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PI3-kinase Pathway Biomarkers in Oral Cancer and Tumor Immune Cells

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Abstract

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Conflict of interest statement

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Background—This study investigated the hypothesis that PI3-kinase pathway dysregulation in either head and neck cancer cells and/or tumor infiltrating immune cells would influence outcomes of patients with surgically treated oral tongue squamous cell carcinomas (SCC).

Methods—We constructed tissue microarrays containing 123 oral tongue SCC samples and performed immunohistochemistry using antibodies against seven PI3-kinase pathway markers (PTEN, Akt, p-Akt, mTOR, p-mTOR, survivin, and Ki67). Expression levels in cancer cells or tumor infiltrating immune cells were correlated with outcomes.

Results—Higher levels of PTEN expression in immune cells were significantly associated with improved recurrence-free (HR=0.45, 95% CI 0.23–0.90, P=0.03), and overall survival (HR=0.34, 95% CI 0.15–0.76, P=0.01) in univariate and multi-covariate models.

Conclusions—We identified a novel, negative prognostic role of PI3-kinase activation (as determined by PTEN loss) in oral SCC infiltrating immune cells. These findings could be relevant for clinical development of PI-3 kinase inhibitors for this disease.

Keywords

PI3-kinase pathway; PTEN; Immunology; Oral squamous cell carcinomas; Tumor infiltrating immune cells

Introduction

Phosphoinositide 3-kinase (PI3-kinase) is an enzyme composed of two subunits (an 85 k-Da regulatory subunit and a 110-kDa catalytic subunit) involved in signal transduction and regulation of a variety of cellular functions, including cell growth, proliferation, metabolism, migration and secretion. In humans, four class I catalytic isoforms of PI3-kinase have been described (p110-alpha, p110-beta, p110-gamma, and p110-delta – the former two ubiquitously expressed and the latter two enriched in immune cells). PI3-kinase catalyzes the phosphorylation of PtdIns-4,5-P₂ to generate PtdIns-3,4,5-P₃, which works as a second messenger to recruit cytoplasmic proteins to specific membrane locations. Downstream effectors of PI3-kinase include the Akt / mammalian target of rapamycin (mTOR) pathway, which is responsible for anabolic metabolism, cell growth, and proliferation. The tumor suppressor PTEN terminates PI3-kinase signaling by removing 3'-phosphate from PtdIns-3,4,5-P₃, and serves as a negative regulator of the activity of the pathway (1).

PI3-kinase is one of the most commonly mutated and/or amplified genes in head and neck squamous cell carcinomas. In The Cancer Genome Atlas project, mutations in the p110-alpha gene (PIK3CA) were identified in 18% of human papilloma virus (HPV)-negative and 37% of HPV-positive head and neck cancers (2). When including genetic changes (mutation or DNA copy number variation) of any of the PI3-kinase pathway components, 66% of the TCGA-studied samples have been found to have an alteration (3). Furthermore, reduced PTEN expression has been observed in approximately 30% of head and neck cancers (4) increasing the pool of patients that may exhibit PI3-kinase pathway dysregulation. Of note, PTEN loss can result from genetic, as well as epigenetic events, such as promoter methylation, alternative splicing of pre-messenger mRNA, and post-translational modification (5).

PI3-kinase activation has been considered one of the hallmarks of cancer and there have been several efforts to target this pathway as a therapeutic strategy for head and neck cancers and other tumor types (3). Identifying a prognostic role of potentially targetable molecular alterations may serve as a useful approach to assist in cancer drug development, and has been used, for example, to support the decision to study epidermal growth factor receptor (EGFR) inhibitors for head and neck cancer treatment (6). Differently than EGFR, however, PI3-kinase biology is not mostly centered in epithelial cells, and modulation of this pathway in host tissues (e.g. immune cells) may significantly affect the natural history of cancer. Given the recent clinical development of immune checkpoint inhibitors, it is now recognized that the immune system may be successfully harnessed for head and neck cancer treatment, which illustrates the importance of characterizing oncogenic pathways not only in cancer cells but also in the microenvironment.

Although previous studies have evaluated the associations of PI3-kinase pathway activation and head and neck cancer prognosis, none of these efforts have included a comprehensive analysis of multiple components of the pathway in both cancer cells and immune cells (7–12). We therefore designed this study to investigate the hypothesis that PI3-kinase pathway dysregulation in either head and neck cancer cells and/or tumor infiltrating immune cells would influence long-term outcomes of patients with oral tongue squamous cell carcinomas treated with surgical resection. To test this hypothesis, we evaluated protein expression levels of PTEN, Akt, phospho-Akt (pAkt), mTOR, phospho-mTOR (p-mTOR), Ki67, and survivin in cancer cells and immune cells using tissue microarrays and correlated them with recurrence-free and overall survival.

Methods

Patient Selection

For this retrospective study, we selected 144 consecutive cases of oral tongue squamous cell carcinoma patients that were treated surgically in the Head and Neck Cancer Program at The University of Texas MD Anderson Cancer Center (MDACC). Chart review was performed to retrieve demographic, clinical, histopathological and outcome information. A complete data set was available for 123 patients, and their surgical resection tissue specimens were selected from the Head and Neck tissue bank for tissue microarray construction. The study was approved by the MD Anderson Institutional Review Board, which granted a waiver of consent.

Tissue Microarray Construction and Immunohistochemistry

Tissue microarrays were constructed using three 1-mm diameter cores for each sample, and 5-micron formalin-fixed, paraffin-embedded tissue sections were obtained, stained with hematoxylin-eosin and reviewed by a pathologist to verify accuracy of sampling. Immunohistochemical staining was performed on deparaffinized and hydrated sections. We used a DAKO antigen retrieval bath at 121 °C for 30 seconds and 90 °C for 10 seconds in a decloaking chamber (Biocare, Concord CA) for heat-induced epitope retrieval, followed by a 30-min cool-down period. Endogenous peroxidase activity was blocked prior to antibody immunostaining with 3% H₂O₂ in methanol for 30 min, followed by a 30-minute incubation

with 10% fetal bovine serum in Tris-buffered saline solution with Tween 20 to block non-specific antibody binding. The slides were then incubated with the primary antibody at ambient temperature for 60 minutes, followed by incubation with biotin-labeled secondary antibody (Envision Dual Link +, DAKO) for 30 minutes. The staining was developed with 0.05% 3',3'-diaminobenzidine tetrahydrochloride, after which the slides were counterstained with hematoxylin, dehydrated and mounted. The following primary antibodies and dilutions were used, as previously standardized: PTEN (Cell Signaling, 1:100), Akt (Cell Signaling, 1:100), p-Akt S473 (Cell Signaling, 1:50), mTOR (Cell Signaling, 1:100), p-mTOR Ser2448 (Cell Signaling 1:50), survivin (Lab Vision, 1:75), Ki67 (DAKO, 1:200). These antibodies had been shown to be specific by Western blot analysis using non-small cell lung cancer cell lines and human bronchial epithelial cell line models, and had been optimized for immunohistochemistry using cell line pellets fixed in formalin and embedded in paraffin, as previously reported by our group (13–15).

Expression of each biomarker was jointly quantified by two pathologist (MN and IW) in three cores per sample and the average was used for downstream statistical analysis. Scoring of three cores allowed us to account for, and potentially minimize (but not completely eliminate) interpretation bias resulting from tumor heterogeneity that certainly exists when assessing protein expression levels in different regions of the tumor. Markers were evaluated in epithelial cancer cells, as well as in lymphocytes and macrophages (herein collectively referred to as immune cells). To assess markers in immune cells, we first localized lymphocytes and macrophages in hematoxylin-eosin-stained slides, and then quantified the staining in these specific cells in adjacent sections used for immunohistochemistry. As illustrated in Appendix Figure 1, the vast majority of immune cells (>95%) consisted of lymphocytes. For each marker, samples were classified as negative or positive, and when appropriate, a 2- or 3-point scale was used to semi-quantify the staining. H-scores (0–300 scale) were also obtained by multiplying the percent of stained cells (0–100) x intensity (0–3), when feasible.

Statistical Analysis

Spearman rank correlation was used to assess correlation between markers. Survival distributions were estimated by the Kaplan-Meier method. Overall survival was defined as the time of surgery to death due to any cause (event) or to the time of last follow up (censored). Recurrence-free survival was defined as the time from surgery to first recurrence or death due to all causes, whichever occurred first (event). For recurrence-free survival, patients who were still alive and recurrence-free at the last follow-up date were censored. Regression analyses of survival data based on the Cox proportional hazards model were conducted on recurrence-free and overall survival. Log-rank test was performed to test the difference in time-to-event variables among groups. Multi-covariate analyses of survival were performed for patient characteristics that were significant in the univariate models. Multi-covariate analyses were performed for each marker variable that was significant in the univariate model adjusting for patient characteristics that were still significant in the multi-covariate model using only patient characteristics. The final model consisted of these significant patient characteristics and the marker variables that remained significant. All

statistical tests were two-sided. P-values <0.05 were considered statistically significant. SAS version 9.3 and S-Plus version 8.2 were used for all analysis.

Results

Patient Characteristics

Table 1 describes the clinicopathological and treatment characteristics of 123 patients with surgically treated oral tongue squamous cell carcinomas for which tissue and long term outcomes data were available. At the time of the final analysis, 37 patients (30%) had developed recurrence, of which 12, 11, 8, 5, 1 were local, regional, distant, local and regional, and local and distant, respectively. There were 55 deaths (45%), of which 29 were related to oral cancer. The median recurrence-free and overall survival times for the entire cohort were 6.0 and 8.8 years, respectively. The median follow up time for the censored observations (i.e., patients alive and recurrence-free at the time of the analysis) was 6.4 years.

Marker Distribution and Correlations

In Table 2 and Appendix Table 1, we describe the distribution and correlations of markers in cancer cells, respectively. To allow for a more detailed analysis, and to account for potential functional significance according to subcellular localization of each marker, expression levels were semi-quantified in the membrane, cytoplasm and nucleus. Despite the well characterized canonical signaling pathway of PI3K activation followed by downstream phosphorylation of Akt and mTOR, leading to cancer cell proliferation and survival, we did not observe any robust correlations between PTEN, Akt, p-Akt, mTOR, p-mTOR, survivin, or Ki67 expression in our data set (with the exception of a strong correlation of cytoplasmic p-AKT and p-mTOR, $Rho=0.61$, $P<0.0001$ – Appendix Table 1).

In Table 3 and Appendix Table 2, we describe the distribution and correlations of markers in immune cells, respectively. Subcellular localization was not possible due to limitations in delineating cell subregions on non-automated semi-quantitative analysis, and results are presented as total cell staining. Similar to the findings in cancer cells, no strong correlations were identified between PTEN, Akt, p-Akt, mTOR, p-mTOR, or Ki67 expression (Appendix Table 2).

Association of Markers with Recurrence-Free and Overall Survival

To assess whether our patient cohort would be representative of oral cavity cancer patients in general, we first determined the impact of known prognostic clinical and histopathological factors on recurrence-free and overall survival. As expected, higher T/N stage, perineural invasion, and extracapsular lymph node spread were positively and significantly associated with recurrence and/or death (Table 4). Next, we evaluated whether any of our biomarkers assessed in cancer cells and immune cells were associated with recurrence-free survival or overall survival. Although there was a trend towards markers reflecting PI3 kinase pathway activation being associated with worse prognosis (e.g., cytoplasmic PTEN loss, high nuclear/cytoplasmic AKT, nuclear/membrane p-Akt, cytoplasmic p-mTOR, nuclear survivin, nuclear Ki67), none of the associations reached statistical significance at a P-value<0.05 (Table 5).

By contrast, we identified a strong association between PTEN loss in immune cells and inferior recurrence-free and overall survival (Table 6, Appendix Figure 2). To further investigate this phenomenon, we assessed whether PTEN loss in immune cells was associated with any known prognostic clinical or histopathological variable. No robust correlations were identified (data not shown). We then performed multivariate analysis of overall survival including in the final model PTEN expression in immune cells, N classification, and tobacco use. Results demonstrated that both N classification ($P<0.01$) and PTEN loss ($P=0.01$) remained independent prognostic factors associated with worse overall survival (Appendix Table 3). Appendix Figure 3 shows representative tissue sections of no/low or high PTEN staining in immune cells and cancer cells.

Discussion

In this study, we identified a non-statistically significant trend towards inferior recurrence-free and overall survival of patients with resected oral tongue squamous cell carcinoma exhibiting higher levels of cancer cell expression of p-Akt (nuclear and membrane), p-mTOR (cytoplasmic), Ki67 (nuclear), and lower levels of cancer cell expression of PTEN (cytoplasmic). There were no strong correlations of expression levels, in cancer cells, among these PI3-kinase pathway components, with the exception of cytoplasmic p-mTOR and p-Akt. Likewise, no correlations between these markers were identified in immune cells. Nonetheless, we demonstrated that higher levels of PTEN protein expression in immune cells were significantly associated with improved recurrence-free (HR=0.45, 95% CI 0.23–0.90, $P=0.03$), and overall survival (HR=0.34, 95% CI 0.15–0.76, $P=0.01$) in univariate and multi-covariate models including clinicopathological factors, suggesting that downregulation of the PI3-kinase pathway in immune cells could contribute to better outcomes.

Our findings that activation of the PI3-kinase pathway in cancer cells (as demonstrated by either PTEN loss, or increased p-AKT and/or p-mTOR) were associated with a trend towards higher risk of recurrence and/or death are in line with previous observations that evaluated the pathway at the protein level in head and neck squamous cell carcinomas. For example, in a previous, independent analysis of 41 patients treated at MD Anderson, loss of PTEN expression in tongue cancer cells was associated with shorter overall survival and event-free survival (7). In 90 Chinese patients with oral tongue squamous cell carcinomas, low PTEN protein expression also predicted for shorter overall survival (12). Overexpression of p-Akt has been shown to be associated with shorter disease-free survival independently of stage and nodal status in a cohort of 52 tongue cancer cases (10) and shorter overall survival in two cohorts of 84 (9) and 191 (8) oral squamous cell carcinoma cases. Likewise, Monteiro et al. demonstrated lower disease-free and overall survival in patients with oral squamous cell carcinoma with high p-mTOR expression (11). Despite these various observations, a definitive prognostic role of PI3-kinase pathway activation in oral cavity squamous cell carcinomas is yet to be established. We recognize that most of the reports (including this one) have analyzed a limited number of samples, have used different antibodies and methods of tissue staining and scoring, and often lacked validation in independent data sets. Furthermore, the biomarker-outcomes associations described in our work failed to reach statistical significance, which may indicate that evaluation of a single marker alone may not be sufficient to fully capture the magnitude of activity of the pathway and its effect in

driving cancer progression. This is further illustrated by the lack of strong correlations among the well-established components of the PI3-kinase pathway in our analysis (Appendix Table 1), which may indicate the existence of cross talks with other signaling molecules not evaluated herein that ultimately contribute to cancer cell proliferation and survival [e.g., Ras/Raf/Mek/Erk (3), p53/sestrin, p63/REDD1 (16), etc]. Indeed, Molinolo et al. have established one of the largest head and neck squamous cell carcinomas tissue microarrays through an international consortium, and have demonstrated the existence of subgroups of tumors in which immunohistochemistry analyses have shown mTOR pathway activation independently of Akt (17). Unfortunately, outcomes data for that cohort are not available and the prognostic significance of this finding is unknown. In our study, we also failed to evaluate other components of the PI3-kinase pathway that could further impact outcomes and assist in refining our prognostic models, such as expression of the p110 catalytic subunit itself (and its four isoforms), as well as additional PtdIns-3,4,5-P₃-dependent signaling proteins that have been shown to be pro-carcinogenic through downstream effectors that bypass Akt. These include the phosphoinositide-dependent kinase 1 (PDK1) and Rac pathways. PDK1 activation has been shown to activate p70s6k in an Akt-dependent and independent manner, and to induce anchorage-independent growth of cancer cell lines through a mechanism mediated by the downstream effector SGK3, but not Akt (18,19). Rac promotes Akt-independent cytoskeletal remodeling and glycolysis coordination (20). Overexpression of p110- α , PDK1, and Rac1 have been found to be associated with head and neck squamous cell carcinoma progression (21,22).

Despite the aforementioned shortcomings of tissue-based analyses, extensive pre-clinical work have further validated the importance of the PI3-kinase/Akt/mTOR pathway targeting for treatment of head and neck squamous cell carcinomas (23–25). This was recently corroborated by a randomized phase 2 clinical trial in pre-treated recurrent/metastatic head and neck squamous cell carcinoma patients showing improved progression-free survival, overall survival, and response rates by the addition of the PI3-kinase inhibitor buparlisib to paclitaxel (26). While a major effect of PI3-kinase inhibitors *in vivo* is thought to be derived from activity in cancer cells directly, the PI3-kinase pathway is also central to the physiology of host cells in the microenvironment, raising the possibility that its modulation in non-epithelial cells could influence cancer progression.

The most innovative aspect of our work was the evaluation of the PI3-kinase pathway not only in cancer cells, but also in the tumor immune infiltrate. Notably, we identified that no/low PTEN protein expression in immune cells (which consisted primarily of lymphocytes) was associated with inferior recurrence-free and overall survival on univariate (Table 6, Appendix Figure 2) and multi-covariate analysis (Appendix Table 3). Based on these findings, one could postulate that PI3-kinase activation in these cells might contribute to tumor immune escape leading to worse prognosis, and that PI3-kinase inhibition *in vivo* could have anti-cancer effects through mechanisms involving immune modulation. Indeed, PI3-kinase activation is key to innate and adaptive immunity, and may enhance or impair immune function depending on the biologic context (1). As regards to the innate immune system, PI3-kinase activation is involved in phagocytosis (27), production of reactive oxygen species (27), toll-like receptors 7 and 9-induced type I interferon production (28), and attenuation of the inflammatory response triggered by other toll-like receptors (29). In tumor

associated macrophages, the PI3-kinase catalytic isoform p110-gamma promotes an immunosuppressive phenotype characterized by upregulation of IL-10 and TGF-beta (30). inhibition of p110-gamma in animal models delays tumor growth and enhances the efficacy of immune checkpoint blockade through mechanisms related to reprogramming of the immune microenvironment, as opposed to a direct effect on cancer cells (30,31). Participation of the PI3-kinase pathway in the process of adaptive immunity includes downstream signaling after T-cell receptor engagement and activation by costimulatory molecules (27). proliferation of T regulatory cells (32), and control of CD8-positive T cell fate (33). The overall effect of PI3-kinase modulation will therefore depend on opposing actions in different subset of lymphocytes (e.g., T regulatory cells and T effector cells) that might ultimately influence adaptive anti-tumor immunity (1).

Our study was not designed to characterize the status of the PI3-kinase pathway activation on the subpopulations of the immune cells. As such, we could not determine whether the inferior outcomes associated with PTEN loss in immune cells could result from impaired T effector cell function, enhanced T regulatory cell activity, and/or modulation of the innate immune system. However, the expression levels of immune cell markers quantified in this study were primarily driven by expression of these markers in lymphocytes, since these represented the most abundant cell population observed in the immune infiltrate, as evidenced by hematoxylin-eosin staining (Appendix Figure 1). Indeed, a recent study by Partlová et al. characterized immune infiltrates in HPV-negative head and neck squamous cell carcinomas by flow cytometry, and demonstrated that the ratio of lymphocytes to macrophages/monocytes/dendritic cells was approximately 20:1 (34). Despite the limitations of lack of deep characterization of the immune infiltrate, our observations serve as basis for further investigations of the interactions between PI3-kinase dependent signaling pathways and immune editing in oral cavity squamous cell carcinomas, especially if our findings can be replicated in independent data sets.

In summary, to our knowledge, this is the first report demonstrating a negative prognostic role of PI3-kinase activation (as determined by PTEN loss) in head and neck squamous cell carcinoma infiltrating immune cells. PI3-kinase inhibitors have now been found to be active to treat this disease (26), as well as PD-1 inhibitors (35), and pre-clinical work demonstrate a synergism of combined PI3-kinase and immune checkpoint targeting in multiple tumor models (31). As such, detailed evaluation of the mechanisms of PTEN loss in immune cells in oral tongue cancers, its consequence to the process of immune evasion, as well as its effects in determining response to targeted agents and immunotherapy could lead to the development of improved treatment strategies for patients with oral squamous cell carcinomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PI3-kinase	Phosphoinositide 3-kinase
mTOR	/ mammalian target of rapamycin
SCC	Squamous Cell Carcinoma
HPV	human papilloma virus
EGFR	epidermal growth factor receptor
MDACC	MD Anderson Cancer Center
PDK1	phosphoinositide-dependent kinase 1

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Table 1

Clinicopathological characteristics

Variable	Number of patients*	Percent
Sex		
Male	75	61
Female	48	39
Age (median: 59 years)		
> 59 years	64	52
≤ 59 years	59	48
Tobacco use		
> 30 pack years	54	50
≤ 30 pack years	55	50
Stage**		
1	14	12
2	50	42
3	38	32
4	18	15
T classification**		
1	50	41
2	51	42
3	20	16
4	1	1
N classification**		
0	68	55
1	26	21
2	29	24
Number of positive lymph nodes		
0	68	55
1–2	37	30
3	18	15
Extracapsular lymph node spread		
No	30	55
Yes	25	45
Perineural invasion		
No	82	67
Yes	41	34
Lymphatic invasion		

Variable	Number of patients [*]	Percent
No	115	94
Yes	8	7
Vascular invasion		
No	115	94
Yes	8	7
Post-operative radiation therapy		
No	69	57
Yes	53	43

^{*} Missing values are not included.

^{**} According to the American Joint Committee on Cancer 7th edition.

Table 2

Distribution of markers in cancer cells *

	Staining No. of Patients (%)			H-score**
	Negative	Weak	Strong	Median (inter-quartile range)
PTEN nuclear				30 (0–60)
PTEN cytoplasmic	N=10 (9%)	N=44 (38%)	N=61 (53%)	
Akt nuclear	N=3 (3%)	N=30 (26%)	N=82 (71%)	
Akt cytoplasmic				200 (152–250)
p-Akt nuclear	N=55 (47%)	N=61 (53%)	N=1 (1%)	
p-Akt cytoplasmic				35 (0–90)
p-Akt membrane	N=97 (83%)	N=20 (17%)		
mTOR cytoplasmic				180 (129–210)
p-mTOR cytoplasmic				170 (100–230)
Survivin nuclear				238 (209–261)
Survivin cytoplasmic	N=60 (52%)	N=53 (46%)	N=2 (2%)	
Ki67 nuclear				50 (23–91)**

Abbreviations: N (number of patients).

* Missing values are not included.

** For Ki-67, data are reported as proliferative index (i.e., % of cells with positive nuclear staining).

Black boxes represent not applicable data.

Table 3

Distribution of markers in immune cells *

Marker	Staining No. of Patients (%)			
	Negative	1+	2+	3+
PTEN	N=45 (40%)	N=30 (26%)	N=39 (34%)	
Akt	N=6 (5%)	N=25 (22%)	N=38 (33%)	N=46 (40%)
p-Akt	N=113 (97%)	N=4 (3%)		
mTOR	N=49 (43%)	N=35 (30%)	N=31 (27%)	
p-mTOR	N=104 (89%)	N=13 (11%)		
Ki67	N=105 (91%)	N=10 (9%)	N=1 (1%)	

Abbreviations: N (number of patients).

* Missing values are not included.

Black boxes represent not applicable data.

Table 4

Prognostic effects of clinical and histopathological markers on RFS and OS

Marker	Hazard Ratio RFS (95% CI)	P-Value	Hazard Ratio OS (95% CI)	P- Value
Age (median: 59 years)				
> 59 years vs. 59 years	1.24 (0.75–2.04)	0.40	1.46 (0.85–2.50)	0.17
Tobacco use				
> 30 vs. 30 pack years	1.57 (0.92–2.69)	0.10	1.86 (1.03–3.35)	0.04
Stage*				
2 vs. 1	1.33 (0.54–3.28)	0.45	0.87 (0.34–2.22)	0.06
3 vs. 1	1.68 (0.68–4.15)		1.40 (0.56–3.49)	
4 vs. 1	2.05 (0.76–5.54)		2.11 (0.78–5.72)	
T classification*				
2 vs. 1	1.45 (0.82–2.56)	<0.01	1.75 (0.94–3.26)	<0.01
3 vs. 1	1.90 (0.97–3.72)		2.57 (1.24–5.31)	
4 vs. 1	52.66 (5.33–520.62)		45.88 (4.93–427.08)	
N classification*				
1 vs. 0	1.15 (0.57–2.32)	<0.01	0.94 (0.42–2.10)	<0.01
2 vs. 0	4.40 (2.51–7.69)		5.88 (3.27–10.59)	
Extracapsular lymph node spread				
Yes vs. No	2.61 (1.52–4.49)	<0.01	2.77 (1.56–4.93)	<0.01
Perineural invasion				
Yes vs. No	1.96 (1.19–3.25)	0.01	1.80 (1.05–3.10)	0.03
Lymphatic invasion				
Yes vs. No	1.47 (0.63–3.43)	0.37	1.38 (0.55–3.49)	0.49
Vascular invasion				
Yes vs. No	1.84 (0.79–4.29)	0.16	1.31 (0.47–3.64)	0.60

Abbreviations: CI (confidence interval); OS (overall survival); RFS (recurrence-free survival).

* According to the American Joint Committee on Cancer 7th edition.

Table 5

Prognostic effects of tumor cell biomarkers on RFS and OS

Marker	Hazard Ratio RFS (95% CI)	P-Value	Hazard Ratio OS (95% CI)	P- Value
PTEN nuclear				
High vs. Low *	1.07 (0.63–1.81)	0.81	0.93 (0.53–1.66)	0.82
PTEN cytoplasmic				
Weak vs. Negative	0.96 (0.39–2.35)	0.27	1.24 (0.43–3.63)	0.87
Strong vs. Negative	0.64 (0.26–1.54)		0.92 (0.32–2.64)	
Akt nuclear				
Weak vs. Negative	0.55 (0.12–2.43)	0.65	0.39 (0.09–1.75)	0.46
Strong vs. Negative	0.68 (0.16–2.85)		0.47 (0.11–1.99)	
Akt cytoplasmic				
High vs. Low *	1.36 (0.82–2.26)	0.24	1.31 (0.76–2.27)	0.82
p-Akt nuclear				
Weak vs. Negative	0.87 (0.52–1.45)	0.63	0.93 (0.53–1.61)	0.45
Strong vs. Negative	2.12 (0.29–15.61)		3.41 (0.46–25.47)	
p-Akt cytoplasmic				
High vs. Low *	0.97 (0.58–1.62)	0.91	0.94 (0.55–1.64)	0.84
p-Akt membrane				
Positive vs. Negative	1.55 (0.84–2.87)	0.16	1.89 (1.01–3.55)	0.05
mTOR cytoplasmic				
High vs. Low *	0.82 (0.49–1.37)	0.44	0.92 (0.53–1.59)	0.74
p-mTOR cytoplasmic				
High vs. Low *	1.24 (0.75–2.06)	0.41	1.47 (0.85–2.54)	0.17
Survivin nuclear				
High vs. Low *	1.10 (0.66–1.83)	0.73	1.37 (0.79–2.37)	0.27
Survivin cytoplasmic				
Weak vs. Negative	1.27 (0.76–2.13)	0.59	1.02 (0.58–1.78)	0.96
Strong vs. Negative	0.70 (0.10–5.18)		0.76 (0.10–5.59)	
Ki67 nuclear				
High vs. Low *	1.31 (0.78–2.20)	0.31	1.36 (0.78–2.37)	0.28

Abbreviations: CI (confidence interval); OS (overall survival); RFS (recurrence-free survival).

* High (≥ median); Low (< median).

Table 6

Prognostic effects of immune cell biomarkers on RFS and OS

Marker	Hazard Ratio RFS (95% CI)	P-Value	Hazard Ratio OS (95% CI)	P- Value
PTEN				
Weak vs. Negative	1.12 (0.63–2.02)	0.03	1.15 (0.63–2.12)	0.01
Strong vs. Negative	0.45 (0.23–0.90)		0.34 (0.15–0.76)	
Akt				
1+ vs. Negative	1.81 (0.52–6.28)	0.50	1.35 (0.38–4.77)	0.53
2+ vs. Negative	1.21 (0.36–4.07)		1.07 (0.32–3.63)	
3+ vs. Negative	1.12 (0.33–3.76)		0.78 (0.23–2.70)	
p-Akt				
Positive vs. Negative	1.91 (0.60–6.13)	0.28	2.56 (0.79–8.29)	0.12
mTOR				
Weak vs. Negative	0.48 (0.25–0.91)	0.07	0.47 (0.23–0.95)	0.09
Strong vs. Negative	0.66 (0.35–1.23)		0.64 (0.33–1.27)	
p-mTOR				
Positive vs. Negative	0.72 (0.31–1.68)	0.45	0.82 (0.35–1.92)	0.64
Ki67				
Weak vs. Negative	0.55 (0.17–1.77)	0.60	0.67 (0.21–2.14)	0.79
Strong vs. Negative	N/A		N/A	

Abbreviations: CI (confidence interval); N/A (not applicable); OS (overall survival); RFS (recurrence-free survival).