Expression of thrombospondin-1 in conjunctival squamous cell carcinoma is correlated to the Ki67 index and associated with progression-free survival

Expression of thrombospondin-1 in conjunctival squamous cell carcinoma is correlated to the Ki67 index and associated with progression-free survival

Mizuki Tagami, Anna Kakehashi, Atsushi Sakai, Norihiko Misawa, Atsuko Katsuyama-Yoshikawa, Hideki Wanibuchi, Atsushi Azumi & Shigeru Honda

Tagami, M., Kakehashi, A., Sakai, A. *et al.* Expression of thrombospondin-1 in conjunctival squamous cell carcinoma is correlated to the Ki67 index and associated with progression-free survival. *Graefes Arch Clin Exp Ophthalmol* **259,** 3127–3136 (2021). https://doi.org/10.1007/s00417-021-05236-7

Expression of thrombospondin-1 in conjunctival squamous cell carcinoma is correlated to the Ki67 index and associated with progression-free survival

Mizuki Tagami[1,3], Anna Kakehashi[2], Atsushi Sakai[1], Norihiko Misawa[1], Atsuko Katsuyama-Yoshikawa[3], Hideki Wanibuchi[2], Atsushi Azumi[3], Shigeru Honda[1]

[1] Department of Ophthalmology and Visual Sciences, Graduate School of Medicine, Osaka City University, Osaka, Japan

[2] Department of Molecular Pathology, Graduate School of Medicine, Osaka City University, Osaka, Japan

[3] Ophthalmology Department and Eye Center, Kobe Kaisei Hospital, Kobe, Hyogo, Japan

Corresponding author:

Mizuki Tagami

Department of Ophthalmology and Visual Sciences

Graduate School of Medicine, Osaka City University

1-5-7 Asahimachi, Abeno-ku, Osaka-shi, Osaka 545-8586, Japan

Tel: +81- 6-6645-3867, Fax: +81-6645-3867

E-mail: tagami.mizuki@med.osaka-cu.ac.jp

Word count: 2725

Key Message

- In this study, there were significant differences in 45 genes between conjunctival carcinoma in situ (Tis) and advanced squamous cell carcinoma (SCC). Thrombospondin-1 had the highest frequency of different expression.
- Thrombospondin 1 showed significantly different expression between Tis and advanced SCC (≤T1). There was a significant correlation between thrombospondin-1 expression and the Ki67 index associated with tumor progression.
- Thrombospondin-1 expression correlated with progression-free survival and final orbital exenteration.

ABSTRACT

Purpose: Conjunctival squamous cell carcinoma (SCC) is primarily treated with surgical resection. SCC has various stages, and local recurrence is common. The purpose of this study was to investigate thrombospondin-1 expression and its association with prognosis.

Methods: In this retrospective study, a gene expression array along with immunohistochemistry were performed for the evaluation of thrombospondin-1 expression, localization, as well as Ki67 labeling cell indices in carcinoma in situ (Tis) and advanced conjunctival SCC (Tadv). The presence or absence and intensity of cytoplasmic and nuclear staining in tumor cells were also divided into groups with a score of 0-3 and semi-quantitatively analyzed to investigate intracellular staining patterns. The association between thrombospondin-1 expression and tumor progression in a series of 31 conjunctival SCCs was further investigated.

Results: All 31 patients in the cohort (100%) were East Asian. A simple comparison between Tis and Tadv demonstrated significant differences in expressions of 45 genes, including thrombospondin-1 ($p<0.01$). In this cohort, 30/31 tumors were positive (96%) for thrombospondin-1. Furthermore, thrombospondin-1 intracellular staining pattern analysis scores were 2.12 and 0.96 for nuclear and cytoplasmic staining, respectively, with a significant difference observed between Tis and Tadv ($p<0.01$). Alteration of the Ki67 labeling index was significantly correlated with that of the thrombospondin-1 cytoplasmic score ($p=0.030$). Furthermore, univariate Cox regression analysis showed a significant correlation between thrombospondin-1 staining and progression-free survival ($p=0.026$) and final orbital exenteration($p=0.019$).

Conclusions: The present results demonstrated that thrombospondin-1 is a potential molecular target in the pathology of conjunctival SCC, in addition to serving as a prognostic factor.

Keywords: Conjunctival squamous cell carcinoma, thrombospondin-1, Ki67 index, immunohistochemistry, Asian population

INTRODUCTION

Ocular surface squamous neoplasia (OSSN) includes several diseases, such as conjunctival premalignant dysplasia, carcinoma *in situ*, and invasive conjunctival squamous cell carcinoma (SCC).[1, 2]

Scholz et al. examined clinicopathological factors and biomarkers and identified promoter mutations in telomerase reverse transcriptase in 44% of 48 samples of conjunctival OSSN associated with sun exposure, and mutations were present in 4 of 7 (57.1%) patients with local recurrence of disease and all patients with metastases (100%).[3]

In molecular biological area, recent research demonstrated that programmed death ligand-1 (PD-L1) was expressed in almost half of conjunctival SCC cases and additionally noted the potential application of immune checkpoint blockade as a treatment strategy for conjunctival SCC.[4] In addition, investigation of molecular biological alterations in the field of conjunctival SCC has become a special point of interest, with numerous studies being focused on analysis of molecular targeting drugs. We also examined the molecular expression and intracellular localization of potential molecular target in conjunctival SCC in East Asian patients.[5]

In 1978, thrombospondin-1 was initially reported to be a member of extracellular matrix (ECM) proteins that are widely present in normal human tissues and tumors.[6] Although it is well known that thrombospondin-1 suppresses angiogenesis, there are both reports that its expression is associated with tumor growth and the opposite in which it is negatively associated with tumor development, thereby indicating it could be a multifaceted molecular model.[7] In addition, there have been many different conflicting Relationship between expression and prognosis observed in different tumors.

Sargiannidou et al. reported that high levels of thrombospondin-1 secreted by tumors, which were engineered to overexpress thrombospondin-1, inhibited tumor growth, whereas glioblastoma and thyroid cancer areas were described that correlate with tumor infiltration and metastasis in relation to tumor growth.[8-10]

There have been some reports targeting ocular surface thrombospondin-1 expression, which has been shown to be associated with chronic inflammatory diseases. However, a literature search identified no tumor case series.[11, 12] In our preliminary experiment, we compared the gene expression in carcinoma *in situ* and advanced stage SCC and found that thrombospondin-1 was most likely associated with conjunctival SCC development. In the present study, thrombospondin-1 expression in tumor cells and its association with prognosis were investigated in conjunctival SCC patients.

MATERIAL AND METHODS

Selection of cases and collation of clinicopathologic data

This study was approved by the Institutional Review Boards of Osaka City University and Kobe Kaisei Hospital and adhered to the tenets of the 1964 Declaration of Helsinki. Written, informed consent was obtained from all patients before enrollment. After identifying 31 patients treated by ophthalmologists (AA, MT) between November 2007 and January 2020, we were able to procure formalin-fixed paraffin-embedded (FFPE) tissue blocks stored at normal temperature with residual tumor for each of these cases.

For each patient, we collected demographic features (age at initial diagnosis and at presentation to our institution, and sex) and primary tumor features (disease status at presentation and in situ versus invasive disease). There were no cases of radiation

therapy or chemotherapy (including local treatment) before surgery. The American Joint Committee on Cancer (AJCC) stage, local recurrence (anatomic site and date), metastases (regional or distant and date), vital status at the last follow-up, cause of death, type of surgery, and adjuvant therapy were also recorded.

Immunohistochemistry

Sections (3-μm-thick) from the FFPE tissue samples were deparaffinized and treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. For heat-induced antigen retrieval, tissue sections were immersed in 0·01 mol/L citrate buffer (pH 6·0) and treated in a microwave oven for 20 min at 620 W. Immunohistochemical analyses for thrombospondin-1 and Ki67 were performed on tissue sections using the following antibodies: anti-thrombospondin-1 rabbit monoclonal antibody (1:50 ab85762; Abcam, Cambridge, UK), anti-Ki67 rabbit monoclonal antibody (clone: SP6; ab16667; Abcam), horseradish peroxidase-conjugated anti-rabbit IgG (H+L) goat polyclonal antibody (Histofine #424134, Nichirei Corporation, Tokyo, Japan), and horseradish peroxidase-conjugated anti-mouse IgG (H+L) goat polyclonal antibody (Histofine #424144, Nichirei Corporation), with Mayer's hematoxylin as the counterstain. Stained sections were viewed with an Olympus BX53+DP74 (Tokyo, Japan). Immunohistochemical staining was optimized by testing different negative controls (with and without primary antibody and with and without secondary antibody) and antigen retrieval methods.

Image analysis (including Ki67 index) and scoring

The histopathological samples of all cases were carefully reviewed and representative

blocks were chosen for immunohistochemistry. The pathological diagnoses, immunohistochemical analyses, evaluation, and counting were carried out by at least two pathologists (MT and AK) in our hospital in a blinded manner using the thrombospondin-1 score and the KI67-index shown below. Thrombospondin-1 expression was visually estimated as the localization of tumor cells with complete or partial membranous staining. The presence or absence and intensity of cell cytoplasmic staining were also divided into groups with a score of 0-3 and then semi-quantitatively analyzed (graded as: 0, none; 1, 0–25%; 2, 26–50%; 3, 51–100%). The presence or absence and intensity of nuclear staining were semi-quantitatively divided into groups with a score of 0-3 (graded as: 0, none; 1, 0–25%; 2, 26–50%; 3, 51–100%).

To evaluate the growth rates of the tumors, the percentage of tumor cells expressing the proliferation marker Ki67 was measured, according to past reports.[13-15]

The proliferation index was calculated for each tumor lesion by counting the total number of tumor cell nuclear profiles and the number of Ki67-positive nuclear profiles in randomly and systematically selected fields. The first field in each tumor lesion was selected randomly, with the following fields then sampled systematically using a mesh. On average, 3000 nuclear profiles per 10 fields were counted per tumor lesion. Counting was performed using a semiautomatic image analysis program.

Gene expression in tumors

Gene expression in the tumor was analyzed using nCounter analysis (NanoString, Seattle, WA, USA). Archival formalin-fixed paraffin-embedded tumor tissues were retrieved and manually macrodissected. Total mRNA was isolated from the macrodissected tumor tissues using a Qiagen miRNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA sample was quantified using NanoDrop (Thermo Scientific, Wilmington, DE, USA) and regarded as adequate if it contained a minimum of 400 ng. The sample was subsequently analyzed using the nCounter PanCancer Progression Panel (NanoString) according to the manufacturer's instructions [16]. NanoString data processing was done in the R statistical programming environment (v3.4.2). To obtain the counts for the positive control probe sets, raw NanoString counts for each gene were subjected to technical factorial normalization, which was carried out by subtracting the mean counts plus two times the standard deviation from the CodeSet inherent negative controls. Subsequently, biological normalization using the included mRNA reference genes was performed. Additionally, all counts with $p>0.05$ after a one-sided *t*-test versus negative controls plus two times the standard deviation were interpreted as not being expressed over the basal noise.

Statistical analysis

The clinical and histopathological characteristics were summarized using descriptive statistics. Correlations between immunohistochemical, demographic, and clinicopathological factors were assessed using the Wilcoxon rank sum and Fisher's exact tests. The interobserver agreement was assessed using the κ statistic for two

pathologist raters [17]. It was defined as $\kappa<0$ indicating no agreement; $\kappa = 0.0$ to 0.19, poor; $\kappa = 0.20$ to 0.39, fair; $\kappa = 0.40$ to 0.59, moderate; and $\kappa = 0.60$ to 0.79, substantial; and $\kappa = 0.80$ to 1.0, almost perfect agreement [18]. Progression-free survival (PFS) was defined as the time from surgery to disease recurrence or death from any cause. We assigned the Ki67 labeling cell index for each thrombospondin-1 staining pattern tested by evaluating the results using Spearman's rank correlation test. Cox regression modeling was used to evaluate correlations between clinicopathological and immunohistochemical features (age, sex, AJCC T stage, thrombospondin-1 scores) and survival outcomes. Statistical analyses were performed using SPSS Statistics version 22 software (IBM Japan, Tokyo, Japan). Values of $p<0.05$ were considered significant.

RESULTS

Table 1 summarizes the clinicopathological findings of our cohort. All 31 patients in the cohort (100%) were East Asian, and included 17 men and 14 women, with a mean age at presentation of 77.9 years. There were 16 patients (52%) with invasive SCC, and 15 (48%) with an *in situ* tumor. Primary orbital exenteration was necessary for local disease control in 3 patients (9%), whereas 2 patients (6%) underwent additional orbital exenteration. There were 9 patients (31%) who underwent adjuvant therapy, which is referred to as the most common additional local surgery. Topical chemotherapy (topical mitomycin C) and radiation therapy were performed in 1 patient in the adjuvant therapy group. In this group, 2 patients died with disease at 6 and 11 months after diagnosis of regional and lung metastases. One patient had a moderately differentiated SCC with keratinization (AJCC stage 3). Another patient had an undifferentiated cancer, which

was diagnosed as SCC because of positive epithelial staining (AE-1/AE-3, keratin) (AJCC stage 4b). Histology of both patients were upper eyelid origin primary tumor and the lung metastasis lesions were not histology-proved. Therefore, possibilities such as double cancer remain. In addition, the other patient was still alive without disease at 44 months after diagnosis of regional metastases. Of the 3 patients (6%) who died, 2 died due to conjunctival SCC (described above). There were 9 patients (31%) who developed local recurrence after curative surgery (Table 1).

The nCounter PanCancer Progression Panel was performed on tumorous tissue of 8 surgically resected SCCs. There was an association between the gene expression levels of the tumor and AJCC T staging (Tis: tumor carcinoma *in situ* vs. Tadv: tumor advanced carcinoma). The simple comparison between Tis and Tadv showed significant differences of 45 genes ($p<0.01$) (Table 2). However, no significant differences were detected by the multiple comparison test. The present analysis demonstrated that thrombospondin-1 was the most biologically important molecule detected under the two rate change conditions, with the difference shown to be significant (Figure 1).

In our cohort, 30/31 tumors were positive (96%) for thrombospondin-1. Furthermore, analysis of intracellular staining patterns of thrombospondin-1 showed scores of 2.12 and 0.96 for nuclear and cytoplasmic staining, respectively. The total thrombospondin-1 staining agreement was Substantial ($\kappa=0.68$). The agreement in the nuclear staining category was moderate ($\kappa=0.53$) and the agreement in the cytoplasmic staining category was Substantial ($\kappa=0.79$). In addition, calculation of the Ki67 labeling index demonstrated significant differences between Tis and Tadv ($p<0.01$)(Figure 2); the average Ki67 labeling index was 30.58%, and there was a significant correlation with the thrombospondin-1 cytoplasmic score ($p=0.030$) (Figure 3).

The COX regression model was used to examine and analyze the clinicopathological status, AJCC T stage, and thrombospondin-1 score for the PFS. Univariate Cox regression analyses showed significant correlations between thrombospondin-1 total staining and PFS (hazard ratio (HR): 1.873, p=0.026) (Table 3).

Local recurrence, the distant metastasis rate, and the overall survival rate, included in the multivariate Cox regression analysis, were not significantly different by various parameters (age: p=0.126, sex: p=0.126, T stage (AJCC): p=0.987, thrombospondin-1 scores: p=0.075, n=31).

In addition, thrombospondin-1 score was significantly correlated with final orbital exenteration (P=0.019) (Table.4)

.

DISCUSSION

To the best of our knowledge, this is the first study to survey the prevalence of thrombospondin-1 expression and intracellular localization, and to evaluate its prognostic significance in conjunctival SCC.

The present results showed significant differences between Tis and Tadv in 45 genes. Of these genes, thrombospondin-1 had the highest frequency of differential expression. Since there were no significant differences due to the small number of cases, we additionally examined the tumors by immunohistochemistry.

In our cohort, thrombospondin-1 immunohistochemistry showed that 30/31 tumors were positive (96%) for thrombospondin-1. Furthermore, the staining intensity demonstrated that there was a significant correlation between thrombospondin-1 expression and the Ki67 index, which is used to evaluate the level of cell proliferation and growth rate of tumors.

Previous studies have confirmed the expression of thrombospondin-1 on the ocular surface, which was shown to be related to homeostasis via the cell surface receptor CD36 and the immunomodulatory cross-talk between the conjunctival goblet cells and dendritic cells on the ocular surface. Thus, thrombospondin-1 has become a target molecule for the treatment of Sjögren's syndrome.[11] Moreover, as long as the molecule is involved in homeostasis on the ocular surface, its expression may affect tumor development in the microenvironment of cancer, similar to conjunctival SCC.

The present results showed that the expression of thrombospondin-1 was increased in correlation with the expression of Ki67, which is a well-known marker of cell proliferation reflecting the tumor growth rate. Furthermore, PFS was correlated with the expression of thrombospondin-1. In a previous conjunctival SCC study that was performed in Japan, Ohara et al. reported that the Ki67 labeling index may become a sensitive marker for ocular malignant tumor grading [19], and it appears to have a positive correlation with tumor progression. With regard to prognosis and treatment associated with molecular biological markers, other studies have examined the use of hypoxia-inducible factor-α (HIF-α) as a potential marker.[20] However, in the present study, HIF-α staining was not confirmed in preliminary our experiment(data not shown) and thus could not be used to obtain additional knowledge. Daubon et al. reported that transforming growth factor β1 induced thrombospondin-1 expression via Smad3, which contributes to the invasive behavior during expansion found in glioblastoma development.[8] On the other hand, contrary results have also been reported.[21-23]

Tzeng et al. reported that Rab37-mediated thrombospondin-1 secretion in cancer cells suppressed metastasis and angiogenesis via a cross-talk with endothelial cells. The authors additionally reported finding a novel component of the vesicular exocytic

machinery in the tumor microenvironment and tumor progression.[24]

Although there is a contradiction in its effects, we believe that thrombospondin-1 itself may both suppress and promote the growth of tumors. As previously reported, suppression of angiogenesis is disadvantageous for tumors because it reduces the feeding vessels of tumors. However, the reduction in the number of feeding vessels may prevent access to anti-tumor immune cells such as the NKT cells.[25] A previous study reported that PD-L1 was also overexpressed in almost half of the conjunctival SCC cases, with the tumor itself having the ability to escape tumor immunity.[4]

Regarding the correlation with the Ki67 labeling index, these findings indicated that the expression of thrombospondin-1 was stronger in the advanced cancer that is characterized by the faster cell growth, and thus, it is possible that the scaffold for the tumor progression is created as ECM. Furthermore, as described above, the increase in thrombospondin-1 expression may reflect the ability of cancerous cells to protect themselves from systemic tumor immunity while they are proliferating.

One of the possible clinical implications of the present study is that these results could be indicative of a new prognostic marker for conjunctival SCC, with the ability to predict PFS and final orbital exenteration based on the results of immunohistochemistry. Furthermore, although its role is poorly understood, treatments that affect the expression level of thrombospondin-1 may be options for treatment of conjunctival SCC. In particular, since it is associated with OSSN, it is possible that an intervention method could be developed for thrombospondin-1, in which eye drop administration could be used for treatment, since this would not burden the patient. In addition to the reported existing possibilities of topical application of interferonα-2b, there could be a place for using an agent directed at thrombospondin-1 [26] Previous reports have not shown

significant differences in recurrence or metastasis.[2, 27] However, it was suggested that the pathological background was poor, and it might be difficult to investigate prognosis in advanced cases. Above all, gene array analysis, which allowed us to identify a new finding related to thrombospondin-1 between the advanced cases and carcinoma in situ. Moreover, the results were in agreement with the previously reported grading of Ki67. [19] Therefore, the results of the present research using Ki67 and thrombospondin-1 may be further examined in the future.

There were several important limitations in the present study. First, regarding thrombospondin-1 expression on the ocular surface, it should be noted that changes in benign diseases and age-related changes in normal tissues still need to be sufficiently investigated. In addition, variation in RNA quality results in inaccurate and misleading changes in molecular profiling, underpinning the need for reliable and reproducible protocols for the processing of tissue and extraction of RNA.[28, 29] Numerous studies have reported the effect of RNA modification for the isolation of nucleic acids from FFPE tissue, which is important for researchers prior to undertaking experimental processes and interpreting results, including the present experiment. Furthermore, due to the small number of events, the COX models of PFS and Orbital exenteration were univariates analysis, and statistical confounding factors could not be excluded. We will need adding cases and multivariate analysis in the future.

In the present study, thrombospondin-1 expression might be correlated with PFS and necessity of final orbital exenteration. Further studies, including other multi-institutional centers, along with an increase in the number of cases need to be conducted in the future. In addition, the size of our study cohort was relatively small (n=31). Therefore, additional studies will be needed to corroborate our findings.

In conclusion, the results of this study indicate that thrombospondin-1 may be worth considering in the future and is a potential prognostic factor in the pathology of OSSN, including SCC.

Acknowledgements

We gratefully acknowledge the technical assistance of the Research Support Platform, Osaka City University Graduate School of Medicine and the Clinical Laboratory Department of Kobe Kaisei Hospital.

Funding

None of the authors has any proprietary or financial interests to declare.

Competing interests

The authors declare that they have no competing interests.

Ethics declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Approval for this study was obtained prior to the start of the study from the institutional review board at Osaka City University, Japan (IRB-4236). Written, informed consent for the storage of patient information in the hospital database and use in research was provided by all patients enrolled in the study.

Consent for publication

Not applicable.

Author information

Affiliations

Department of Ophthalmology and Visual Science, Graduate School of Medicine, Osaka City University, 1-5-7 Asahimachi, Abeno-ku, Osaka-shi, 545-8586, Japan

Norihiko Misawa, Atsushi Sakai & Shigeru Honda

Department of Molecular Pathology, Graduate School of Medicine, Osaka City University, 1-5-7 Asahimachi, Abeno-ku, Osaka-shi, 545-8586, Japan

Anna Kakehashi & Hideki Wanibuchi

Ophthalmology Department and Eye Center, Kobe Kaisei Hospital, 3-11-15, Shinohara-kita-machi, Kobe, Hyogo, Japan

Atsuko Katsuyama-Yoshikawa& Atsushi Azumi

Contributions

MT wrote the main text of the manuscript and prepared the figures. Datasets were prepared by NM and SA. AK, HW, and SH reviewed the manuscript and checked the statistical analysis. All authors have read and approved the manuscript in its final form.

Corresponding author: Mizuki Tagami.

Data Sharing

All data relevant to the study are included in the article or uploaded as online supplementary material.

References

1. Lee GA, Hirst LW (1995) Ocular surface squamous neoplasia. Surv Ophthalmol 39: 429-450 DOI 10.1016/s0039-6257(05)80054-2
2. Cruzado-Sanchez D, Tellez WA, Villarreal-Aguilar B, Melendez M, Olivera A, Moran F, Serpas-Frias S, Cordero-Garcia R (2020) Conjunctival squamous cell carcinoma: prognostic factors for the recurrence and metastasis and clinicopathological characteristics at an oncological hospital in Peru. Br J Ophthalmol 104: 1010-1015 DOI 10.1136/bjophthalmol-2019-314058
3. Scholz SL, Thomasen H, Reis H, Moller I, Darawsha R, Muller B, Dekowski D, Sucker A, Schilling B, Schadendorf D, Steuhl KP, Paschen A, Westekemper H, Meller D, Griewank KG (2015) Frequent TERT Promoter Mutations in Ocular Surface Squamous Neoplasia. Invest Ophthalmol Vis Sci 56: 5854-5861 DOI 10.1167/iovs.15-17469
4. Nagarajan P, El-Hadad C, Gruschkus SK, Ning J, Hudgens CW, Sagiv O, Gross N, Tetzlaff MT, Esmaeli B (2019) PD-L1/PD1 Expression, Composition of Tumor-Associated Immune Infiltrate, and HPV Status in Conjunctival Squamous Cell Carcinoma. Invest Ophthalmol Vis Sci 60: 2388-2398 DOI 10.1167/iovs.19-26894
5. Sakai A, Tagami M, Kakehashi A, Katsuyama-Yoshikawa A, Misawa N, Wanibuchi H, Azumi A, Honda S (2020) Expression, intracellular localization, and mutation of EGFR in conjunctival squamous cell carcinoma and the association with prognosis and treatment. PLoS One 15: e0238120 DOI 10.1371/journal.pone.0238120
6. Lawler JW, Slayter HS, Coligan JE (1978) Isolation and Characterization of a High Molecular-Weight Glycoprotein from Human-Blood Platelets. J Biol Chem 253: 8609-8616
7. Streit M, Velasco P, Brown LF, Skobe M, Richard L, Riccardi L, Lawler J, Detmar M (1999) Overexpression of Thrombospondin-1 Decreases Angiogenesis and Inhibits the Growth of Human Cutaneous Squamous Cell Carcinomas. The American Journal of Pathology 155: 441-452 DOI 10.1016/s0002-9440(10)65140-1
8. Daubon T, Leon C, Clarke K, Andrique L, Salabert L, Darbo E, Pineau R, Guerit S, Maitre M, Dedieu S, Jeanne A, Bailly S, Feige JJ, Miletic H, Rossi M, Bello L, Falciani F, Bjerkvig R, Bikfalvi A (2019) Deciphering the complex role of thrombospondin-1 in glioblastoma development. Nat Commun 10: 1146 DOI 10.1038/s41467-019-08480-y
9. Nucera C, Porrello A, Antonello ZA, Mekel M, Nehs MA, Giordano TJ, Gerald D, Benjamin LE, Priolo C, Puxeddu E, Finn S, Jarzab B, Hodin RA, Pontecorvi A, Nose V, Lawler J, Parangi S (2010) B-Raf^{V600E} and thrombospondin-1 promote thyroid cancer progression. Proceedings of the National Academy of Sciences 107: 10649-10654 DOI 10.1073/pnas.1004934107

10. Sargiannidou I, Zhou J, Tuszynski GP (2001) The Role of Thrombospondin-1 in Tumor Progression. Exp Biol Med 226: 726-733 DOI 10.1177/153537020222600803
11. Sekiyama E, Nakamura T, Cooper LJ, Kawasaki S, Hamuro J, Fullwood NJ, Kinoshita S (2006) Unique distribution of thrombospondin-1 in human ocular surface epithelium. Invest Ophthalmol Vis Sci 47: 1352-1358 DOI 10.1167/iovs.05-1305
12. Contreras-Ruiz L, Ryan DS, Sia RK, Bower KS, Dartt DA, Masli S (2014) Polymorphism in THBS1 Gene Is Associated with Post-Refractive Surgery Chronic Ocular Surface Inflammation. Ophthalmology 121: 1389-1397 DOI https://doi.org/10.1016/j.ophtha.2014.01.033
13. Tisell LE, Oden A, Muth A, Altiparmak G, Molne J, Ahlman H, Nilsson O (2003) The Ki67 index a prognostic marker in medullary thyroid carcinoma. Br J Cancer 89: 2093-2097 DOI 10.1038/sj.bjc.6601453
14. Cheang MCU, Chia SK, Voduc D, Gao D, Leung S, Snider J, Watson M, Davies S, Bernard PS, Parker JS, Perou CM, Ellis MJ, Nielsen TO (2009) Ki67 Index, HER2 Status, and Prognosis of Patients With Luminal B Breast Cancer. JNCI: Journal of the National Cancer Institute 101: 736-750 DOI 10.1093/jnci/djp082
15. Hall PA, Levison DA, Woods AL, Yu CC, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, et al. (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 162: 285-294 DOI 10.1002/path.1711620403
16. Cesano A (2015) nCounter((R)) PanCancer Immune Profiling Panel (NanoString Technologies, Inc., Seattle, WA). J Immunother Cancer 3: 42 DOI 10.1186/s40425-015-0088-7
17. Posner KL, Sampson PD, Caplan RA, Ward RJ, Cheney FW (1990) Measuring interrater reliability among multiple raters: An example of methods for nominal data. Stat Med 9: 1103-1115 DOI https://doi.org/10.1002/sim.4780090917
18. Landis JR, Koch GG (1977) The Measurement of Observer Agreement for Categorical Data. Biometrics 33: 159-174 DOI 10.2307/2529310
19. Ohara M, Sotozono C, Tsuchihashi Y, Kinoshita S (2004) Ki-67 labeling index as a marker of malignancy in ocular surface neoplasms. Jpn J Ophthalmol 48: 524-529 DOI 10.1007/s10384-004-0129-0
20. Nuessle S, Soriano D, Boehringer D, Mittelviefhaus H, Lange C, Reinhard T, Atzrodt L, Auw-Haedrich C (2020) HIF-1alpha, HIF-2alpha, and ProExC: diagnostic or prognostic relevance in conjunctival intraepithelial neoplasia? Graefes Arch Clin Exp Ophthalmol 258: 2023-2030 DOI 10.1007/s00417-020-04734-4

21. Huang TT, Sun L, Yuan XL, Qiu H (2017) Thrombospondin-1 is a multifaceted player in tumor progression. Oncotarget 8: 84546-84558 DOI 10.18632/oncotarget.19165
22. Albo D, Rothman VL, Roberts DD, Tuszynski GP (2000) Tumour cell thrombospondin-1 regulates tumour cell adhesion and invasion through the urokinase plasminogen activator receptor. Br J Cancer 83: 298-306 DOI DOI 10.1054/bjoc.2000.1268
23. Huang CH, Huang TY, Chang WJ, Pan YS, Chu HR, Li ZL, Unson S, Chin YT, Lin CY, Huang HM, Hsiung CN, Gionfra F, De Vito P, Pedersen JZ, Incerpi S, Chen YR, Lee SY, Lin HY, Davis PJ, Whang-Peng J, Wang K (2020) Combined Treatment of Heteronemin and Tetrac Induces Antiproliferation in Oral Cancer Cells. Mar Drugs 18 DOI 10.3390/md18070348
24. Tzeng HT, Tsai CH, Yen YT, Cheng HC, Chen YC, Pu SW, Wang YS, Shan YS, Tseng YL, Su WC, Lai WW, Wu LW, Wang YC (2017) Dysregulation of Rab37-Mediated Cross-talk between Cancer Cells and Endothelial Cells via Thrombospondin-1 Promotes Tumor Neovasculature and Metastasis. Clin Cancer Res 23: 2335-2345 DOI 10.1158/1078-0432.CCR-16-1520
25. Moodycliffe AM, Nghiem D, Clydesdale G, Ullrich SE (2000) Immune suppression and skin cancer development: regulation by NKT cells. Nat Immunol 1: 521-525 DOI 10.1038/82782
26. Kanaya R, Kase S, Ishijima K, Ishida S (2019) Usefulness of Topical Interferon Alpha-2b Eye Drop as an Adjunctive Therapy Following Surgical Resection in Ocular Surface Squamous Neoplasia. In Vivo 33: 2211-2215 DOI 10.21873/invivo.11724
27. McKelvie PA, Daniell M, McNab A, Loughnan M, Santamaria JD (2002) Squamous cell carcinoma of the conjunctiva: a series of 26 cases. Br J Ophthalmol 86: 168-173 DOI 10.1136/bjo.86.2.168
28. Yip L, Fuhlbrigge R, Atkinson MA, Fathman CG (2017) Impact of blood collection and processing on peripheral blood gene expression profiling in type 1 diabetes. BMC Genomics 18: 636 DOI 10.1186/s12864-017-3949-2
29. Seiler C, Sharpe A, Barrett JC, Harrington EA, Jones EV, Marshall GB (2016) Nucleic acid extraction from formalin-fixed paraffin-embedded cancer cell line samples: a trade off between quantity and quality? BMC Clin Pathol 16: 17 DOI 10.1186/s12907-016-0039-3

Table 1. Clinicopathological findings of 31 cases of conjunctival squamous cell carcinoma

	All (n=31) n (%)
Age, y	
Mean (range)	77.9 (63-98)
Sex	
Male	17 (54)
Female	14 (46)
Follow-up duration after primary surgery	
Months (range)	36.1 (12-135)
T-stage (AJCC)	
Tis	15 (49)
T1	4 (12)
T2	3 (10)
T3	7 (23)
T4	2 (6)
Primary surgery type	
Local excision	28
Orbital exenteration	3
Adjuvant therapy	
No	22 (70)
Yes	9 (30)
Additional excision	7
Topical chemotherapy	1
Radiation therapy	1
Immunohistochemical markers	
Thrombospondin-1 expression in tumors	
Positive	30 (96)
Negative	1 (4)
Thrombospondin-1 nuclear expression in tumors (score)	
Very strong (3)	11 (36)
Strong (2)	12 (38)
Weak (1)	7 (22)

Negative (0)	1 (3)
Thrombospondin-1 cytoplasm expression in tumors (score)	
Very strong (3)	6 (20)
Strong (2)	2 (6)
Weak (1)	6 (20)
Negative (0)	17 (54)
Ki67 labeling index	
≤50%	8(25)
>50%	23(75)
Average (%)	30.5
Orbital exenteration	
Yes	5 (16)
No	26 (84)
Local recurrence after curative therapy	9 (31)
Yes	18 (69)
No	
Metastasis	
Distant	0 (0)
Regional + Distant	2 (6)
Regional	1 (3)
None	27 (91)
Status at last follow-up	
Dead	3 (10)
Alive	28 (90)
Cause of death	
Conjunctival SCC (metastasis)	2 (75)
Other	1 (25)

Table 2. Forty-five significantly different genes between Tis and Tadv in the nCounterPanel

	Gene name	Log2 fold change	Std error (log2)	p-value	probe.ID	
1	THBS1-mRNA	2.74	0.324	0.000147	NM_003246.2:3465	
2	POSTN-mRNA	4.54	0.55	0.000171	NM_001135935.1:910	
3	COL1A1-mRNA	4.07	0.516	0.000219	NM_000088.3:4272	
4	BGN-mRNA	3.62	0.538	0.000525	NM_001711.3:1935	
5	COL3A1-mRNA	3.78	0.597	0.000727	NM_000090.3:180	
6	CXCR4-mRNA	3.93	0.668	0.00106	NM_003467.2:1335	
7	NFKB1-mRNA	-2.32	0.344	0.00108	NM_003998.2:1675	
8	PRF1-mRNA	3.57	0.627	0.00127	NM_005041.3:2120	
9	COL5A2-mRNA	3.08	0.548	0.00136	NM_000393.3:4075	
10	COL1A2-mRNA	3.47	0.623	0.00142	NM_000089.3:2635	
11	VSIG4-mRNA	2.45	0.459	0.00178	NM_001100431.1:160	
12	SRGN-mRNA	2.61	0.493	0.00186	NR_036430.1:65	
13	TMPRSS4-mRNA	-2.38	0.459	0.00206	NM_019894.3:1146	
14	SPARC-mRNA	2.2	0.428	0.00216	NM_003118.2:910	
15	ERMP1-mRNA	-1.14	0.228	0.0024	NM_024896.2:852	
16	ARHGAP32-mRNA	-1.32	0.27	0.0027	NM_001142685.1:2100	
17	CD163-mRNA	1.96	0.414	0.00322	NM_004244.4:1630	
18	FN1-mRNA	3.42	0.726	0.00328	NM_212482.1:1776	
19	ARHGDIB-mRNA	1.92	0.418	0.00369	NM_001175.4:833	
20	COL6A3-mRNA	2.89	0.631	0.00373	NM_004369.3:2782	
21	VAV3-mRNA	-1.82	0.4	0.00391	NM_001079874.1:352	
22	ID1-mRNA	-1.19	0.262	0.00393	NM_002165.2:345	
23	RORA-mRNA	0.563	0.125	0.00404	NM_134261.2:1715	
24	RAC2-mRNA	3.05	0.681	0.00419	NM_002872.3:1069	
25	CCL5-mRNA	3.37	0.756	0.0043	NM_002985.2:280	
26	CD46-mRNA	-0.968	0.22	0.00459	NM_172350.1:365	
27	MMP1-mRNA	3.45	0.802	0.00506	NM_002421.2:700	
28	OLFML2B-mRNA	2.13	0.495	0.00513	NM_015441.1:2920	
29	PLXNC1-mRNA	1.77	0.415	0.00533	NM_005761.2:3705	
30	STAB1-mRNA	1.75	0.416	0.00565	NM_015136.2:95	
31	PPL-mRNA	-2.2	0.529	0.00593	NM_002705.4:2435	
32	CRISPLD2-mRNA	2.54	0.614	0.00605	NM_031476.3:2575	
33	LUM-mRNA	2.38	0.575	0.00608	NM_002345.3:1285	
34	WIPF1-mRNA	1.98	0.485	0.00651	NM_001077269.1:1660	
35	HIF1A-mRNA	1.62	0.413	0.00774	NM_001530.2:1985	
36	NR4A1-mRNA	2.29	0.584	0.00774	NM_173157.1:1575	
37	ID4-mRNA	-1.61	0.411	0.0078	NM_001546.2:588	
38	ELF3-mRNA	-1.95	0.497	0.00781	NM_001114309.1:460	
39	CYBB-mRNA	2.61	0.679	0.00847	NM_000397.3:2686	
40	PRKCB-mRNA	2.82	0.738	0.00868	NM_212535.1:1750	
41	GTF2I-mRNA	-0.662	0.174	0.00899	NM_033001.2:920	
42	COL4A1-mRNA	1.91	0.509	0.00945	NM_001845.4:780	
43	SERPINE1-mRNA	2.83	0.756	0.00956	NM_001165413.1:1670	
44	THBS2-mRNA	3.24	0.875	0.01	NM_003247.2:4460	
45	NRP1-mRNA	1.39	0.374	0.01	NM_003873.5:370	

Table 3. Relationships between progression-free survival and clinicopathological and molecular factors

Variable	HR	95%CI	p
Age per year	1.305	0.904-1.882	0.155
Male sex	3.294	0.339-32.035	0.304
T-stage (AJCC)	3.428	0.356-33.031	0.287
Thrombospondin-1 total score	1.873	1.079-3.251	0.026*

CI: confidence interval, HR: hazard ratio

Statistical significance is underlined.

*p value based on a Cox proportional hazards model.

Table 4. Relationship between Orbital Exenteration and clinicopathologic and molecular factors

Variables	HR	95%CI	p
Age per year	1.091	0.944-1.260	0.241
Male SEX	2.957	0.223-39.251	0.411
T-stage (AJCC)	0.126	0.008-2.115	0.126
Thrombospondin-1 total score	2.797	1.183-6.612	0.019*

CI; indicates confidence interval, HR; hazard rate

Statistical significance is underlined. *p value based on a COX proportional hazard model.

Figure Legends

Figure 1. Volcano plot displaying differentially expressed genes between squamous cell carcinoma in situ (Tis) and invasive squamous cell carcinoma (Tadv). The vertical axis (y-axis) corresponds to the mean expression value of log 10 (q-value), and the horizontal axis (x-axis) displays the log 2-fold change value.

Figure 2. Photomicrograph of conjunctival squamous cell carcinoma (AJCC stage T2N0M0) (A) Hematoxylin-eosin (HE) staining (bar=50 μm). (B) Expression of Ki67 staining. Ki67 index 67% (bar=50 μm). (C) Expression of thrombospondin-1 staining. Nuclear score: 3 Cytoplasmic score: 3 (bar=50 μm). (D) Expression of thrombospondin-1 staining. High magnification (bar=20 μm).

Figure 3. Linear regression analysis between the Ki67 index and the thrombospondin-1 staining pattern for three groups

(Thrombospondin-1 Total Score, Thrombospondin-1 Cytoplasmic Score, Thrombospondin-1 Nuclear Score). There is a significant correlation with the thrombospondin-1 cytoplasmic score (p=0.030).