C73F8, Cell Signaling # 4249) at 1:100 dilution, rabbit monoclonal anti-Human phospho-Akt (Ser473) Phosphorylation Site Specific (Clone 14-5; Dako # M3628) at 1:20 dilution, or rabbit anti-phospho-S6 Ribosomal Protein (Ser235/236) (Cell Signaling # 2211) at 1:200 dilution, using the Dako EnVision Flex + Visualization System (Dako

Autostainer) and diaminobenzidine chromogen as substrate. Negative controls were prepared by omitting the primary antibody. Counterstaining with hematoxylin was the final step.

The IHC results were independently evaluated by two observers (JPR and JMG-P), blinded to clinical data. Nuclear YAP1 immunostaining was scored from 0 to 2, with a score of zero if 0-10% of tumor cells were stained, 1 if 11-50% of tumor cells were stained, and 2 if >50% stained cells. The staining intensity was scored from 0 to 2 (0 = negative, 1 = weak, 2 = strong). The raw data were then converted to an Immunoreactive Score (IRS) by multiplying the quantity and staining intensity scores. Theoretically, the scores could range from 0 to 4. Nuclear TAZ staining was evaluated as a percentage of stained cells and dichotomized as negative expression (0-10% stained cells) versus positive expression (>10% stained tumor cells). For cytoplasmic YAP1, p110α, and p-AKT (Ser473) expression, a semiquantitative scoring system based on staining intensity was applied, and divided into three categories: negative (absence of staining, score 0), weak to moderate (some cytoplasmic staining in tumor areas, score 1), and strong protein expression (intense and homogeneous cytoplasmic staining in tumor areas, score 2). For p-S6 (Ser235) immunostaining, the percentage of tumor cells positively stained was scored from 0 to 3 (0% = 0, 1-10% = 1, 11-50% = 2, and >50% = 3), as previously described (de Vicente et al., 2017). For the analyses of combined expression of cytoplasmic and nuclear YAP1, nuclear staining IRS=4 and cytoplasmic staining score=2 were used as cut-off points for high nuclear YAP1 and high cytoplasmic YAP1, respectively.

2.3 Statistical analysis

Statistical analysis was performed using IBM SPSS for Windows (version 27.0.1, IBM-SPSS Inc., USA). Bivariate analysis by χ^2 and Fisher's exact tests were used for comparison between protein immunohistochemical evaluation and clinicopathological categorical variables, and the Spearman correlation test was used to measure the relationship between two ranked variables. The endpoints of the survival analysis were overall survival (OS) and disease-specific survival (DSS). OS was measured as the time interval from the initial treatment to the time of death from any cause or the last date of observation, and DSS was determined from the date of treatment completion to death of the tumor. DSS and OS were estimated using the Kaplan-Meier method and compared with the log-rank test. Hazard ratios (HR) with their 95% confidence intervals (CI) for clinicopathological variables were calculated using univariate and multivariate Cox proportional hazards model. Independent variables (covariates) that were statistically significant in the univariate analysis were included in the multivariate analysis, and introduced in the model using the forward stepwise method. All tests were two-sided and *p* values less than 0.05 were considered statistically significant.

3. RESULTS

3.1 Immunohistochemical analysis of YAP1, TAZ, and PI3K/mTOR pathway components in OSCC tissue specimens

The expression of YAP1, TAZ, and three key components of the PI3K/mTOR pathway, i.e. PI3K p110α catalytic subunit, p-AKT (Ser473), and p-S6 (Ser235) was jointly evaluated by immunohistochemistry in 165 OSCC tissue specimens. YAP1 expression was detected in both the cytoplasm and the nucleus of tumor cells. Cytoplasmic YAP1 expression was observed in 149 (90%) OSCC samples (1 case was not evaluable): 88 (53.3%) tumors showed weak cytoplasmic staining (score 1), and 61 tumors strong cytoplasmic staining (score 2). Nuclear YAP1 staining (IRS) was detected in 123 (74%) OSCC samples: 47 cases (29.1%) showed a score of 1, 30 (18.2%) a score of 2, and 5 (27.3%) a score of 4. TAZ protein exclusively exhibited a nuclear expression pattern in cancer cells, which was detected in 42 (25%) tumor samples (4 cases were not evaluable).

p110α expression was detected in 93 (56.3%) tumor samples (6 cases were not evaluable): 74 cases (44.8%) with a score of 1 and 19 (11.5%) a score of 2. p-AKT (Ser473) immunostaining was only found in two (1.2%) out of 165 OSCC samples (6 cases were not evaluable). By contrast, p-S6 (Ser235), which is a surrogate marker of mTORC1 activation, was detected in 106 (69%) tumors: 33 cases (20%) scored as 1, 48 cases (29.1%) scored as 2 and 25 cases (15.2%) scored as 3. Figure 1 shows representative examples of YAP1, TAZ, p110α, p-AKT (Ser473), and p-S6 (Ser235) immunostaining in OSCCs.

There was a strong positive correlation between the expression of cytoplasmic and nuclear YAP1, and also between the expression of nuclear YAP1 and nuclear TAZ (Table 2). Regarding the relationship between the activation of Hippo-YAP and PI3K pathway, we found that both cytoplasmic and nuclear YAP1 were significantly correlated with the expression of PI3K p110α catalytic subunit (Table 2). There was also a significant correlation between the surrogate marker of mTORC1 activation p-S6 (Ser235), p-AKT (Ser473), and cytoplasmic YAP1 expression (a tendency with nuclear YAP1), but not with TAZ expression (Table 3).

3.2 Associations between protein expression, clinicopathological variables and patient prognosis

Cytoplasmic YAP1 expression was significantly correlated with the presence of perineural (Fisher's exact test, p = 0.02) and vascular invasion (Fisher's exact test, p = 0.004) (Table 3). Nuclear YAP1 (IRS) was dichotomized into two groups: IRS scores of 0-3 versus a score of 4. Thus, high nuclear YAP1 expression (IRS=4) was found to significantly associate with pT classification (χ^2 test, p = 0.03), the presence of neck node metastasis (χ^2 test, p = 0.02; Odds ratio = 2.6), advanced disease stage (Fisher's exact test, p = 0.02), and with tumors moderate or poorly differentiated (χ^2 test, p = 0.04) (Table 3). Nuclear TAZ expression was significantly associated with both tobacco and alcohol consumption as well as with moderately or poorly differentiated tumors (χ^2 test, p = 0.03, 0.04, and 0.04, respectively) (Table 3). No significant relationships were observed between p110α or p-AKT (Ser473) with any of the clinicopathologic parameters studied (Table 4). In marked contrast, positive expression of p-S6 (Ser235) was inversely associated with pT classification (χ^2 test, p = 0.06), perineural and vascular invasion (Fisher test, p = 0.03 and 0.03, respectively), and clinical status (Fisher's exact test, p = 0.01).

At the time of the last follow-up (range: 1 to 221 months), 67 (40.6%) deaths occurred. The mean and median follow-up times were 62.58 (SD: 53.60), and 59.0 months, respectively. The 5- and 10-year disease-specific survival rates were 61% and 53%, respectively, and the mean survival time was 132.73 months (95% CI: 116.49 to 148.96 months). In the survival analyses, tumor size and local extension (T1-2 vs 3-4), neck node status (N0 vs N+), and disease stage were significantly correlated to survival (p < 0.0005; HR = 3.9, HR = 3.8; HR = 4.3, respectively). In univariate Kaplan-Meier analysis, patients harboring positive p-S6 (Ser235) exhibited a significantly better disease-specific survival (DSS) (Log-rank test, p = 0.02, HR = 0.561) and overall survival (OS) (Log-rank test, p = 0.008, HR = 0.558) (Figure 2). None of the other proteins studied showed significant associations with survival.

We further investigate the contribution of YAP1 activation on OSCC prognosis by analyzing the impact of combined expression of cytoplasmic and nuclear YAP1 on patient survival. Thus, cytoplasmic and nuclear YAP1 expression scores were combined into three categories, as follows: 1) inactive YAP1: negative/low nuclear staining (IRS<4) and any cytoplasmic staining (scores 0-2); 2) partially active YAP1: high nuclear staining (IRS=4) and high cytoplasmic staining (score 2); and 3) fully active YAP1: high nuclear staining (IRS=4) and negative/low cytoplasmic staining (scores 0 and 1). As shown in Figure 3, fully active YAP1 was significantly and consistently associated with a worse DSS (Log-rank test, p = 0.023, HR = 2.27) and worse OS (Log-rank test, p = 0.036, HR = 1.84). There were no differences on HR and survival rates between patients harboring partially active and inactive YAP1 (Table 5).

Multivariate Cox analysis including the clinical stage, perineural and vascular invasion, treatment, status of surgical margin, YAP1 activation, and p-S6 expression further showed that disease stage, perineural invasion, vascular invasion, treatment, and YAP1 activation were significant independent predictors of poor OS (p = 0.03, 0.01, 0.03, 0.002 and 0.01, respectively) and stage, perineural invasion, and treatment were predictors of poor DSS (*p* = 0.006, 0.005 and 0.003, respectively) (Table 6).

4 DISCUSSION

The Hippo pathway acts through a kinase cascade to inhibit YAP1 and TAZ activity (Wang et al., 2021). The present study was focused on the expression analysis of YAP1 and TAZ in a large homogeneous OSCC patient cohort, based on the hypothesis that the YAP1/TAZ complex was able to promote an aggressive behavior in OSCC. In support of this hypothesis we found a significant relationship between nuclear YAP1 expression and various well-known prognostic factors in OSCC, such as pT classification, neck lymph node metastasis, disease stage, and histological grade of differentiation. This is consistent with previous reports (Qiu et al., 2017) which suggest that YAP1 may contribute to tumor progression by favoring the acquisition of motility and invasive abilities by tumor cells and therefore more aggressive phenotypes. In addition, YAP1 expression seems to be predominant in OSCC compared to TAZ expression, which is less frequent and showed no clinical relevance. Multiple signaling events restrict YAP1/TAZ to the nucleus, the best characterized of which are signals mediated by the Hippo pathway (Hiemer et al., 2015), but also mechanical cues that affect cytoskeletal dynamics, cell shape, and extracellular matrix stiffness control YAP1/TAZ localization (Dupont et al., 2011; Hasegawa et al., 2021). Experimental studies have shown that in dense cell populations with maintained contacts among cells, YAP1 and TAZ are located in the cytoplasm and remained inactive, whereas in the sparse cell populations YAP1 and TAZ are located in the nucleus and trigger proliferation (Dupont et al., 2011). Thus, YAP1 subcellular localization either in the cytoplasm or in the nucleus could respond to both extra- and intracellular clues. Cytoplasmic YAP1 has been associated with a better prognosis in OSCC (Szelachowska et al., 2019). We did not find a significant correlation between cytoplasmic YAP1 and improved survival; however, cytoplasmic YAP1 expression was significantly correlated with positive p-S6 (Ser235), which was actually significantly associated with a better prognosis in our OSCC cohort. In line with previous findings (García-Escudero et al., 2018), we found a strong correlation between the expression of cytoplasmic and nuclear YAP1, nuclear TAZ and PI3K p110α catalytic subunit.

YAP1 overexpression has been shown to support EMT (Shao et al. 2014), which suggests that hyperactivated YAP1 could be involved in neck lymph node metastasis (Ge et al. 2011), as we found here with an odds ratio of 2.6. Consequently, we could therefore infer that nuclear YAP1 may play a role in the local and regional invasiveness of OSCC. In corcondance with our findings, Liu et al. (Liu et al. 2021) reported that the expression of YAP1 and TAZ proteins was closely related to TNM stage and lymph node metastasis, and the expression of both proteins was positively correlated. Previous studies showed that TAZ overexpression was significantly associated with high histological grade in breast cancer, malignant glioma, and tongue squamous cell carcinoma (Wei et al., 2013). This is similar to what we found here; however, we did not find a significant relationship between TAZ expression and tumor size or other potentially relevant clinicopathological variables. Interestingly, we found that TAZ expression was most frequently found in cases of smoking or alcohol consumption, which suggests that the Hippo pathway can be one way by which tobacco and alcohol demonstrate their well-known oncogenic potential.

Analysis of the correlation between YAP1 and TAZ revealed that their expression was positively correlated, but at least in OSCC, YAP1 has a more prominent transcriptional role than TAZ (Santos-de-Frutos et al., 2019). Novel cancer therapies based on the molecular mechanisms underlying OSCC tumorigenesis are awaited (Hasegawa et al., 2021). YAP1 therapies could be a promising treatment choice for YAP1-sustained cancers; however, YAP1 has been shown to be a key gene in normal tissue development, homeostasis, and tissue regeneration after damage (Yu et al., 2015), and consequently, these therapies might cause side effects in the normal tissues. Based on breast cancer studies (Sorrentino et al., 2015), it has been suggested that statins (that repress TAZ expression) may have beneficial effects for oral cancer patients (Li et al., 2015).

Hiemer et al. (2015) performed a hierarchical clustering analysis of the YAP1/TAZ-induced genes in OSCC, and found two different clusters. One cluster was enriched in genes critical for cell cycle progression, likely explaining the pro-proliferation roles of YAP1/TAZ. A second cluster was enriched in genes that respond to cancer-related signaling pathways, such as those regulated by TGF-β and Wnt growth factors. Amplification or mutation of *PIK3CA* gene is one of the most common oncogenic drivers in HNSCC (Cancer Genome Atlas Network, 2015), and although the interaction between PI3K signaling pathway and YAP1/TAZ in HNSCC has not been clearly demonstrated, activated PIK3CA was shown to promote YAP1 activity by inactivating Hippo signaling (Shin et al., 2020). Based on this, we sought a potential correlation between these two pathways, and found that both cytoplasmic and nuclear YAP1 were

concordantly strongly correlated with the p110α/PIK3CA expression, although neither of them showed prognostic relevance in our series. However, importantly, combined expression of nuclear and cytoplasmic YAP1 was prognostic in both univariate and multivariate analyses. Remarkably, patients harboring fully active nuclear YAP1 (i.e. high nuclear YAP1 and low cytoplasmic YAP1) significantly and consistently exhibited poor disease-specific and overall survival. In this study, TAZ staining was only detected in the nucleus but not the cytoplasm, and to a much lower extent and at a lower frequency than YAP1. This could be partly attributable to a lower stability of TAZ compared to YAP1, since it has been reported that TAZ has a higher tendency to be degraded than YAP1 (Liu et al., 2010; Huang et al., 2012; Reggiani et al., 2021).

Our findings strongly support a tight link between YAP1/TAZ activation and PI3K/mTOR pathway. In agreement with this, García-Escudero et al. (2018) found that in tumors overexpressing *PIK3CA* oncogene there was a decrease in the phosphorylated or inactive form of YAP1, and a concomitant activation of YAP1-target gene expression. Moreover, a target of PI3K is the phosphoinositide-3-dependent protein kinase (PDK1), and YAP1 is activated via PI3K/PDK1 (Fan et al., 2013). Despite the fact that the oncogenic role of PIK3CA has been extensively and widely demonstrated in multiple cancers and p110α expression was frequently detected in our OSCC cohort, consistent with other studies (Starzyńska et al., 2020), no associations of p110α protein with clinicopathological variables and OSCC patient prognosis were observed. Nevertheless, we found a positive significant correlation between the two markers of PI3K activation assessed, p-AKT (Ser473) and p-S6 (Ser235). The limited number of positive p-AKT cases detected in our OSCC series prevents us from drawing conclusions regarding this marker. It is however noteworthy that positive p-S6 (Ser235), a downstream effector of PI3K/mTOR pathway and a surrogate marker of mTORC1 activation, was detected in 69% of OSCC samples and was strongly and consistently associated with a good prognosis (both DSS and OS). Similarly, the immunohistochemical analysis of different components of the PI3K/AKT/mTOR pathway in over 400 HNSCC patients also revealed p-S6 (Ser235) expression as a biomarker of good prognosis and inverse predictor of lymph node and distant metastasis (García-Carracedo et al., 2016). Furthermore, we specifically found a strong positive correlation between p-S6 (Ser235) and cytoplasmic YAP1, which is the inactive form, but not with the active nuclear forms of YAP1/TAZ.

This study has the following limitations: i) its retrospective nature may be prone to lead time and ascertainment biases, ii) tissue microarrays were used by selecting three small tumor

areas from paraffin blocks but not the entire tumor, iii) protein expression may vary across the entire tumor (intratumoral protein heterogeneity) (Bartels et al., 2018).

In summary, we have demonstrated that nuclear YAP1 and TAZ are closely associated with each other. Moreover, nuclear YAP1 expression shows a higher prevalence than TAZ in OSCC, and it presents clinical and biological relevance related to tumor aggressiveness and advanced disease. More importantly, nuclear YAP1 activation emerges as an independent predictor of poor patient survival. Our findings also support a tight link between Hippo-YAP signaling and PI3K/mTOR pathway, specifically evidencing strong correlations between YAP1 and p110α/PIK3CA protein expression, and between p-S6 (Ser235) and inactive YAP1.

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TABLE 1. Clinical and pathological characteristics of the cohort of 165 OSCC patients selected for study.

Variable	Number (%)
Age (year) (mean ±SD; median; range)	63.80 ± 12.65; 64; 30 - 92
Gender	
Men	113 (68.5)
Women	52 (31.5)
Tobacco use	
Smoker	107 (65)
Non-smoker	58 (35)
Alcohol use	
Drinker	89 (54)
Non-drinker	76 (46)
Location of oral squamous cell carcinoma	
Tongue	75 (45)
Floor of the mouth	34 (21)
Other sites within the oral cavity	56 (34)
Tumor status	
pT1	42 (25)
pT2	72 (44)
pT3	24 (14)
pT4	19 (11)
Unknown	8 (6)
Nodal status	
pN0	95 (58)
pN+	63 (38)
No neck dissection	7 (4)
Clinical stage	
Stage I	37 (23)
Stage II	51 (31)
Stage III	30 (18)
Stage IV	47 (28)
Treatment	
Surgery	83 (50)
Surgery + radiotherapy	65 (39)
Surgery + radiotherapy + chemotherapy	17 (11)
Status of resection margin	
Negative	
Positive	133 (80)

	32 (20)
Pathological grading	
Well differentiated	105 (64)
Moderately differentiated	51 (31)
Poorly differentiated	9 (5)
Perineural invasion	
No	156 (95)
Yes	9 (5)
Vascular invasion	
No	158 (96)
Yes	7 (4)
Clinical status at the end of the follow-up	
Alive and without recurrence	77 (47)
Dead of index cancer	67 (40)
Lost or died of other causes (censored)	18 (11)
Second primary carcinoma	3 (2)

TABLE 2. Spearman correlation among the expression of YAP1, TAZ, p110α, p-AKT and p-S6 proteins.

	Nuclear YAP1	**Nuclear TAZ**	**p-AKT (Ser473)**	**p110α**	**p-S6 (Ser235)**
Cytoplasmic YAP1	rho = 0.414 *p* < 0.0005	rho = 0.05 *p* = 0.49	rho = 0.04 *p* = 0.62	rho = 0.25 *p* = 0.001	rho = 0.226 *p* = 0.005
Nuclear TAZ	rho = 0.162 *p* = 0.04		rho = 0.06 *p* = 0.45	rho = 0.135 *p* = 0.09	rho = 0.069 *p* = 0.40
p-AKT (Ser473)	rho = 0.06 *p* = 0.39			rho = 0.126 *p* = 0.11	rho = 0.176 *p* = 0.03
p110α	rho = 0.237 *p* = 0.003				rho = 0.087 *p* = 0.29
p-S6 (Ser235)	rho = 0.143 *p* = 0.07				

TABLE 3. Associations between YAP1 and TAZ expression and clinicopathological variables in the cohort of 165 OSCC patients.

Variable	No cases	Cytoplasmic YAP1 (%)			*p*	Nuclear YAP1 (%)		*p*	Nuclear TAZ (%)		*p*
		0	1	2		IRS (0-3)	4		0	1	
Gender											
Men	113	13 (11)	62 (55)	38 (34)	0.18	81 (72)	32 (28)	0.70	80 (73)	30 (27)	0.66
Women	52	2 (4)	26 (51)	23 (45)		38 (75)	13 (25)		38 (76)	12 (24)	
Tobacco use											
Smoker	107	10 (9)	58 (54)	39 (37)	0.96	78 (73)	29 (27)	0.89	71 (68)	33 (32)	**0.03**
Non-smoker	58	5 (9)	30 (53)	22 (38)		41 (72)	16 (28)		47 (84)	9 (16)	
Alcohol use											
Drinker	89	9 (10)	48 (54)	32 (36)	0.86	63 (71)	26 (29)	0.57	59 (68)	28 (32)	**0.04**
Non-drinker	76	6 (8)	40 (53)	29 (39)		56 (75)	19 (25)		59 (81)	14 (19)	
pT											
pT1	42	3 (7)	26 (65)	11 (28)	0.47	35 (88)	5 (12)	**0.03**	33 (85)	6 (15)	0.10
pT2	72	5 (7)	39 (54)	28 (39)		51 (71)	21 (29)		50 (71)	20 (29)	
pT3	24	3 (12)	11 (46)	10 (42)		16 (67)	8 (33)		16 (70)	7 (30)	
pT4	19	3 (16)	7 (37)	9 (47)		10 (53)	9 (47)		11 (58)	8 (42)	
pN											
pN0	95	8 (9)	54 (57)	32 (34)	0.52	76 (81)	18 (19)	**0.02**	70 (75)	23 (25)	0.22

pN+	63	7 (11) 29 (46) 27 (43)		39 (62) 24 (38)		42 (69) 19 (31)		
Clinical stage								
I	37	2 (6) 22 (63) 11 (31)	0.59	32 (91) 3 (9)	**0.02**	29 (85) 5 (15)	0.59	
II	51	3 (6) 28 (55) 20 (39)		35 (69) 16 (31)		36 (72) 14 (28)		
III	30	4 (13) 17 (57) 9 (30)		22 (73) 8 (27)		20 (71) 8 (29)		
IV	47	6 (12) 20 (44) 20 (44)		29 (61) 18 (39)		32 (70) 14 (30)		
Pathological grading								
Well differentiated	105	10 (10) 54 (52) 40 (38)	0.83	81 (78) 23 (22)	**0.04**	80 (79) 21 (21)	**0.04**	
Moderately or poorly differentiated	60	5 (8) 34 (57) 21 (35)		38 (63) 22 (37)		38 (64) 21 (36)		
Tumor location								
Tongue	75	5 (7) 38 (51) 31 (42)	0.41	57 (77) 17 (23)	0.24	53 (73) 20 (27)	0.76	
Other sites	90	10 (11) 50 (56) 30 (33)		62 (69) 28 (31)		65 (75) 22 (25)		
Tumor location								
Floor of the mouth	34	3 (9) 20 (59) 11 (32)	0.84	26 (76) 8 (24)	0.56	25 (76) 8 (24)	0.76	
Other sites	131	12 (9) 68 (52) 50 (39)		93 (71) 37 (29)		93 (73) 34 (27)		
Perineural invasion								
No	156	13 (8) 88 (56) 56 (36)	**0.02**	114 (73) 41 (26)	0.26	111 (73) 40 (26)	0.99	
Yes	9	3 (33) 2 (22) 4 (44)		5 (56) 4 (44)		7 (78) 2 (22)		
Vascular invasion								
No	158	13 (8) 88 (56) 56 (36)	**0.004**	115 (73) 42 (27)	0.39	112 (73) 41 (27)	0.67	

Yes	7	2 (29)	0 (0)	5 (71)		4 (57)	3 (43)	6 (87)	1 (14)	
Clinical status at the end of the follow-up										
Alive without recurrence	77	5 (7)	40 (53)	31 (41)	0.15	56 (74)	20 (26)	0.17	58 (80)	15 (20)	0.38
Dead of index cancer										
Censored	67	7 (10)	35 (52)	25 (37)		44 (66)	23 (34)		47 (70)	20 (30)	
Second primary	18	1 (6)	12 (67)	5 (28)		16 (89)	2 (11)		11 (65)	6 (35)	
cancer	3	2 (67)	1 (33)	0 (0)		3 (100)	0 (0)		2 (67)	1 (33)	

TABLE 4. Associations between the expression of p110α, p-AKT (Ser 473), p-S6 (Ser235) and the clinicopathological variables in the cohort of 165 OSCC patients.

Variable	No cases	p110α (%)			*p*	p-AKT (Ser473) (%)		*p*	p-S6 (Ser235) (%)		*p*
		0	1	2		0	1		0	1-3	
Gender											
Men	113	46 (43)	50 (46)	12 (11)	0.86	106 (98)	2 (2)	1.0	37 (35)	69 (65)	0.26
Women	52	20 (39)	24 (47)	7 (14)		51 (100)	0 (0)		13 (26)	37 (74)	
Tobacco use											
Smoker	107	40 (40)	51 (50)	10 (10)	0.34	100 (99)	1 (1)	1.0	34 (33)	68 (67)	0.63
Non-smoker	58	26 (45)	23 (40)	9 (15)		57 (98)	1 (2)		16 (29)	38 (71)	
Alcohol use											
Drinker	89	35 (41)	42 (49)	9 (9)	0.74	86 (100)	0 (0)	0.20	30 (35)	55 (65)	0.34
Non-drinker	76	31 (42)	32 (44)	10 (14)		71 (97)	2 (3)		20 (28)	51 (72)	
pT											
pT1	42	15 (37)	18 (45)	7 (17)	0.31	39 (97)	1 (3)	1.0	14 (36)	25 (64)	0.06
pT2	72	24 (34)	39 (56)	7 (10)		69 (99)	1 (1)		16 (24)	52 (76)	
pT3	24	12 (57)	8 (38)	1 (5)		21 (100)	0 (0)		7 (30)	16 (70)	
pT4	19	10 (53)	6 (31)	3 (16)		19 (100)	0 (0)		10 (56)	8 (44)	
pN											
pN0	95	37 (40)	44 (48)	11 (12)	0.95	91 (99)	1 (1)	1.0	24 (27)	66 (73)	0.22

pN+	63	26 (43) 26 (43) 8 (13)		59 (98) 1 (2)		24 (41) 35 (59)		
Clinical stage								
I	37	13 (37) 18 (51) 4 (11)	0.44	33 (94) 2 (6)	0.1	10 (29) 24 (71)	0.14	
II	51	16 (32) 26 (52) 8 (16)		50 (100) 0 (0)		11 (22) 38 (78)		
III	30	12 (44) 13 (48) 2 (8)		27 (100) 0 (0)		9 (32) 19 (68)		
IV	47	25 (55) 16 (34) 5 (11)		45 (100) 0 (0)		20 (44) 25 (56)		
Pathological grading								
Well differentiated	105	45 (45) 47 (47) 9 (9)	0.26	100 (99) 1 (1)	1.0	30 (31) 68 (69)	0.61	
Moderately or poorly differentiated	60	21 (36) 27 (47) 10 (17)		57 (98) 1 (2)		20 (35) 38 (65)		
Tumor location								
Tongue	75	26 (36) 37 (51) 9 (13)	0.44	71 (99) 1 (1)	1.0	26 (37) 45 (63)	0.26	
Other sites	90	40 (46) 37 (42) 10 (12)		86 (99) 1 (1)		24 (26) 61 (72)		
Tumor location								
Floor of the mouth	34	18 (55) 14 (42) 1 (3)	0.10	33 (100) 0 (0)	1.0	10 (30) 23 (70)	0.80	
Other sites	131	48 (38) 60 (48) 18 (14)		124 (98) 2 (2)		40 (32) 83 (68)		
Perineural invasion								
No	156	62 (41) 70 (47) 18 (12)	1.0	148 (99) 2 (1)	1.0	44 (30) 103 (70)	**0.03**	
Yes	9	4 (44) 4 (44) 1 (12)		9 (100) 0 (0)		6 (67) 3 (33)		
Vascular invasion								
No	158	63 (41) 72 (47) 17 (12)	0.27	150 (99) 2 (1)	0.39	45 (30) 104 (70)	**0.03**	

Yes	7	3 (44) 2 (28) 2 (28)		7 (100) 0 (0)		5 (71) 2 (29)		
Clinical status at the end of the follow-up								
Alive without recurrence								
Dead of index cancer	77	27 (36) 38 (51) 9 (12)	0.90	72 (97) 2 (3)	0.61	14 (20) 57 (80)	**0.01**	
Censored								
Second primary	67	29 (45) 28 (43) 8 (12)		65 (100) 0 (0)		28 (43) 37 (57)		
cancer	18	8 (47) 7 (41) 2 (12)		17 (100) 0 (0)		7 (39) 11 (61)		
	3	2 (67) 1 (33) 0 (0)		3 (100) 0 (0)		1 (50) 1 (50)		

Table 5. Univariate Cox analysis of OS and DSS for the three categories of YAP1 activation and clinicopathological variables. HR, 95% Confidence Intervals, and *p* values are shown.

Variables	OS			DSS		
	P	**HR**	**95% CI**	***p***	**HR**	**95% CI**
Gender (men vs. women)	0.22	1.34	0.83 – 2.14	0.47	1.21	0.71 – 2.06
Tobacco (yes vs. no)	0.30	1.26	0.80 – 2.00	0.96	0.99	0.59 – 1.64
Alcohol (yes vs. no)	0.10	1.42	0.92 – 2.18	0.35	1.26	0.77 – 2.04
Location						
Tongue vs. rest	0.35	1.22	0.80 – 1.86	0.64	1.12	0.69 – 1.81
Floor of the mouth vs. rest	0.87	0.95	0.56 – 1.63	0.45	1.28	0.67 – 2.45
Clinical stage (I + II vs. III + IV)	**< 0.0001**	2.89	1.88 – 4.46	**< 0.0001**	4.31	2.51 – 7.40
Pathological grading (Well differentiated vs moderately or poorly differentiated)	0.09	1.46	0.93 -2.28	0.09	1.38	0.83 -2.31
Perineural invasion (no vs. yes)	**< 0.0001**	5.47	2.67 – 11.18	**< 0.0001**	5.25	2.47 – 11.18
Vascular invasion (no vs. yes)	**< 0.0001**	4.59	2.10 – 10.04	**< 0.0001**	4.92	2.11 – 11.49
Treatment	**< 0.0001**			**< 0.0001**		

Surgery		Reference			Reference	
Surgery + radiotherapy	**<0.0001**	3.38	2.05 – 5.57	**< 0.0001**	3.79	2.07 – 6.93
Surgery + radiotherapy + chemotherapy	**< 0.0001**	6.40	3.37 – 12.15	**<0.0001**	7.85	3.84 – 16.04
Surgical margin (without vs with tumor)	**< 0.0001**	3.00	1.80 – 4.99	**<0.0001**	2.09	1.44 – 3.04
YAP1 activation	**0.04**			**0.03**		
Inactive		Reference			Reference	
Partially active	0.28	0.70	0.37 – 1.33	0.98	0.99	0.49 – 1.97
Fully active	**0.03**	1.84	1.03 – 3.31	**0.01**	2.27	1.22 – 4.23
pS6 expression (negative vs. positive)	**0.008**	0.55	0.36 – 0.85	**0.02**	0.56	0.34 – 0.91

TABLE 6. Multivariate Cox analysis of OS and DSS for the three categories of YAP1 activation, clinical stage, perineural invasion, vascular invasion, surgical margin status, treatment, and pS6. HR, 95% CI and *p* values are shown.

Variables	**OS**			**DSS**		
	p	**HR**	**95% CI**	***p***	**HR**	**95% CI**
Clinical stage						
I + II	0.03	1.75	1.03 – 2.97	0.006	2.39	1.28 – 4.44
III + IV						
Perineural invasion (no vs yes)	0.01	2.90	1.28 – 6.55	0.005	3.07	1.40 – 6.69
Treatment	0.002			0.003		
Surgery		Reference			Reference	
Surgery + radiotherapy	0.002	2.54	1.42 – 4.54	0.01	2.28	1.15 – 4.54
Surgery + radiotherapy + chemotherapy	0.002	3.28	1.56 – 6.90	0.001	4.02	1.79 – 8.99
Vascular invasion (no vs yes)	0.03	2.38	1.06 – 5.35			
YAP1 activation	0.01					
Inactive		Reference				
Partially active	0.02	0.44	0.22 – 0.88			
Fully active	0.01	2.54	1.25 – 5.19			

Figure legends

Figure 1. Immunohistochemical analysis of YAP1, TAZ and PI3K/mTOR pathway components in OSCC tissue specimens. Representative images of tumors showing negative (A) and positive (B) nuclear and cytoplasmic YAP1 staining, negative (C) and positive (D) nuclear TAZ staining at the periphery of tumor nests, negative (E) and positive (F) cytoplasmic PIK3CA/p110α staining, negative (G) and positive (H) p-AKT (Ser473) staining, and negative (I) and positive (J) p-S6 (Ser235) staining. Magnification 20x. Scale bar 20 µm.

Figure 2. Kaplan-Meier disease-specific survival and overall survival curves in the cohort of 165 OSCC patients categorized by p-S6 (Ser235) expression.

Figure 3. Kaplan-Meier disease-specific survival and overall survival curves in the cohort of 165 OSCC patients categorized by combined expression of nuclear and cytoplasmic YAP1 into three categories: inactive, partially active and fully active YAP1.

Figure 1

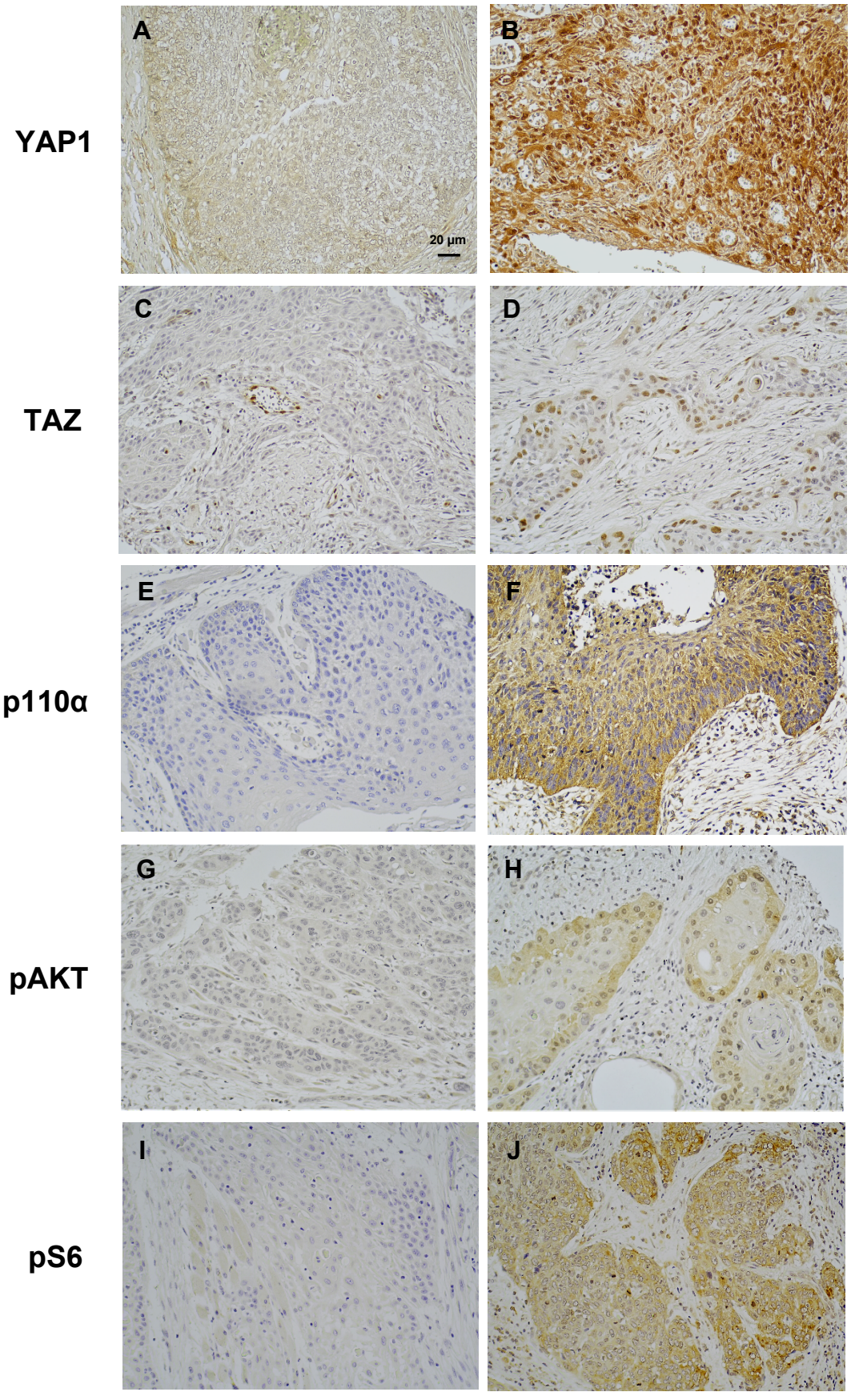
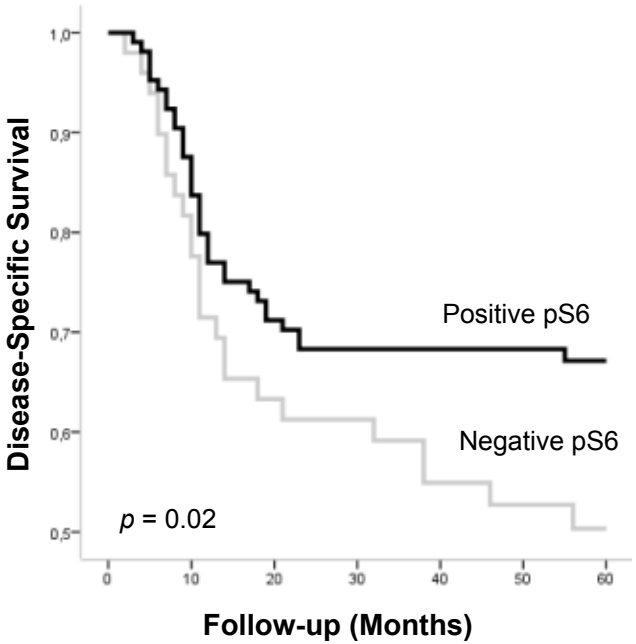


Figure 2


Disease-Specific Survival
1,0
0,9
0,8
0,7
0,6
0,5
Positive pS6
Negative pS6
p = 0.02
0
10
20
30
40
50
60
Follow-up (Months)

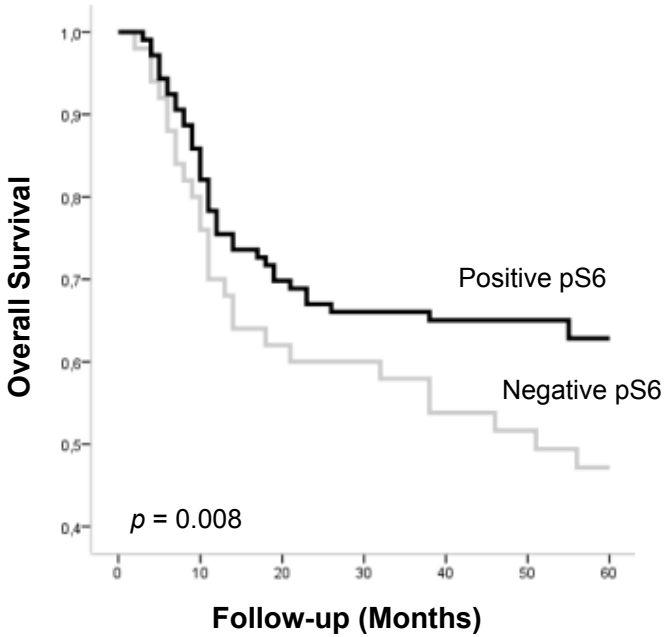
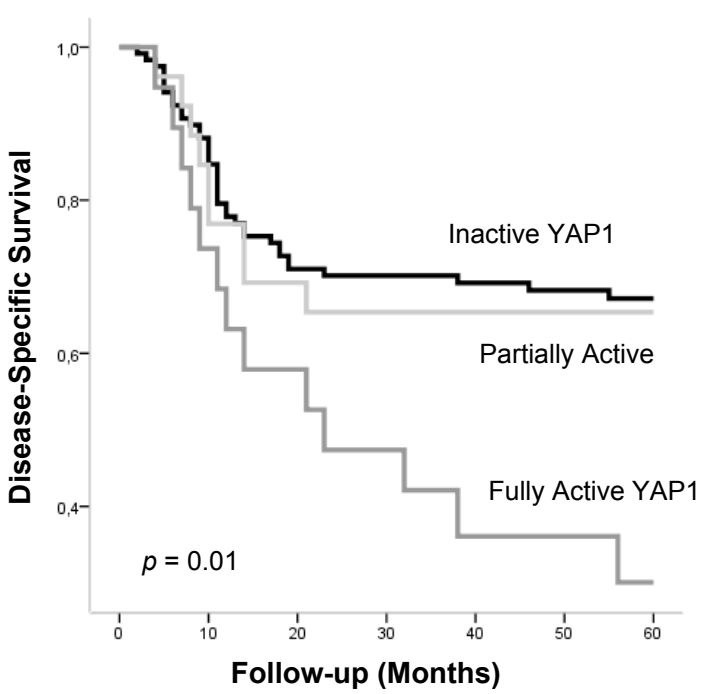
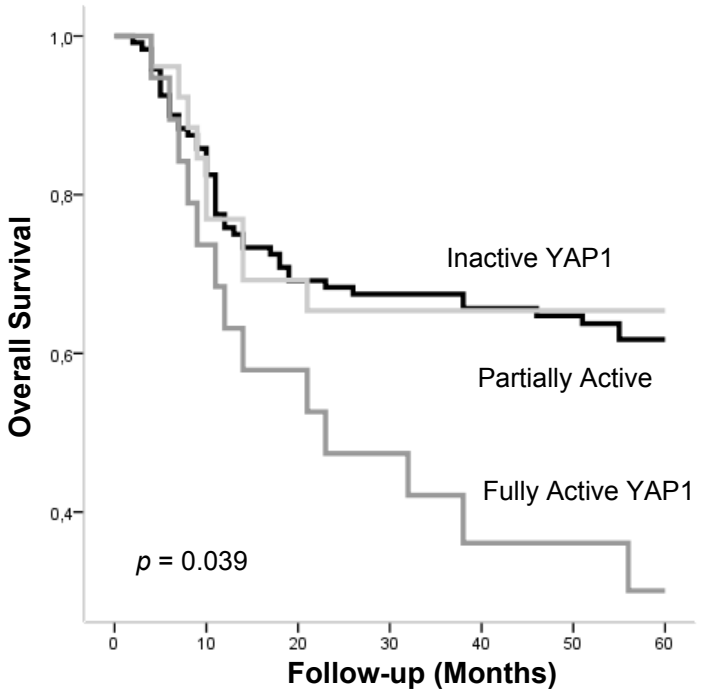

Overall Survival
1,0
0,9
0,8
0,7
0,6
0,5
0,4
Positive pS6
Negative pS6
p = 0.008
0
10
20
30
40
50
60
Follow-up (Months)

Figure 3


Disease-Specific Survival
1,0
0,8
0,6
0,4
Inactive YAP1
Partially Active
Fully Active YAP1
p = 0.01
0
10
20
30
40
50
60
Follow-up (Months)


Overall Survival
1,0
0,8
0,6
0,4
Inactive YAP1
Partially Active
Fully Active YAP1
p = 0.039
0
10
20
30
40
50
60
Follow-up (Months)