Graefe's Archive for Clinical and Experimental Ophthalmology (2018) 256:989–995
https://doi.org/10.1007/s00417-017-3864-x

PATHOLOGY

Conjunctival melanoma: association of cyclooxygenase-2 tumor expression to prognosis

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Received: 8 May 2017 / Revised: 11 November 2017 / Accepted: 11 December 2017 / Published online: 2 January 2018

Abstract

Purpose Conjunctival melanoma is a rare but potentially lethal tumor. Its biologic profile is still largely unknown, with recent studies aiming at establishing histopathological and genetic tumor profiles. The aim of this study was to analyze the association between clinicopathological characteristics and tumor expression of cyclooxygenase-2 (COX-2) to prognosis, assessing its usefulness as a possible prognostic marker.

Methods Case series of 50 patients from 1991 to 2008 with pathologically proven conjunctival melanoma. Demographic, clinical, and pathological characteristics were evaluated by reviewing clinical files and pathology. Expression of COX-2 was studied by immunohistochemistry of formalin-fixed paraffin-embedded tissue samples of 20 melanomas. Samples were classified in a score which included intensity of staining and percentage of cells with positive reactivity.

Results Clinicopathological features significantly associated ($p < .05$) with a poor prognosis (death) included involvement of fornix and tarsal conjunctiva, tumor thickness exceeding 2 mm, local tumor recurrence, lymph node, and systemic metastasis. In the immunohistochemistry study ($n = 20$), 18 cases expressed COX-2 although with different scores. However, only cases with a high score were associated with a poor outcome. Multivariate association analysis revealed that recurrence rate, metastasis, corneal invasion, and tumor thickness were associated with high score cases and, therefore, with a clinical profile with a higher risk of death.

Conclusions Results suggest that higher COX-2 expression may be a negative prognostic factor in conjunctival melanoma. Further studies can address the potential use of anti-COX-2 drugs as adjuvant therapy of this disease.

Keywords Conjunctival melanoma · COX-2 · Cyclooxygenase-2 · Prognostic factor

Introduction

Conjunctival melanoma is a rare but potentially sight- and life-threatening malignancy of the eye [1, 2]. It has an estimated incidence of approximately 0.24 to 0.8 cases per 1 million and some studies suggest that it is increasing [1, 2]. Nevertheless, it is extremely rare, representing only 2–7% of all ocular melanomas [1–3]. Risk factors for conjunctival melanoma are not well understood because of the rarity of the disease and lack of large population-based studies [1, 2, 4]. Conjunctival melanoma tends to recur locally, seed to distant parts of the conjunctiva, and systemically metastasize to regional lymph nodes [5]. Once metastatic disease has occurred, outcomes are often fatal. In fact, after 10 years, mortality rates reach 30%, as much as 50% of cases recur locally, and 25% show evidence of distant metastasis [6].

Ocular predictors of systemic metastatic disease and mortality include large basal tumor diameter, increased tumor thickness, and nonbulbar conjunctival involvement [7–13]. Genetic predictors of metastasis have not been identified, although some mutations have been reported [14]. *BRAF*

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V600E mutation occurs in about 50% of cases of conjunctival melanomas [15–17] and approximately 50% of such melanomas respond to systemic BRAF inhibitors [18].

COX-2 has been shown to be expressed on nonepithelial malignancies such as cutaneous melanomas and melanoma cell lines and seems to play a role in tumor invasion [19]. COX-2 expression in uveal melanoma has been recently studied with about 58% of these tumors expressing COX-2 and this expression being associated to histopathological markers of poor prognosis [20]. The relevance of this finding is the potential interest of COX-2 inhibitors as adjuvant therapy in uveal melanoma [21].

Up to now, the role of COX-2 in conjunctival melanomas remains largely unknown, as there are no data focusing on COX-2 expression in this tumor. The aim of this study is to report the clinicopathological findings in a cohort of cases and the results of the immunohistochemical COX-2 expression in conjunctival melanomas, discussing its potential use as a prognostic factor.

Material and methods

Materials

Retrospective study of patients treated from 1991 to 2006. Fifty clinical charts of patients from the Onco-Ophthalmology National Reference Center, Ophthalmology Department, Hospital and University Center of Coimbra, with pathologically proven conjunctival melanoma and known outcome were reviewed. Minimum follow-up of patients was 60 months. Formalin-fixed and paraffin-embedded tissue specimens were obtained from the archives of the Ophthalmic Pathology Laboratory. This study followed the WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, and was approved by the ethics committee of the Faculty of Medicine, University of Coimbra, Portugal.

Methods

Demographic and clinical characteristics including pre-existing lesions, localization, diameter, thickness, local invasion, follow-up, treatments performed, recurrences, metastasis, and tumor-related death were evaluated by reviewing clinical files.

All 50 formalin-fixed and paraffin-embedded tissue specimens were cut and stained with hematoxylin and eosin for histopathologic assessment. Diagnosis was reviewed by two experienced pathologists from the Pathology Department and by two ophthalmic pathologists from the Ophthalmic Pathology Laboratory. The tumor, node, and metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) [22] was used to stage conjunctival melanomas. Hematoxylin and eosin (HE)-stained slides were reviewed in order to determine the predominant cell type (spindle-shaped, epithelioid, or mixed) and the tumor thickness (measured from the epithelial surface to the deepest tumor cell), in line with the current TNM classification. The origin (primary acquired melanosis, nevus, or de novo) was established by reviewing HE-stained slides, staining against melan-A and cytokeratin and clinical information.

COX-2 immunohistochemistry

Immunohistochemistry for COX-2 was performed in 20 specimens using a monoclonal rabbit IgG anti COX-2 ready-to-use antibody (SP21, 1:500 dilution; Cell Marque Corporation, Rocklin, CA, USA). As a positive control, colon adenocarcinoma samples known to be positive for COX-2 were used.

From the archived paraffin blocks, 3-μm tissue sections were cut and placed on positively charged slides and allowed to dry overnight at 58 °C. COX-2 staining was performed using the Ventana BenchMark® XT (Roche, Ventana Medical Systems, Inc.) automated staining system using two different detection kits. The UltraView™ DAB Detection Kit (Roche, Ventana®) and the UltraView™ Universal Alkaline Phosphatase Red Detection Kit (Roche, Ventana®), which are both indirect biotin-free systems for detecting antibodies, were used according to the manufacturer's instructions.

The slides, antibody, and detection kits were loaded onto the BenchMark® instrument. After a mild pre-treatment (Cell Conditioning Solution 1, pH 8 for 30 min at 95 °C), the antibody was incubated at 37 °C for 16 min. After the staining run, the slides were moved from the instrument and rinsed well with wash buffer. The XT UltraView™ DAB v3 protocol was used on all 20 samples, as seen in Fig. 1, and the XT UltraView™ Red v3 protocol was used on two samples. All procedures were performed by experienced histology technicians.

Staining analysis

All samples were classified in terms of intensity of staining and percentage of cells with positive reactivity separately in a masked manner using a modified adaptation of the German Immunoreactive Scoring System [23, 24]. Scoring for the number of positive tumor cells was defined as: 0 (no positive cells); 1 (1–10% positive cells); 2 (11–50% positive cells); 3 (51–80% positive cells); and 4 (> 80% positive cells). Intensity scoring was defined as: 0 (no staining); 1 (weak staining); 2 (moderate staining); and 3 (intense staining). Multiplying both variables yielded a product (immunoreactivity score) that allowed categorization into negative (score 0–1), low (score 2–9), high (score 12) immunoreactivity.

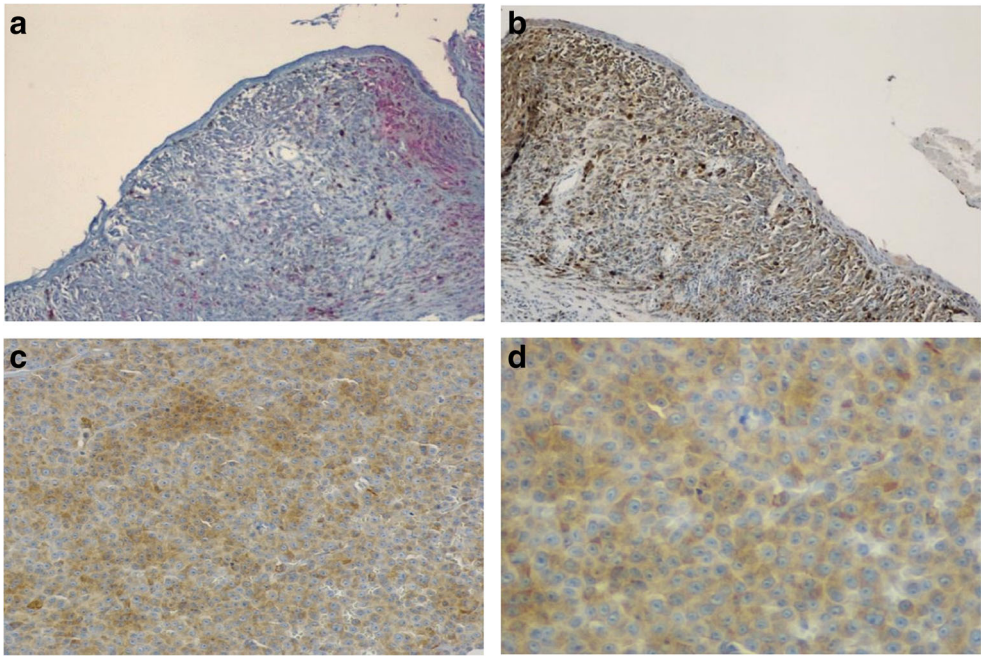

Fig. 1 Intensity staining for COX-2 in conjunctival melanoma tissues: **a** case 9: weak staining (score 1), XT UltraView™ Red v3 protocol, 100×; **b** case 11: intense staining (score 12), XT UltraView™ DAB v3 protocol, 100×; **c** case 11: moderate staining (score 6), XT UltraView™ DAB v3 protocol, 200×; and **d** case 11: moderate staining (score 6), XT UltraView™ DAB v3 protocol, 400×. The percentage of COX-2 positive tumor cells was assessed under 400 magnification

Statistical analysis

For statistical data analyses purposes, all data was analyzed using SPSS version 24.0.0.0 software. Non-parametric Mann–Whitney tests were performed for group comparison. Results were considered to be statistically significant at an alpha level of < 0.05.

Results

Clinical features

The clinical data of the patients are summarized in Table 1; 76% of tumors were clinically classified with TNM as T1, 19 (38%) as T1a, 13 (26%) as T1b, and six (12%) as T1c; 20% were classified as T2, two (4%) as T2a, five (10%) as T2b, and three (6%) as T2c. Only two (4%) cases T3b were observed. Local recurrences were observed in 26% of the patients (6% T1a, 8% T1b, 2% T1c, and 10% T2), after a median of 1.2 years. In nine cases, there was only one recurrence, and it was treated with a new surgical excision and adjuvant therapy. Two patients had a second recurrence, one had four recurrences, and another had nine recurrences. Four of these patients underwent secondary exenteration.

Ten patients (20%) were diagnosed with lymph node metastasis (4% T1a, 6% T1b, 2% T1c, 6% T2b, and 2% T3b) and from these, nine developed systemic metastasis. One patient is alive and well after excision of a cervical node metastasis. One patient developed systemic metastasis without regional nodal involvement. Treatment with chemotherapy or radiotherapy was provided to selected patients.

Tumor-related death was observed in ten cases (20%) with systemic metastasis after a mean time of 18 ± 19.3 months (range, 3–72 months) after surgical excision.

Pathological features

The lesions predominantly involved the bulbar conjunctiva (80%) and other sites that were less involved included the palpebral conjunctiva (20%), conjunctival fornix (18%), and lacrimal caruncle (8%). Conjunctival melanoma arose from pre-existing nevi in 29 cases (58%), primary acquired melanosis in ten (20%), and as de novo lesions in 11 (22%). Tumor-related death occurred in melanoma arising from pre-existing nevi in four cases (40%), primary acquired melanosis in three (30%), and as de novo lesions in three (30%).

Tumor-related death was associated (Mann–Whitney U test; $p < 0.05$) with mean large basal tumor, mean tumor thickness, tarsal conjunctiva invasion, fornix invasion, recurrences, mean number of recurrences, lymph node metastasis, systemic metastasis, and exenteration (Table 2). No statistically significant association was found between death and gender, age, right or left eye, previous lesions, development time, tumor localization, corneal or limbus involvement, treatment, or adjuvant chemotherapy.

Expression of COX-2

Expression of COX-2 was studied by immunohistochemistry of formalin-fixed paraffin-embedded tissue samples of 20 selected specimens. We were not able to evaluate two specimens since one was insufficient to be appropriately classified and in

Table 1 Clinical data of patients

Patient group	Conjunctival melanoma
Total	50
Females	27 (54%)
Males	23 (46%)
Age range (years)	23–88 years
Mean age (years) ± SD	54.64 ± 17.64 years
Females	48.7 ± 16.9 years
Males	66.8 ± 20.3 years
Follow-up (months) ± SD	79.2 ± 46.4 months
Range	60–204 months
Localization	
Bulbar conjunctiva	40 (80%)
Temporal	23 (46%)
Nasal	14 (28%)
Superior	7 (14%)
Inferior	6 (12%)
Bulbar and tarsal conjunctiva	10 (20%)
Involvement of:	
Limbus	30 (60%)
Cornea	13 (26%)
Fornix	9 (18%)
Caruncle	4 (8%)
Mean large basal tumor diameter (mm) ± SD	5.02 ± 1.97 mm
Range	2–10 mm
Mean tumor thickness (mm) ± SD	2.16 ± 1.51 mm
Range	1–8 mm
Predisposing lesions	
Pre-existing nevi	29 (58%)
Primary acquired melanosis	10 (20%)
De novo melanomas	11 (22%)

the other, melanin pigmentation was too intense to be able to be differentiated from the chromogen.

Table 2 Clinical factors associated with tumor-related death

Factor	μ	*p* value
Mean large basal tumor	64.500	< 0.01
Mean tumor thickness	41.000	< 0.01
Tarsal conjunctiva invasion	104.500	< 0.01
Fornix invasion	104.500	< 0.01
Recurrences	43.000	< 0.001
Mean number of recurrences	34.000	< 0.001
Lymph node metastasis	29.500	< 0.001
Systemic metastasis	4.500	< 0.001
Exenteration	50.000	< 0.001

Samples were classified in terms of intensity of staining and percentage of cells with positive reactivity. Only one specimen revealed absence of COX-2 immunoreactivity, five specimens showed weak staining, five moderate staining, and seven intense staining (Fig. 1). Regarding the percentage of COX-2 positive cells, four specimens had 1–10% positive cells, three had 11–50% positive cells, six had 51–80% positive cells, and four had > 80% positive cells (Table 3).

Staining pattern was homogeneous in half of the cases and patchy in the other half. When a patchy pattern was present, an enhanced expression was observed in the periphery of the tumor. Intraepithelial-positive tumor cells were observed in cases associated with primary acquired melanosis.

Overall, 94.4% of conjunctival melanomas presented positive expression for COX-2, 23.5% with an intense immunoreactivity score. Tumor-related death was associated with a high immunoreactivity for COX-2. None of the cases with a negative or low score had a negative outcome (death).

Discussion

Clinicopathological features and prognosis

Ocular predictors of systemic metastatic disease and mortality in conjunctival melanoma include large basal tumor diameter, increased tumor thickness, and nonbulbar involvement (particularly the tarsal conjunctiva, fornix, and caruncular tumor location) [7]. Two studies have validated the current AJCC [22] staging criteria, and both have demonstrated that higher-staged tumors have a higher risk of local recurrence, local and distant metastasis, and death [25, 26]. The current staging system is being revised to describe the circumferential tumor extent in quadrants irrespective of whether the tumor crosses the horizontal or vertical meridians [27, 28].

Conjunctival melanoma cases involving nonbulbar conjunctiva were associated with a higher risk of metastasis and melanoma-related mortality. This is in agreement with other studies [10, 11, 29, 30]. However, an increased mortality rate for cases involving the caruncle could not be confirmed [30]. Our results are similar to those from a population-based study from Finland in which an adverse prognosis for conjunctival melanoma with caruncular involvement could not be demonstrated either [31]. Patients with tumors located in the forniceal conjunctiva, caruncle, plica semilunaris, or eyelid margins have been shown to have a poorer prognosis [14, 29]. Local tumor recurrence is reported to occur in more than 50% of these cases [10, 14]. Our rate of recurrence was only 26%.

Tumor thickness has previously been claimed to be the sole sovereign prognosticator in conjunctival melanoma [19, 32]. We assessed initial tumor thickness and observed that the thickest tumors were more frequently located in the bulbar

Table 3 Expression of COX-2 in conjunctival melanomas

Patient	COX-2 + cells (%)*	Intensity score**	Immunoreactivity score***	Tumor-related death	TMN class	Tumor thickness (mm)
1	3	2	Low	No	pT1c	2
2	3	1	Low	No	pT1b	1
3	1	3	Low	No	pT1c	5
4	0	0	Negative	No	pT1b	1
5	2	2	Low	No	pT2c	2
6	3	2	Low	No	pT1b	2
7	2	1	Low	No	pT1b	1
8	3	3	Low	No	pT1c	1
9	1	1	Low	No	pT1c	2
10	4	3	High	Yes	pT2c	5
11	4	3	High	Yes	pT1c	2
12	1	2	Low	No	pT1c	1
13	1	3	Low	No	pT1b	1
14	3	1	Low	No	pT1c	2
15	2	1	Low	No	pT1c	2
16	3	2	Low	No	pT1c	2
17	Not able to evaluate			No	pT1c	1
18	Not able to evaluate			No	pT1c	1
19	4	3	High	Yes	pT1b	2
20	4	3	High	Yes	pT1c	7

*Scoring for the number of positive tumor cells was defined as: 0 (no positive cells); 1 (1–10% positive cells); 2 (11–50% positive cells); 3 (51–80% positive cells); and 4 (> 80% positive cells)

**Intensity scoring was defined as: 0 (no staining); 1 (weak staining); 2 (moderate staining); and 3 (intense staining)

***Immunoreactivity score: negative (score 0–1), low (score 2–9) and high (score 12)

conjunctiva. In addition, tumors exceeding 2 mm were found to be associated with a significantly higher proportion of melanoma-related death. Some studies have shown a correlation between the latest TNM classification and survival, especially regarding local recurrence [25, 33]. In this study, we observed an association with T1c and T2C tumors, confirming thickness as an important prognostic factor.

The nomenclature and classification of conjunctival melanoma have changed considerably over the years. In order to optimally assess tumor origin, we therefore re-evaluated its primary origin in all cases. We observed no differences in tumor-related mortality between de novo, primary acquired melanosis, or nevus-derived melanomas. However, 30% of patients with previous primary acquired melanosis have died, compared to 18.2% of patients with de novo melanomas and 13.8% of patients with nevus-derived melanomas.

We observed 20% of metastatic disease, which is in accordance with most studies pointing to a 20–30% rate [27, 29, 34]. Clinical risk factors included: disease recurrence, involvement of non-bulbar conjunctiva, medial bulbar conjunctiva, caruncle and plica semilunaris, and tumor thickness of more than 2 mm [10, 29, 35, 36]. The estimated 5-year mortality rate of conjunctival melanoma is 12–19%, whereas the 10-year mortality rate is about 30% [10, 33, 35, 36].

Expression of COX-2 and prognosis

There are several studies showing COX-2 overexpression in epithelial tumors such as gastric adenocarcinomas, esophageal tumors, pancreatic adenocarcinomas, bladder cancers, pulmonary adenocarcinomas, and tumors of the colon and rectum [20, 37]. Furthermore, COX-2 has also been shown to be expressed in nonepithelial malignancies such as cutaneous melanomas and melanoma cell lines. In fact, Chwirot and Kuzbicki reported that it may be possible to distinguish between cutaneous melanomas and benign lesions using a threshold percentage of COX-2-positive cells [19]. Moreover, recent studies by the same authors have shown that a higher expression of COX-2 in primary cutaneous melanoma lesions is associated with a poorer prognosis and a shorter survival [35].

The importance of COX-2 in human tumors arises from epidemiological and experimental studies using non-steroidal anti-inflammatory drugs in chemoprevention and

adjuvant chemotherapy. The use of NSAIDs has been demonstrated to decrease mortality in colon, breast, and lung cancer, and it has been approved for the adjuvant treatment of familial adenomatous polyposis by the US Food and Drug Administration [38].

Several studies assessed the role of COX-2 in carcinogenesis such as in cell growth, resistance to apoptosis, radio resistance, immunosuppression, angiogenesis, invasion, and metastasis. Muraki et al. [39] studied COX-2 inhibition in angiogenesis and concluded that COX-2 inhibitors (COX-2is) prevent proliferation and migration of tumor endothelial cells in malignant melanoma.

Tumor-associated macrophages (TAMs) have been shown to be linked to poorer prognosis and COX-2 expression in TAMs has also been investigated. It has been shown that TAMs are a predominant source of COX-2 expression in experimental models of colorectal carcinogenesis and colon adenomas. In fact, a study showed that from 100 cases of uveal melanomas, all contained TAMs, 58 contained COX-2-positive TAMs and 53 of these co-expressed COX-2 in malignant melanoma cells [39, 40].

Regarding uveal melanoma, COX-2 expression has been widely described, although its biological role has not been totally elucidated. Cryan et al. [41] showed moderate or intensive positive immunoreactivity for COX-2 in 90.6% of uveal melanoma specimens in their series and Figueiredo et al. [20] described COX-2 expression in 58% of cases.

Accordingly, our series showed positive expression of COX-2 in 17 out of 18 melanoma specimens (94.4%). In the present study, tumor-related death was associated with a high score expression for COX-2 ($n = 6$; four deaths). None of the cases showing a negative score has died. None of the cases showing a low score has died ($\mu = 4.000$; $p < 0.01$).

The work of Figueiredo et al. [20] showed that choroid and ciliary body melanomas express COX-2 and its expression is correlated with histologic poor prognostic factors, thus reflecting a more malignant phenotype. Cryan et al. [41] associated the expression of COX-2 with reduced survival rates, showing a positive association between metastatic death and both the intensity and extent of COX-2 staining.

Marshall et al. [21] have recently studied COX-2 expression and inhibition on human uveal melanoma cell proliferation, using Amfenac, an active metabolite of Nepafenac, a COX-2 inhibitor agent. This study showed that cell lines transfected to express COX-2 had higher proliferation rates than those who did not. The addition of Amfenac, a topical COX-2 inhibitor, to uveal melanoma cell lines decreased the proliferation rate of these cells, their immunohistochemical expression of COX-2 and increase their radiosensitivity [20, 21]. As for in vivo studies, Marshall et al. were able to show that the use of topical nepafenac delayed progression of intraocular tumors and the development of distant metastasis in a xenograft animal model of human uveal melanoma [37].

Based on previous reports, we evaluated the expression of COX-2 in conjunctival melanomas and, to the best of our knowledge, this was the first study on this type of tumor. All cases of tumor-related death expressed a high score. However, considering the small cohort in this study and the technical difficulties in assessing expression in two of our 20 specimens, we cannot exclude the possibility that this result may change in larger studies.

In conclusion, this study has opened a new door for research in conjunctival melanomas. We have shown that COX-2 could have a role in conjunctival melanoma and that it could be used as a prognostic factor. We hope that further studies with larger specimen numbers, and eventually an animal model using anti-COX 2, can address the potential use of anti-COX-2 drugs as adjuvant therapy in conjunctival melanoma.

Acknowledgements This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. None of the authors employed writing assistance for this manuscript.

Funding No funding was received for this research.

Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval 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.

Informed consent Informed consent was obtained before treatment from all individual participants included in the study.

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