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Title: Clinicopathologic significance of ROCK2 expression in oral squamous cell carcinomas

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Abstract

Background: Rho-associated coiled-coil kinase 2 (ROCK2) is an oncoprotein that controls cytoskeleton organization and acts as prognostic marker in different types of solid tumors. ROCK2 overexpression is also observed in cancer-associated fibroblasts (CAF), which suggests its relevance within the tumor microenvironment. This study aimed to access the prognostic value of ROCK2 in oral squamous cell carcinomas (OSCC) and its association with CAF density.

Methods: ROCK2 immunohistochemical analysis was applied in 93 OSCC samples from 2 centers in Brazil and Finland. The samples were also stained for isoform α of smooth muscle actin (α-SMA) to characterize the presence of CAF in the tumor stroma. Clinicopathologic associations were analyzed using chi-square test, survival curves were constructed according to the Kaplan-Meier method, and Cox proportional hazard model was applied for multivariate survival analysis.

Results: Advanced clinical stage (p=0.002) and increased density of CAF (p=0.002) were significantly associated with high ROCK2 expression. The high expression of ROCK2 was also associated with shortened disease-specific survival (HR: 2.22, 95% CI: 1.15-4.38, p=0.04), but the association did not withstand the Cox multivariate survival analysis.

Conclusions: The findings suggest that high ROCK2 expression in OSCC is associated with advanced disease and follows the increase in CAF density, which may be important for tumor progression.

Introduction

Oral squamous cell carcinoma (OSCC), one of the 10 most prevalent cancers worldwide, has a global annual incidence of approximately 300,000 new cases and 145,000 deaths, with considerable geographic and environmental risk factor differences[1]. Although the overall incidence of OSCC has been decreasing in some areas of the world, which is consistent with decreases in tobacco use, the global incidence of oral cavity cancers is predicted to rise in the next 20 years

because of demographic changes[2]. Clinical features including tumor size and cervical lymph node metastasis are the most consisting prognostic factors for OSCC, but it frequently shows unpredictable prognosis. Among the histological characteristics, depth of invasion has shown prognostic importance for OSCC[3], leading to its incorporation in the T stage classification in the new edition of the stating manual of the American Joint Cancer Committee[4]. However, OSCC still shows significant morbidity and mortality rates, which have remained unchanged over recent decades[5]. Therefore, better prognostic markers are required to better understand and select more aggressive tumors.

Rho-associated coiled-coil kinase 2 (ROCK2) is an oncoprotein with important roles in tumor proliferation, apoptosis, adhesion, migration and invasion, and its deregulated expression has been reported in tumor cells from hepatocellular carcinomas[6], pancreatic adenocarcinomas[7], colorectal cancers[8], gastric cancers[9], breast carcinomas[10], esophageal cancers[11], renal cell carcinomas[12] and oral cancer[13]. In most of those studies, overexpression of ROCK2 has been related to tumor progression, metastasis and poor clinical outcome[7,9,10,11,12], and its inhibition could suppress tumor invasion *in vitro*[8]. ROCK2 expression is also observed in the tumor microenvironment cells, including inflammatory cells, endothelial cells and cancer-associated fibroblasts (CAF)[14,15]. In CAF, expression of ROCK2 is predicted to stimulate Rho kinase signaling pathway, which in turn may contribute to tumor cell survival, proliferation and tumor progression[14]. In OSCC, CAF have been associated with poor outcome and promotion of tumorigenesis *in vitro*[16,17]*.* Owing to this pivotal function in cancer, ROCK2 has been suggested as a promising therapeutic target, but there is no data on the prognostic value for OSCC. On this context, we have evaluated CAF density and the prognostic role of ROCK2 expression in a cohort of OSCCs from Brazil and Finland.

Materials and Methods

This study was carried out following the REMARK guidelines for tumor marker prognostic studies[18].

Patients

This study included samples from 93 patients with OSCC, who underwent radical surgery at the UOPECCAN Cancer Hospital (n=66), Cascavel, Brazil from

1998 to 2008, and at the University Hospital of Oulu, Finland (n=27) between 1979 and 2009. All patients had never received prior therapy before surgery. This is a retrospective study and the complete demographic and clinical data were collected from patient's records, including gender, age, habits such as smoking and alcohol consumption, tumor stage, tumor site, type of treatment post-surgery, histological grade, status of the surgical margins, recurrence and survival. Clinical tumor staging was performed according to the International Union Against Cancer (UICC) 2002 staging system. The outcomes were categorized as disease-specific survival (DSS), time from treatment initiation until death due to cancer or last known date alive, and disease-free survival (DFS), time from treatment initiation until diagnosis of the first recurrence (local, regional or distant) or last follow-up information for those without recurrence. Only survivors with follow-up information of at least 5 years were included. The study was approved by the ethics review board of each of the hospitals affiliated with the collaborative study.

Tissue samples

Paraffin-embedded blocks of all cases were retrieved and new sections were stained with hematoxylin and eosin. Tumors were histologically graded according to the 2017 World Health Organization classification. Surgical margin, identified as the closest distance between the tumor and the surgical resection edge was categorized into 2 groups based on the cut-off value of 5 mm. Margins of less than 5 mm were considered involved, and margins of 5 mm or more were classified as free.

Immunohistochemistry

Immunohistochemistry was performed on 3 μm sections. Slides were dewaxed in xylene and rehydrated by using a graded alcohol series. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution, and antigen retrieval was performed with 10 mM citric acid pH 6.0 in a pressure cooker. Slides were then incubated with primary antibodies against ROCK2 (1:400, ab66320, Abcam, USA) and isoform α of smooth muscle actin (α-SMA, 1:400, clone 1A4, M0851, Dako, USA) at 4°C overnight, followed by biotinylated second antibodies (EnVisionTM+ system, Dako, USA) at 37^{o}C for 30 min., avidin-biotin complex and incubation with 3,3'-diaminobenzidine tetrahydrochloride (Dako, USA) and counterstained with hematoxylin. Control reactions were performed by omission of the primary antibody.

ROCK2 expression was assessed by two pathologists who were not aware of any clinical data. Scores were assigned according to the percentage of positive tumor cells and the intensity of the staining[10,11]. For the percentage of positive tumor cells, the scores were: 0, negative; 1, <25%; 2, 26%-50%; and 3, >51%. For intensity, the scores were: 0, negative; 1, weak; 2, moderate; and 3, strong. Final scores were calculated as the sum of the percentage of positive cells and the intensity of the staining, and for statistical analysis, samples were categorized into two groups. Low expression was defined as a final score <4 points (negative and low expression) and high expression (moderate and strong) was defined as a final score ≥4. CAF score was assessed as previously described by Kellermann et al. (2007)[16]. Tumors were classified as negative if 0% of the fibroblasts were α-SMA positive, scanty if 1% to 50% were α-SMA positive, and abundant if >50% of the stromal fibroblasts were α-SMA positive. For statistical analysis the scores negative and scanty were combined and compared to the abundant cases.

Statistical analysis

Associations between immunohistochemical expression of ROCK2 and clinicopathological parameters of the tumors were performed using cross-tabulation and chi-square test. Survival curves were constructed based on the Kaplan-Meier method and compared with the log-rank test. For multivariate survival analysis, the Cox proportional hazard model with a stepwise method was employed. The level of significance considered was 5% ($p \leq 0.05$).

Results

Out of 93 patients, 70 (75.2%) were men, and the age of the patients ranged from 32 to 86 years, with a median of 60 years. Most of the patients reported smoking habits (84.2%) and alcohol consumption (73.1%). Regarding clinical stage, 37 (39.8%) patients were classified in early-stage (stages I and II) and 56 (60.2%) in advanced-stage (stages III and IV). The tongue was the most commonly affected site (58.7%) and other affected sites included the floor of mouth (29.3%), retromolar area (5.4%), palate (4.4%) and gingiva (2.2.%). Surgery as monotherapy was performed in 37 (40.2%) patients, whereas 47 (51.1%) were treated with a combination of surgery and postoperative radiotherapy and 8 (8.7%) received surgery and

postoperative radio-chemotherapy. Only 4 cases had surgical margins with less than 5 mm. The WHO grading system classified 65 (69.9%) tumors as well differentiated, 18 (19.3%) as moderately differentiated and 10 (10.8%) as poorly differentiated. During follow-up, 34 patients developed recurrence. The overall survival ranged from 1 to 251 months, with a mean of 123 months. Fourth three patients died due to the tumor. The clinicopathological features of patients are depicted in Supplementary Table 1.

Eleven (11.8%) cases were negative for ROCK2. The expression of ROCK2 was localized in both nucleus and cytoplasm of the tumor cells, showing variable distribution and intensity (Fig.1). Immunopositivity was also observed in some inflammatory and firoblast-like cells within the stroma (Supplementary Fig. 1). Regarding the cytoplasmatic ROCK2 immunoreactivity, 24 (25.8%) samples were classified as low expression and 69 (74.2%) as high expression.

High expression of ROCK2 was significantly associated with male gender (p=0.006), drinking habit (p=0.01), high clinical stage (0.002) and abundant CAF density in the stroma (p=0.002) (Table 1). No significant differences were found between ROCK2 expression and smoking habit, tumor site, treatment, histological grade, margin status and recurrence (Table 1). We also determined whether the presence of CAF was associated with clinicopathological features of OSCC patients. As depicted on Supplementary Table 2, a high intensity of CAF was significantly associated with tumor site (p=0.03) and poorly differentiated tumors (p=0.001). A positive but moderate correlation between ROCK2 expression and CAF density was observed in the Spearman test (r=0.52, p=0.002).

Univariate survival analysis based on log-rank test revealed a significant association of age, clinical stage of the tumor, CAF density and ROCK2 expression with DSS, and of age and CAF density with DFS (Table 2). Patients with high expression of ROCK2 had shorter DSS compared with patients with low ROCK2 expression (Fig. 2). High ROCK2 expression was associated with a 5-year DSS of 42.9% compared with 70.3% for patients with low ROCK2 immunoreactivity (p=0.04). Multivariate Cox proportional hazard regression model analysis was built to further evaluate the impact of ROCK2 expression on DSS and DFS (Table 3). In this analysis, ROCK2 did not withstand as an independent prognostic factor, but age and clinical stage remained as independent prognostic factor for DSS, whereas age and CAF density were pointed out as prognostic parameters of DFS in this cohort.

Discussion

Alterations in the expression level of ROCK2 have been reported in several cancers, and the overexpression was an indicator of worse prognosis[7,8,9,10,12]. One early investigation showed that increased ROCK2 expression controls migration and invasion of OSCC cells *in vitro*[13], but the prognostic value of ROCK2 has never been verified in oral cancers. We found in the current study that high ROCK2 expression is associated with the advanced clinical stage, which is well-known affecting tumor aggressiveness. Furthermore, high ROCK2 expression was an indicator of poor DSS, with those patients' 5-year survival being 42.9% compared with 70.3% for patients with low ROCK2 expression. For DFS, the result was similar (56.6% for high ROCK2 vs. 64.9% for low ROCK2), but not statistically significant.

ROCK2 belongs to the serine-threonine kinase AGC family and is a downstream intracellular messenger following small GTPase RhoA activity[19]. Since the description that ROCK2 controls cytoskeleton organization by regulating actin filaments[20], many studies have investigated its roles in cancer, revealing the participation in a wide range of processes related to tumorigenesis *in vitro* such as proliferation, apoptosis, adhesion, migration, and invasion. In consonance, ROCK inhibition due to synthetic drugs/peptides or microRNAs, was found to suppress tumor growth and metastasis *in vivo*[6,7,8]. In a recent study, Vennin et al. (2017)[21] showed that priming with Fasudil, a specific ROCK inhibitor, disrupts the extracellular matrix scaffold, improving the treatment effectiveness in a mouse model of pancreatic cancer. Together, those results suggest that ROCK2 might be a molecular target for preventing cancer progression and metastasis.

The increased ROCK2 expression has been reported in association with metastasis and shorter survival of cancer patients. For example, in pancreatic cancers, ROCK2 levels rose with increasing tumor stage (advanced tumors showed significantly higher levels than early stage and even normal pancreatic tissue) and the survival of patients with genomic amplification or significantly elevated mRNA was significantly shorter than in patients without ROCK2 alterations[7]. Based only on univariate Kaplan-Meier analysis, patients with colorectal cancers and high ROCK2 expression had significantly shorter survival rates than patients whose tumors showed low ROCK2 expression[8]. Our results are in line with those previous ones regarding associations with advanced clinical stage of tumors and shortened

survival. Furthermore, the only published study evaluating ROCK2 in oral cancer demonstrated that in highly metastatic oral tongue SCC cells, enhanced expression of ROCK2, due to a reduction in miR-138 levels, increases the activity of Rho GTPase signaling cascade, which promotes cell migration and invasion[13]. Thus, little has been uncovered regarding the biological mechanisms related to ROCK2 in oral cancer, but our results preliminarily reveal an association with clinical outcome.

Another interesting finding of the present study relies on the CAF analysis. We recently performed a systematic review and meta-analysis to verify the value of CAF in OSCC, and demonstrated that high presence of CAF is frequently associated with parameters that worsen the prognosis, including advanced disease stage, recurrence, tumor grade, depth of invasion, vascular, lymphatic and neural invasion and extranodal metastatic spread, and most important, high presence of CAF significantly predicted shortened time to disease-relapse and an overall decrease in survival[22]. The pro-tumorigenic effects of CAF are related to the vast repertoire of secreted molecules, which can directly influence the behavior of cancer cells, stimulating proliferation, invasion, and metastasis. Among the well-known molecules are stromal derived factor 1 (SDF-1/CXCL12)[23], activin A and matrix metalloproteases[17], some types of collagen and other extracellular matrix proteins such as fibronectin[24], and ROCK2[14]. Our findings in this study revealed ROCK2 positivity in both tumor and stroma cells, and a significant association between ROCK2 expression by tumor cells and the density of CAF was observed. Rath and collaborators (2017)[7] demonstrated that ROCK activation led to significant collagen degradation and remodeling associated with increased invasive growth, and the treatment of ROCK-activated invasive cells with GM6001, a matrix metalloprotease inhibitor, blocks those processes. The association of ROCK2 and CAF suggests the organization of a more invasion-permissive microenvironment, facilitating tumor progression and metastasis.

In this study, the expression of ROCK2 was significantly more common in males and drinkers. Interestingly, ROCK2 was identified as a downstream effector of androgen/androgen receptor pathway, affecting migration and invasion of pancreatic cancer cells through the transcription of miR-135a[25], and inducing endothelial cell migration[26]. In addition, ROCK2 expression was significantly elevated in ethanol-induced gastric ulcer in mice[27]. Even though the association between those

predictors and ROCK2 expression is not widely addressed in the current literature, our results with OSCC clinical samples corroborate with those previous findings.

Besides the predictable associations of clinical stage with DSS and CAF density with DFS, we also found a significant association of age with both DSS and DFS at univariate and multivariate analysis. Those results are in line with previous studies that showed a worse survival in elderly patients with OSCC than in young patients[3,28]. An important feature in favor of a causal effect of advanced age on survival is that previous studies have reported frequent comorbidities in the cardiovascular, gastrointestinal and respiratory systems in elderly people, and demonstrated a significant impact on the prognosis of oral cancer patients[29,30]. Thus, those results suggest that age may affect the survival of oral cancer patients due to the comorbid illnesses.

In closing, the expression of ROCK2 in OSCC is associated with advanced clinical stage of the tumor and poor survival, which indicates that ROCK2 may be involved in OSCC progression. Although ROCK2 may not be an independent prognostic factor, it should be further verified by prospective studies and more comprehensive follow-up.

Conflicts of interest statement

The authors declare that they have no conflict of interests.

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Ethical approvals statement

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and

with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

References

1. Ferlay J, Soerjomataram I, Dikshit R et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;136:E359-386. doi:10.1002/ijc.29210.
2. Shield KD, Ferlay J, Jemal A, Sankaranarayanan R. The global incidence of lip, oral cavity, and pharyngeal cancers by subsite in 2012. *CA Cancer J Clin.* 2017;67:51-64. doi:10.3322/caac.21384.
3. Almangush A, Bello IO, Coletta RD et al. For early-stage oral tongue cancer, depth of invasion and worst pattern of invasion are the strongest pathological predictors for locoregional recurrence and mortality. *Virchows Arch.* 2015;467:39-46. doi:10.1007/s00428-015-1758-z.
4. Ridge JA, Lydiatt WM, Patel SG et al. *Head and Neck*. In: Amin MB, Edge S, Greene FL, Byrd DR, Brookland RK, Washington MK, Gershenwald JE, Compton CC, Hess KR, Sullivan DC, Jessup JM, Brierley JD, Gaspar LE, Schilsky RL, Balch CM, Winchester DP, Asare EA, Madera M, Gress DM, Meyer LR, editors. *AJCC cancer staging manual*, 8th ed. Springer, New York; 2017.pp 79–94.
5. Warnakulasuriya S. Living with oral cancer: epidemiology with particular reference to prevalence and life-style changes that influence survival. *Oral Oncol.* 2010;46:407-10. doi:10.1016/j.oraloncology.2010.02.015.
6. Wong C-M, Wei L, Au SL-K et al. MiR-200b/200c/429 subfamily negatively regulates Rho/ROCK signaling pathway to suppress hepatocellular carcinoma metastasis. *Oncotarget*. 2015;6:13658-70. doi:10.18632/oncotarget.3700.
7. Rath N, Morton JP, Julian L et al. ROCK signaling promotes collagen remodeling to facilitate invasive pancreatic ductal adenocarcinoma tumor cell growth. *EMBO Mol Med.* 2017;9:198-218. doi:10.15252/emmm.201606743.
8. Qiu Y, Yuan R, Zhang S et al. Rock2 stabilizes β-catenin to promote tumor invasion and metastasis in colorectal cancer. *Biochem Biophys Res Commun*. 2015;467:629–37. doi:10.1016/j.bbrc.2015.10.103.

9. Li M, Ke J, Wang Q, et al. Upregulation of ROCK2 in gastric cancer cell promotes tumor cell proliferation, metastasis and invasion. *Clin Exp Med*. 2016;1-11. doi:10.1007/s10238-016-0444-z.
10. Hsu C-Y, Chang Z-F, Lee H-H. Immunohistochemical evaluation of ROCK activation in invasive breast cancer. *BMC Cancer*. 2015;15:943. doi:10.1186/s12885-015-1948-8.
11. Wang X, Liu D, Zhou K et al. Expression of Wnt11 and Rock2 in esophageal squamous cell carcinoma by activation of the WNT/PCP pathway and its clinical significance. *Pathol Res Pract.* 2016;212:880-5. doi:10.1016/j.prp.2016.07.008.
12. Xu Z, Hong Z, Ma M et al. Rock2 promotes RCC proliferation by decreasing SCARA5 expression through β-catenin/TCF4 signaling. *Biochem Biophys Res Commun*. 2016;480:586–93. doi:10.1016/j.bbrc.2016.10.097.
13. Jiang L, Liu X, Kolokythas A et al. Downregulation of the Rho GTPase signaling pathway is involved in the microRNA-138-mediated inhibition of cell migration and invasion in tongue squamous cell carcinoma. *Int J Cancer.* 2010;127:505-12. doi:10.1002/ijc.25320.
14. Bozóky B, Savchenko A, Csermely P et al. Novel signatures of cancer-associated fibroblasts. *Int J Cancer.* 2013;133:286-93. doi:10.1002/ijc.28035.
15. Chen W, Mao K, Liu Z, Dinh-Xuan AT. The role of the RhoA/Rho kinase pathway in angiogenesis and its potential value in prostate cancer. *Oncol Lett.* 2014;8:1907-11. doi:10.3892/ol.2014.2471.
16. Kellermann MG, Sobral LM, da Silva SD et al. Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. *Histopathology*. 2007;51:849-53. doi:10.1111/j.1365-2559.2007.02873.x.
17. Sobral LM, Bufalino A, Lopes MA, Graner E, Salo T, Coletta RD. Myofibroblasts in the stroma of oral cancer promote tumorigenesis via secretion of activin A. *Oral Oncol*. 2011;47:840-6. doi:10.1016/j.oraloncology.2011.06.011.
18. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst.* 2005;97(16):1180-4.

19. Julian L, Olson MF. Rho-associated coiled-coil containing kinases (ROCK): structure, regulation, and functions. *Small GTPases*. 2014;5:e29846. doi:10.4161/sgtp.29846.
20. Amano M, Chihara K, Kimura K. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science.* 1997;275:1308-11.
21. Vennin C, Chin VT, Warren SC et al. Transient tissue priming via ROCK inhibition uncouples pancreatic cancer progression, sensitivity to chemotherapy, and metastasis. *Sci Transl Med.* 2017;9. doi:10.1126/scitranslmed.aai8504.
22. Dourado MR, Guerra ENS, Salo T, Lambert DW, Coletta RD. Prognostic value of the immunohistochemical detection of carcinoma-associated fibroblasts in oral cancer: a systematic review and meta-analysis. *J Oral Pathol Med.* 2017; *in press.* doi: 10.1111/jop.12623.
23. Ishii G, Ochiai A, Neri S. Phenotypic and functional heterogeneity of cancer-associated fibroblast within the tumor microenvironment. *Adv Drug Deliv Rev*. 2016;99:186–96. doi:10.1016/j.addr.2015.07.007.
24. Bagordakis E, Sawazaki-Calone I, Macedo CCS et al. Secretome profiling of oral squamous cell carcinoma-associated fibroblasts reveals organization and disassembly of extracellular matrix and collagen metabolic process signatures. *Tumor Biol*. 2016; 37:9045–57. doi:10.1007/s13277-015-4629-y.
25. Kroiss A, Vincent S, Decaussin-Petrucci M et al. Androgen-regulated microRNA-135a decreases prostate cancer cell migration and invasion through downregulating ROCK1 and ROCK2. Oncogene. 2015; 34(22):2846-55. doi: 10.1038/onc.2014.222.
26. Liao W, Huang W, Guo Y, Xin M, Fu X. Testosterone promotes vascular endothelial cell migration via upregulation of ROCK-2/moesin cascade. Mol Biol Rep. 2013;40(12):6729-35. doi: 10.1007/s11033-013-2788-8.
27. Zhao Z, Gong S, Wang S, Ma C. Effect and mechanism of evodiamine against ethanol-induced gastric ulcer in mice by suppressing Rho/NF-κB pathway. Int Immunopharmacol. 2015;28(1):588-95. doi: 10.1016/j.intimp.2015.07.030.
28. Pontes FSC, Carneiro JT, Fonseca FP, da Silva TSP, Pontes HAR, Pinto D dos S. Squamous cell carcinoma of the tongue and floor of the mouth: analysis of survival rate and independent prognostic factors in the Amazon

region. *J Craniofac Surg*. 2011;22:925-30. doi:10.1097/SCS.0b013e31820fe1cb.

29. Ho H, Lee M, Hsiao S. Squamous Cell Carcinoma of the Oral Cavity in Young Patients: A Matched-Pair Analysis. *Eur Arch Oto-Rhino-Laryngology.* 2008;265:57-61. doi:10.1007/s00405-007-0496-5.
30. Sawazaki-Calone I, Rangel ALCA, Bueno AG et al. The prognostic value of histopathological grading systems in oral squamous cell carcinomas. *Oral Dis.* 2015;21:755–61. doi:10.1111/odi.12343.

Figure Legends

Fig. 1 ROCK2 and α-SMA immunohistochemical staining in OSCC samples. Representative samples classified as negative (A) and positive (B) for ROCK2 immunostaining. (C) High power view revealing that most tumor cells showed a cytoplasmatic positivity for ROCK2. Representative samples classified as (D) negative, (E) scanty, and (F) abundant for α-SMA immunostaining, indicating the absence of presence of CAF within the stroma. It should be highlighted that smooth muscle cells in the blood vessels walls can show positivity for α-SMA, as shown in Fig.D. (Original magnification: C x400; all the other images are x100;)

Fig. 2 Kaplan-Meier curves representing the cumulative 5-year survival of the OSCC patients. Univariate analysis showed an association of advanced age with DSS (A, p=0.02) and DFS (B, p=0.003); CAF density with DSS (C, p=0.02) and DFS (D, p=0.03); advanced clinical stage with DSS (E, p=0.008); and high ROCK2 expression with DSS (F, p=0.04).

Supplementary Fig. 1 Representative images of the immunostaining against ROCK2 in OSCC. A lower magnification shows positivity for tumor and stroma cells (A, x100). In a closer view, is possible to visualize positive fibroblast-like cells surrounding the tumor (B, x400), and a chronic inflammatory infiltrate also positive for ROCK2 (C, x400).

Table 1. Association between the clinicopathological parameters of the oral squamous cell carcinomas and the immunohistochemical expression of ROCK2.

Parameter	ROCK2		p value
	Low expression No. of patients (%)	High expression No. of patients (%)	
Age			
<58 years	9 (37.5)	35 (50.7)	
≥58 years	15 (62.5)	34 (49.3)	0.26
Gender			
Male	13 (54.2)	57 (82.6)	
Female	11 (45.8)	12 (17.4)	0.006
Smoking habit			
No	3 (30)	6 (12.8)	
Yes	7 (70)	41 (87.2)	0.18
Drinking habit			
No	5 (62.5)	9 (20.5)	
Yes	3 (37.5)	35 (79.5)	0.01
Clinical stage			
I/II	16 (66.7)	21 (30.4)	
III/IV	8 (58.4)	48 (69.6)	0.002
Tumor site			
Tongue	10 (41.6)	35 (50.7)	
Others	14 (58.4)	34 (49.3)	0.44
Treatment			
Surgery	12 (52.2)	25 (36.2)	
Surgery + RTX	11 (47.8)	36 (52.2)	
Surgery + RTX + CTX	0	8 (11.6)	0.15
Histological grade			
WD/MD	13 (54.2)	52 (75.4)	
PD	11 (45.8)	17 (24.6)	0.06
Margin status			
> 5 mm	22 (91.7)	67 (97.1)	
< 5 mm	2 (8.3)	2 (2.9)	0.26
Recurrence			
No	16 (66.7)	43 (62.3)	
Yes	8 (33.3)	26 (37.7)	0.70
CAF density			
Negative/Scanty	15 (62.5)	19 (27.5)	
Abundant	9 (37.5)	50 (72.5)	0.002

RTX: radiotherapy, CTX: chemotherapy, WD: well-differentiated; MD: moderately-differentiated; PD: poorly-differentiated; CAF: carcinoma-associated fibroblasts.

Table 2. Univariate analysis for disease-specific survival and disease-free survival of the oral squamous cell carcinoma patients.

Parameter	Disease-Specific Survival		Disease-Free Survival	
	% in 5 years	HR (95% CI) / p value	% in 5 years	HR (95% CI) / p value
Age				
<58 years	56.9	Reference	71.6	Reference
≥58 years	37.4	2.03 (1.11-3.71) / 0.02	44.9	2.84 (1.47-5.47) / 0.003
Gender				
Male	46.8	Reference	61.4	Reference
Female	58.5	0.60 (0.30-1.21) / 0.21	54.3	1.27 (0.58-2.76) / 0.50
Smoking habit				
No	59.3	Reference	65.2	Reference
Yes	43.6	1.35 (0.46-3.93) / 0.52	43.8	1.63 (0.76-15.22) / 0.09
Drinking habit				
No	56.3	Reference	67.1	Reference
Yes	36.9	2.00 (0.90-4.45) / 0.14	59.7	1.01 (0.36-2.82) / 0.98
Clinical stage				
I/II	61.3	Reference	62.0	Reference
III/IV	37.4	2.25 (1.23-4.10) / 0.008	58.9	1.05 (0.54-2.02) / 0.88
Tumor site				
Tongue	55.7	Reference	56.5	Reference
Others	42.8	1.45 (0.79-2.65) / 0.21	62.9	0.75 (0.39-1.45) / 0.41
Treatment				
Surgery	61.7	Reference	61.2	Reference
Surgery + RTX	44.5	1.62 (0.86-3.04) / 0.13	55.8	1.03 (0.51-2.05) / 0.93
Surgery + RTX + CTX	50.0	1.23 (0.38-4.27) / 0.64	62.5	0.83 (0.25-2.66) / 0.75
Histological grade				
WD/MD	43.8	Reference	57.5	Reference
PD	67.3	0.56 (0.29-1.09) / 0.13	63.4	0.93 (0.45-1.91) / 0.84
Margin status				
> 5 mm	49.1	Reference	58.8	Reference
< 5 mm	50.0	0.83 (0.17-3.89) / 0.79	66.7	0.63 (0.13-3.19) / 0.65
Country				
Finland	72.0	Reference	57.0	Reference
Brazil	57.7	2.00 (1.03-3.90) / 0.08	60.0	0.78 (0.37-1.61) / 0.47

CAF density				
Negative/Scanty	64.4	Reference	70.4	Reference
Abundant	41.5	2.14 (1.16-3.94) / 0.02	51.6	1.85 (1.19-3.09) / 0.03
ROCK2				
Low	70.3	Reference	64.9	Reference
High	42.9	2.22 (1.15-4.38) / 0.04	56.6	1.27 (0.71-2.25) / 0.35

RTX: radiotherapy, CTX: chemotherapy, WD: well-differentiated; MD: moderately-differentiated; PD: poorly-differentiated; CAF: carcinoma-associated fibroblasts.

Table 3. Cox multivariate analysis for the risk of death.

Parameter	Disease-Specific Survival		Disease-Free Survival	
	HR (95% CI)	p value	HR (95% CI)	p value
Age				
<58 years	Reference		Reference	
≥58 years	2.44 (1.30-4.56)	0.005	2.98 (1.46-6.08)	0.003
Clinical stage				
I/II	Reference			
III/IV	2.49 (1.32-4.70)	0.004		
CAF density				
Negative/Scanty			Reference	
Abundant			2.08 (1.27-7.43)	0.01

CAF: carcinoma-associated fibroblasts.