“I should have more faith,” he said; “I ought to know by this time that when a fact appears opposed to a long train of deductions it invariably proves to be capable of bearing some other interpretation.”

Sir Arthur Conan Doyle

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List of Abbreviations

CMCs: Circulating Malignant Cells

COXs: Cyclooxygenases

COX-1: Cyclooxygenase-1

COX-2: Cyclooxygenase-2

COX-2is: COX-2 inhibitors

FDA: Food and Drug Administration

HIF-1α: Hypoxia-Inducible Factor 1-α

iNOS: Inducible Nitric Oxide Synthase

MTLN: Mean Diameter of the 10 Largest Nuclei

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs

PGs: Prostaglandins

TAMs: Tumor-associated Macrophages

TECs: Tumor Endothelial Cells

VEGF: Vascular Endothelial Growth Factor

Abstract

Introduction:

Conjunctival melanomas are extremely rare tumours, representing only 2% of all malignant lesions of the eye. Both classification and correct diagnosis of ocular melanocytic lesions can be a challenge and ambiguity in diagnosis can result in severe misguidance of management and prognosis.

Cyclooxygenase-2 is a prostaglandin synthase linked to tumour proliferation and metastization. COX-2 overexpression has been found in many tumours, including uveal melanoma, where it seems to be a marker of poor prognosis. Furthermore, it has also been proposed as a tool in differential diagnosis between cutaneous melanomas and nevi.

Objectives:

The main goal of this study is to report the results of immunohistochemical COX-2 expression in human conjunctival melanomas and nevi and discuss its potential use for differential diagnosis between benign and malignant conjunctival lesions.

Materials and Methods:

Twenty specimens of melanocytic nevi and 20 conjunctival melanomas were collected from the archives of the Ophthalmic Pathology Laboratory from Coimbra University Hospital. Immunohistochemistry detection of COX-2 was performed using a monoclonal antibody and 34 samples were classified, in terms of intensity of staining and percentage of cells with positive reactivity, to achieve a COX-2 total score.

Results:

Comparing the total score values for group, we were able to conclude that a total number of 3 cases presented a score value = 0 (1 melanoma and 2 nevi), 11 cases a score = 1 (6 nevi and 5 melanomas), 12 cases a score = 2 (5 nevi and 7 melanomas) and 8 cases a score = 4 (3 nevi and 5 melanomas). Four nevi and 2 melanoma samples were not evaluated. There was no statistically significant difference for the immunohistochemical expression of COX-2 in the 2 groups. 87.5% of all nevi showed positive expression for COX-2, 62.5% with moderate/intense scores (2 and 4). 94% of all melanomas showed positive expression for COX-2, 67% with moderate/intense scores (2 and 4). Overall, more than 90% of melanocytic conjunctival lesions express COX-2.

Discussion:

Accordingly to what was expected, our series showed positive expression of COX-2 in 17 out of 18 melanoma specimens (94.4%). However, our study we were not able to establish a significant difference in COX-2 expression between conjunctival nevi and melanomas. Consequently, COX-2 expression is not exclusive of conjunctival melanomas and cannot be used as a differentiating tool for the more challenging melanocytic conjunctival lesions.

Keywords:

Immunohistochemistry; Conjunctival Melanoma; Melanocytic Nevi; COX-2; Cyclooxygenase 2 Inhibitors; Angiogenesis

Resumo

Introdução:

Os melanomas da conjuntiva são tumores extremamente raros, que representam apenas 2% de todas as lesões melanocítica malignas do olho. Tanto a classificação como o diagnóstico correcto destas lesões parecem ser ainda pode ser um desafio, e a ambiguidade no diagnóstico pode ter um impacto grave no tratamento e no prognóstico.

A Ciclooxigenase- 2 é uma sintase das prostaglandinas ligada à proliferação tumoral e à metastização. A sobrexpressão de COX-2 tem sido evidenciada em muitos tumores, incluindo o melanoma da úvea, onde parece ser um marcador de prognóstico reservado, e tem sido também proposta como uma ferramenta de diagnóstico diferencial entre melanomas cutâneos e nevus.

Objectivos:

O principal objectivo deste estudo foi a avaliação da marcação imuno-histoquímica de COX- 2 em melanomas da conjuntiva e nevus melanocíticos, discutindo as suas potencialidades no diagnóstico diferencial entre lesões benignas e malignas da conjuntiva.

Materiais e Métodos:

Vinte casos de nevus melanocítico e vinte casos de melanoma da conjuntiva, previamente identificadas por patologia, foram recolhidos dos arquivos do Laboratório de Patologia Oftálmica do Centro Hospitalar e Universitário de Coimbra. Através do uso de um anticorpo monoclonal, foi feita a detecção imunohistoquímica de COX-2 e as 34 amostras foram classificadas em termos de intensidade de coloração e percentagem de células com reactividade positiva, tendo sido aplicado um sistema de pontuação de COX-2.

Resultados:

Comparando os valores totais do score atribuído a cada doente, em ambos os grupos, conseguimos observar que se verificaram 3 casos de score = 0 (1 melanoma e 2 nevus), 11 casos com score = 1 (6 nevus e 5 melanomas), 12 casos de score = 2 (5 nevus e 7 melanomas), e 8 casos de score = 4 (3 nevus e 5 melanomas). Quatro nevus e 2 melanomas não foram avaliados. Não foi encontrada uma diferença estatisticamente significativa entre os scores de expressão imunohistoquímica de COX-2 nos dois grupos. 87.5% de todos os nevus apresentaram uma expressão positiva para COX-2, 62,5% com scores moderados/intensos (2 e 4) e 94% de todos os melanomas apresentaram uma expressão positiva de COX-2, 67% com scores moderados/intensos (2 e 4). No global, mais de 90% das lesões melanocíticas da conjuntiva expressam COX-2.

Discussão:

De acordo com o que era esperado, os nossos resultados mostraram a existência de expressão de COX-2 em 17 de 18 melanoma marcados (94,4%). Ainda assim, o nosso estudo não foi capaz de estabelecer uma diferença estatisticamente significativa entre a expressão de COX-2 entre nevus da conjuntiva e melanomas e por isso não podemos considerar a esta expressão exclusiva dos melanomas da conjuntiva nem usar o score de expressão de COX-2 como uma ferramenta de diagnóstico diferencial para casos mais ambíguos de lesões melanocíticas conjuntivais.

Palavras-Chave:

Imunohistoquímica; Melanoma da Conjuntiva; Nevus Melanocítico; COX-2; Inibidores da Ciclooxigenase 2; Angiogénese

Introduction

The most common melanocytic tumours of the conjunctiva are melanocytic nevi, accounting for roughly one half of these lesions. A wide spectrum of conjunctival lesions derive from melanocytes, ranging from benign nevi (51.7%) to primary acquired melanosis with or without atypia (8,2% and 30,5%, respectively), to the less common malignant melanoma (9.4%).[1] Conjunctival melanoma has an estimated incidence of less than one per million per year [2,3], which makes it extremely rare, representing only 2% of all malignant lesions of the eye.[4] However, its unique characteristics also make treatment extremely challenging. In fact, after 10 years, mortality rates reach 30%, as much as 50% of cases locally recur and 25% show evidence of distant metastization.[3–5]

Both classification and correct diagnosis of ocular melanocytic lesions can still be a challenge even for experienced pathologists, particularly when nevi and melanomas present unusual characteristics. Although their cutaneous counterparts may carry some similarities, there are often unique histological patterns that make diagnosis of a conjunctival melanocytic lesion a more demanding process. Ambiguity in diagnosis can arise both clinically and histopathologically and result in severe misguidance of management and prognosis.[5,6]

Cyclooxygenases (COXs) are prostaglandin synthases that exist as two isoenzymes: cyclooxygenase-1 (COX-1), present in the majority of human tissues and cyclooxygenase-2 (COX-2), an inducible form that responds to several stimuli as growth factors and cytokines, reactive oxygen intermediates and ultraviolet irradiation. The latter is also highly upregulated during inflammation and in tumour development and progression.[7–9] More specifically, this particular enzyme has been proven to be closely related to angiogenesis stimulation, tumor proliferation, inhibition of apoptosis, immunosuppression, and metastization.[8,10,11]

There are several studies that have shown COX-2 overexpression in epithelial tumours such as gastric adenocarcinomas, oesophageal tumours, pancreatic adenocarcinomas, bladder cancers, pulmonary adenocarcinomas and tumours of the colon and rectum.[7,11] Furthermore, COX-2 has also been shown to be expressed on non-epithelial malignancies as cutaneous melanomas and melanoma cell lines and seems to play a role in tumour invasion. In fact, Chwirot and Kuzbicki[7] reported it to be possible to distinguish between cutaneous melanomas and benign lesions using a threshold percentage of COX-2 positive cells.

In what concerns uveal melanomas, a type of ocular tumour in which COX-2 has been recently studied, it seems that 58% express COX-2 and this expression is associated to histopathological markers of poor prognosis. Furthermore, even in cell lines that do not constitutively express the enzyme, when implanted in an animal eye model, they appear to start to express this it, suggesting the need of its induction by the surrounding microenvironment. The relevance of this finding is the rise of a potential interest of COX-2 inhibitors as adjuvant therapy in uveal melanoma. In fact, Marshall JC et al. showed that 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 increased radiosensitivity.[10]

Up to now, the role of COX-2 in conjunctival melanomas remains largely unknown as there is insufficient data focusing on COX-2 expression in conjunctival melanocytic lesions. Moreover, given the need to correctly differentiate conjunctival melanoma from benign melanocytic lesions, the differential immunohistochemical expression of COX-2 can become a useful marker for conjunctival melanoma. The global aim of this study is to report the results of immunohistochemical COX-2 expression in human conjunctival melanomas and nevi and discuss its potential uses in differential diagnosis between benign and malignant conjunctival lesions.

Materials and Methods

Patient Material

Formalin-fixed and paraffin embedded tissue specimens were obtained from the archives of the Ophthalmic Pathology Laboratory of Coimbra University Hospital, Coimbra, Portugal.

The study was based on two groups of specimens, conjunctival melanomas and melanocytic nevi, from patients followed on the Ocular Oncology Unit. The study included a total of 20 conjunctival melanoma specimens from 20 patients who underwent tumour excision from 2001 to 2008, and 20 melanocytic nevi specimens excised from 20 patients between 2011 and 2015.

All sections were stained with haematoxylin-eosin for histopathologic assessment and the diagnosis was made by an experienced ocular pathologist. Clinical records of the patients were consulted.

The project was approved by the committee of ethics of the Faculty of Medicine, University of Coimbra, Portugal.

Immunohistochemistry detection of COX-2

From the histological blocks of paraffin, three-micrometre tissue sections were cut and placed on positive charged slides and allowed to dry overnight at 58ºC.

COX-2 staining was performed using the Ventana BenchMark® XT (Roche, Ventana Medical Systems, Inc.) automated staining system. Two different detection kits were used. The *ultra*View™ DAB Detection Kit (Roche, Ventana®) and the *ultra*View™ Universal Alkaline Phosphatase Red Detection Kit (Roche, Ventana®) are both indirect biotin-free

systems for detecting antibodies, and were used according to standard manufacturer's instructions.

The slides, antibody and detection kits were loaded onto the BenchMark® instrument. After a mild pre-treatment (Cell Conditioning Solution 1, pH8 for 30 minutes at 95ºC), the antibody incubated at 37ºC for 16 minutes. After the staining run, the slides were moved from the instrument and rinsed well with wash buffer.

The XT *ultra*View DAB v3 protocol was used on all 40 the samples and the XT *ultra*View Red v3 protocol was used on 3 samples: two conjunctival melanomas and a nevus.

Immunohistochemistry was performed using a monoclonal rabbit IgG anti COX-2 ready to use antibody (SP21, 1:500 dilution; Cell Marque Corporation, Rocklin, CA).

As a positive control, colon adenocarcinoma samples known to be positive for COX-2 were used.

All procedures were performed by experienced histology technicians.

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Staining analysis

All samples were classified in terms of intensity of staining and percentage of cells with positive reactivity (Table I).

The immunohistochemical staining intensity of cells was graded as: (-) absent; (+) slight to moderate; and (++) intense (similarly to that of positive control). The COX-2 immunoreactivity was also graded according to its extent into two further categories: high, when positivity was observed in >50% of cells; and low, when positive staining was observed in ≤50% of tumour cells.

In order to link the cell's maximum intensity and percentage of staining for COX-2, we applied a scoring system[12] and attributed a final COX-2 score value to each tumor sample. The score was calculated by multiplying the individual percentage of stained cells to an attributed intensity factor (0, 1, 2 or 4) of each tumour.

The analysis of all samples was carried out by two experienced pathologists from the Pathology Department from Coimbra University Hospital - Coimbra, Portugal.

Table I. Staining analysis

Staining intensity grading		
(-)	0	Absent
(+)	1	Slight
(++)	2	Intense
Immunoreactivity		
Low	1	<50% stained cells
High	2	≥50% stained cells

Statistical analysis

For statistical purposes, all data were analysed using SPSS version STATA 13.1 software. The correlations between immunohistochemical COX-2 staining intensity and immunoreactivity for the two groups of specimens were studied using Kruskal-Wallis Test. A p value <0.05 was considered to be significant.

Results

Clinical Data

On the nevi group the patients ages varied between 11 and 79, at the moment of excision, with a mean age of 43.3 years, and 70% of them were female. Patients from the conjunctival melanoma group, on the other hand, presented an age range from 16 to 82, with a mean value of 49.5, and 55% were female (Table II). All patients were Caucasian. Clinical records of the patients, when available, were consulted for data regarding the affected eye, localization of the tumour and relevant histological information (Tables III and IV).

Table II. Demographic characteristics of patients

Patient Group	Melanocytic Nevi	Conjunctival Melanomas
Total	20	20
Female	14 (70%)	11(55%)
Male	6 (30%)	9 (45%)
Age Range	11-79	16-82
Mean Age (years)	43.26	49.5

Table III. Clinical data from the conjunctival nevi patients

Melanocytic Nevi					
Patient nº	Sex	Age	Eye	Localization	Histology
1	M	70	LE	Perilimbic, 9h	Corneal invasion
2	F	79	RE	Palpebral, Superior	NA
3	F	71	LE	Caruncula	NA
4	M	31	LE	Caruncula	NA
5	F	19	RE	Bulbar and Palpebral	NA
6	M	NA	RE	Bulbar, NS	NA
7	F	76	RE	Perilimbic, 7h	Atypical nevus
8	F	42	RE	Bulbar, Nasal	Cysts present
9	M	23	LE	Caruncula	NA
10	F	12	LE	Perilimbic, 9h	Compound nevus
11	F	20	RE	Bulbar, NS	Episcleral Vascular Engorgement
12	M	66	LE	Perilimbic, 3h	Keratinization Present
13	F	52	RE	Caruncula	NA
14	F	34	LE	Perilimbic, 6-9h	NA
15	M	19	RE	Bulbar, Temporal	Elevated, sessile
16	F	11	LE	Perilimbic, 3h	Vascular Engorgement
17	F	58	RE	Bulbar, Temporal	Vascular tortuosity, corneal invasion, cysts present
18	F	38	NA	Bulbar, NS	Pedunculated
19	F	71	LE	Bulbar, NS	Leucoplasic
20	F	30	RE	Perilimbic, 9h	NA

M: masculine; F: feminine; Age: in years; RE: right eye; LE: left eye; NA: information not available; NS: information not specified;

Table IV. Clinical data from the conjunctival melanoma patients

Conjunctival Melanomas					
Patient nº	Sex	Age	Eye	Localization	Histology
1	F	70	RE	Perilimbic, Nasal	Corneal invasion
2	F	76	RE	Perilimbic, Temporal	NA
3	M	76	NA	NA	NA
4	F	78	LE	Palpebral, Superior, External 1/3	NA
5	F	16	RE	Bulbar, NS	NA
6	M	23	LE	Bulbar, Nasal	NA
7	F	NA	NA	NA	NA
8	M	30	LE	Caruncula	NA
9	M	21	NA	NA	NA
10	F	82	LE	Caruncula, Perilimbic, Temporal	Vascular engorgement, Corneal Invasion
11	F	56	RE	Perilimbic, Inferior	PAM, corneal invasion
12	M	29	LE	Perilimbic, Temporal	Corneal invasion
13	F	27	RE	Perilimbic, Temporal	NA
14	F	49	RE	Palpebral, Inferior, Middle 1/3	NA
15	M	30	NA	Perilimbic, Nasal	NA
16	M	55	RE	Perilimbic, Temporal	NA
17	F	35	LE	Bulbar, Nasal	Multiple serous cysts present
18	M	68	RE	Perilimbic, Temporal	NA
19	F	72	NA	Perilimbic, Nasal	NA
20	F	76	RE	Bulbar, Temporal	Corneal invasion

M: masculine; F: feminine; Age: in years; RE: right eye; LE: left eye; NA: information not available; NS: information not specified; PAM: primary acquired melanosis

Expression of COX-2 in Conjunctival Nevi

In the conjunctival nevi group (Table V), 20 specimens were submitted to COX-2 staining. Two specimens revealed absence of COX-2 positive cells, 6 specimens showed slight intensity of COX-2 positive cells, 3 showed moderate intensity, and 5 revealed intense staining. However, 4 specimens were not able to be evaluated. In one of these slides, melanin pigmentation was too intense to allow differentiation from COX-2 immunoexpression, once 3,3-diaminobenzidine tetrahydrochloride (DAB) was the used chromogen. On three other specimens, the lesion was insufficient to be appropriately classified. As far as the percentage of positive cells is concerned, 6 specimens had <50% of COX-2 positive cells and 8 had ≥50%. 87.5% of all nevi presented positive expression for COX-2, 62.5% with moderate/intense scores (2 and 4).

Expression of COX-2 in Conjunctival Melanoma

For the conjunctival melanoma group (Table VI), 20 specimens were submitted to COX-2 staining, only 1 specimen revealed absence of COX-2 immunoreactivity, 5 specimens showed slight intensity, 5 moderate intensity, and 7 intense staining. Nevertheless, 2 specimens were not able to be evaluated since one was insufficient to be appropriately classified and on the other, melanin pigmentation was too intense to be able to be differentiated from the chromogen. Concerning the percentage of COX-2 positive cells, 7 specimens had <50% and 10 had ≥50%. 94% of all melanomas presented positive expression for COX-2, 67% with moderate/intense scores (2 and 4).

Table V. Expression of COX-2 in conjunctival nevi

Melanocytic Nevi			
Patient nº	% of COX-2 positive cells	Intensity of COX-2 positive cells	COX-2 score
1	<50	+ moderate	2
2	>50	+ moderate	2
3	>50	+ slight	1
4	<50	+ slight	1
5	0	- absent	0
6	Not evaluated		
7	Not evaluated		
8	>50	+ slight	1
9	>50	+ slight	1
10	>50	+ moderate	2
11	>50	++ intense	4
12	<50	+ slight	1
13	Not evaluated		
14	<50	++ intense	2
15	<50	++ intense	2
16	>50	++ intense	4
17	<50	+ slight	1
18	>50	++intense	4
19	Not evaluated		
20	0	- absent	0

Table VI. Expression of COX-2 in conjunctival melanoma

Conjunctival Melanomas			
Patient nº	% of COX-2 positive cells	Intensity of COX-2 positive cells	COX-2 score
1	>50	+ moderate	2
2	>50	+ slight	1
3	<50	++ intense	2
4	0	- absent	0
5	<50	+ moderate	2
6	>50	+ moderate	2
7	<50	+ slight	1
8	>50	++ intense	4
9	<50	+ slight	1
10	>50	++ intense	4
11	>50	++ intense	4
12	<50	+ moderate	2
13	<50	++ intense	2
14	>50	+ slight	1
15	<50	+ slight	1
16	>50	+ moderate	2
17	Not able to evaluate		
18	Not able to evaluate		
19	>50	++ intense	4
20	>50	++ intense	4

Differential Expression

Comparing the total score values attributed to each patient in both groups, we were able to conclude that in total there were 3 cases of score value = 0 (1 melanoma and 2 nevi), 11 cases with score = 1 (6 nevi and 5 melanomas), 12 cases of score = 2 (5 nevi and 7 melanomas), and 8 cases of score = 4 (3 nevi and 5 melanomas) (Table VII and Fig.1-7). Overall, more than 90% of melanocytic conjunctival lesions express COX-2.

Table VII. Immunohistochemistry score results in percentages

	Melanocytic Nevi	**Conjunctival Melanomas**
Score	No (%)	No (%)
0	2 (12.5%)	1 (5.56%)
1	6 (37.5%)	5 (27.78%)
2	5 (31.25%)	7 (38.89%)
4	3 (18.75%)	5 (27.78%)

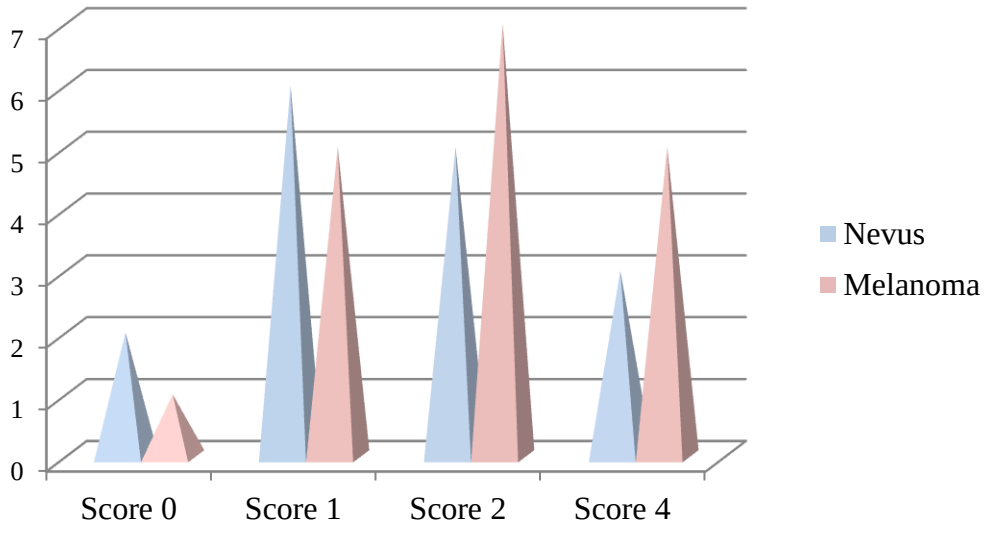

Fig.1. Immunohistochemistry score results by number of cases

A Kruskal-Wallis test was conducted to determine if the immunohistochemical score was significantly different for the two groups, but it showed no statistically significant difference between conjunctival nevi and melanomas, $\chi 2(2) = 0.339$, $p = 0.56$.

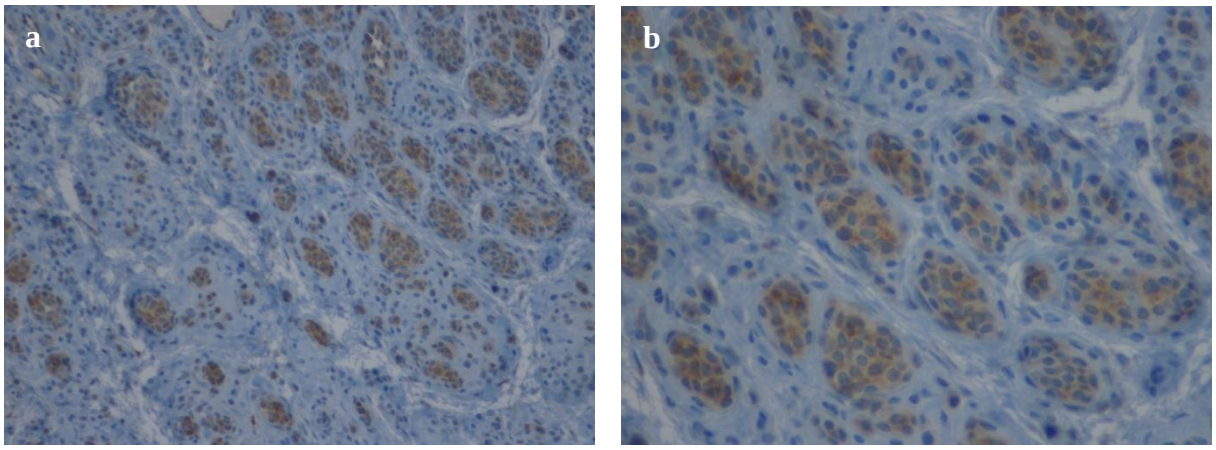

Fig. 2 (a,b). Intense expression in conjunctival nevi of COX-2 using the *ultra*View™ DAB Detection Kit (200x and 400x).

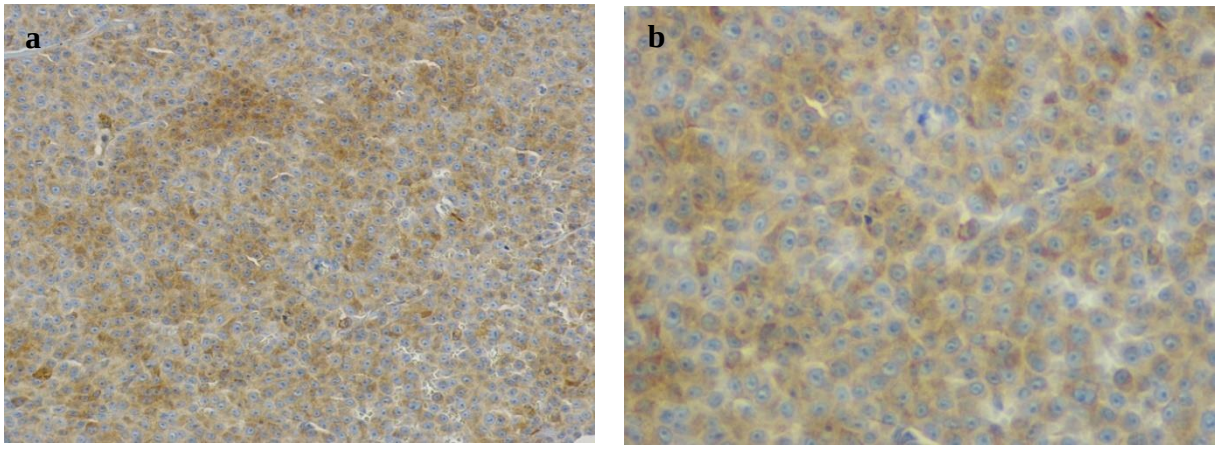

Fig. 3 (a,b). Intense expression in conjunctival melanoma of COX-2 using the *ultra*View™ DAB Detection Kit (200x and 400x).

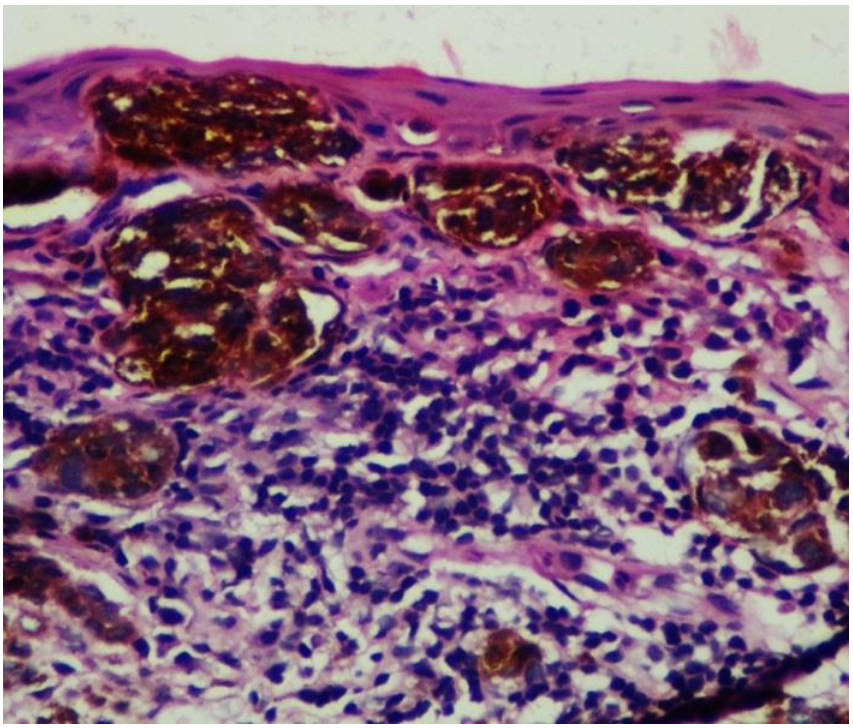

Fig. 4. Intense expression of melanic pigment in conjunctival nevi (400x): we are unable to differentiate the pigment from COX-2 expression using the *ultra*View™ DAB Detection Kit.

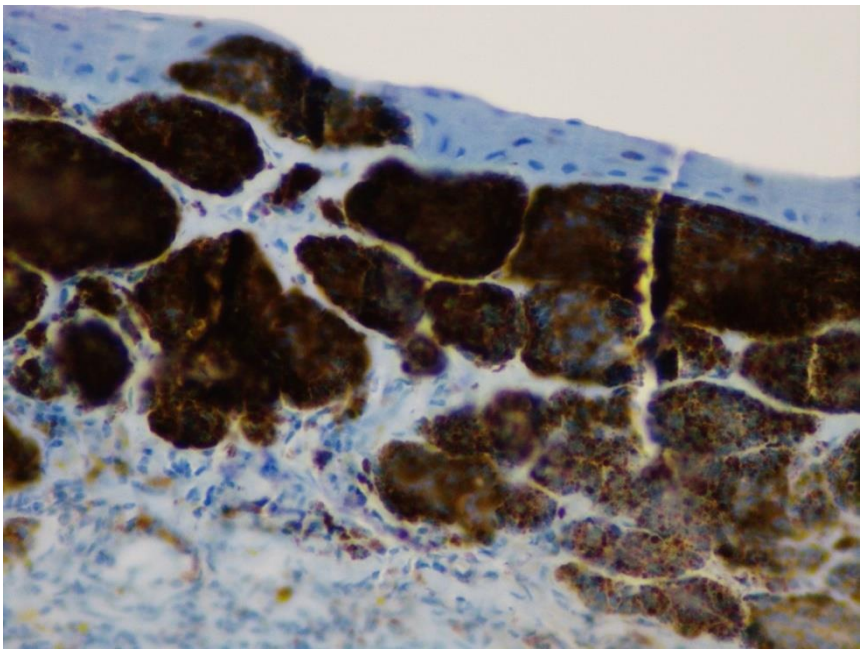

Fig. 5. Intense expression of melanic pigment in conjunctival nevi (400x): we are unable to differentiate the pigment from COX-2 expression using the *ultra*View™ DAB Detection Kit.

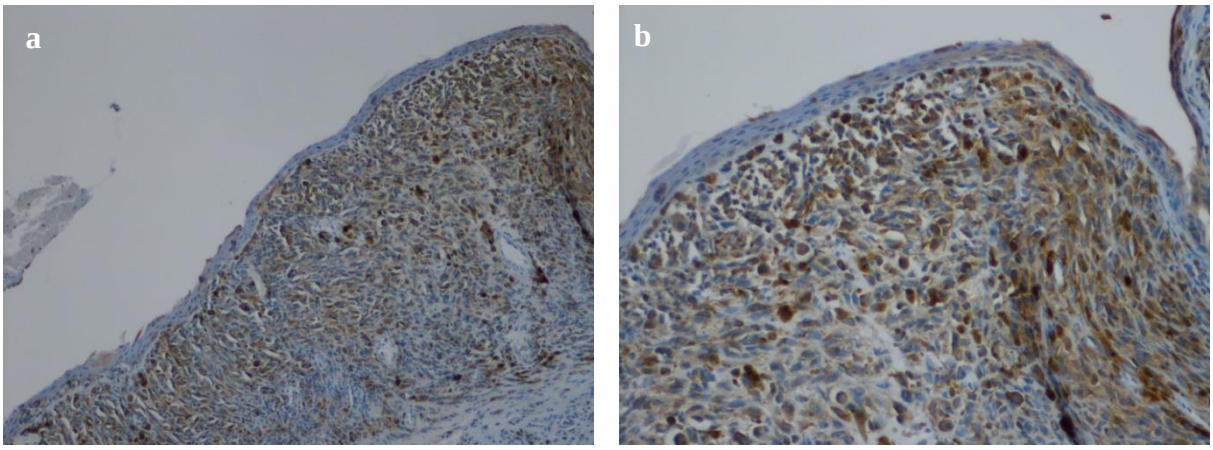

Fig. 6. Expression of melanic pigment in a conjunctival melanoma unable to be differentiated from COX-2 expression using the *ultra*View™ DAB Detection Kit (a:100x and b: 200x).

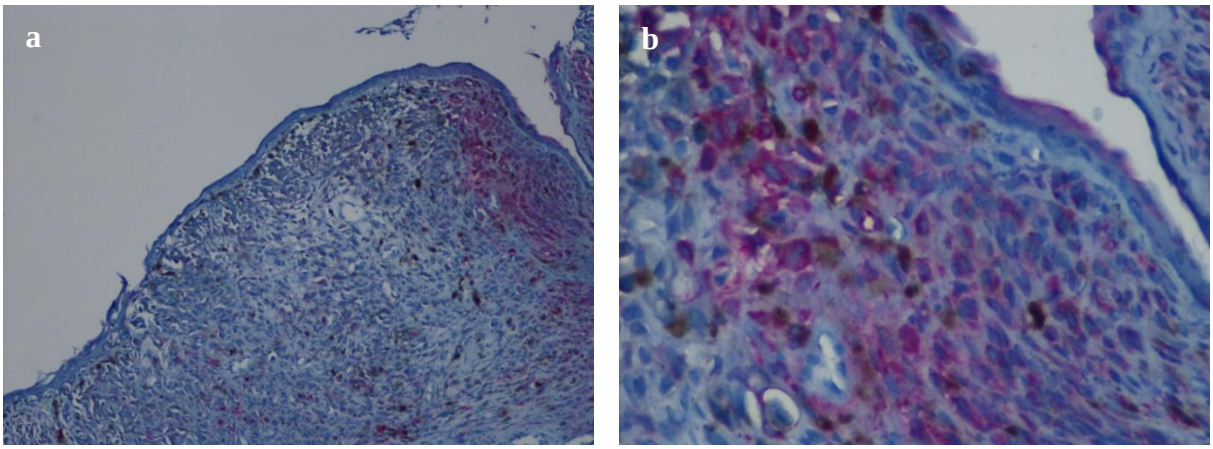

Fig.7. Lack of expression of melanic pigment in the same conjunctival melanoma using the *ultra*View™ Universal Alkaline Phosphatase Red Detection Kit revealing true COX-2 expression (a: 100x, and b: 400x).

Discussion

Cyclooxygenase is an enzyme that is present in mammalian cells in two isoforms: cyclooxygenase-1 and cyclooxygenase-2 (COX-2). The expression of COX-2, the inducible form, has been consistently associated to epithelial and non-epithelial cancers. COX-2 is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandins (PGs) and thromboxane. Moreover, PGE2 has been suggested to be the major mediator of various tumorigenic processes.[9]

The importance of COX-2 in human tumours arises from epidemiological and experimental studies using non-steroidal anti-inflammatory drugs (NSAIDs) in chemoprevention and adjuvant chemotherapy. The use of non-steroidal anti-inflammatory agents 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 (FDA).[13] COX-2 is expressed in neoplastic cells of tumours from stromal and epithelial origin and in the vasculature of various tumours.[12]

Several studies postulated the role of COX-2 in carcinogenesis such as cell growth, resistance to apoptosis, radio resistance, immunosuppression, angiogenesis, invasion and metastization. In what concerns to tumour angiogenesis, vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1-α (HIF-1α) and inducible nitric oxide synthase (iNOS) are known to take part in its promotion. Within the tumour microenvironment, endothelial cells exposed to hypoxic conditions, induce HIF-1α expression, which in turn upregulates COX-2. Conversely, COX-2 stimulates angiogenesis via the induction of VEGF expression in tumour and stromal cells.[9,13]

Muraki et al. studied COX-2 inhibition on angiogenesis and concluded that COX-2 inhibitors (COX-2is) prevent proliferation and migration of tumour endothelial cells (TECs) via Akt phosphorylation, a transduction pathway that promotes survival and growth in response to signals coming from the tumour microenvironment. They were also able to demonstrate that COX-2 is overexpressed in TECs in malignant melanoma specimens and that their growth and angiogenesis were inhibited by COX-2is.[8]

Tumour-associated macrophages (TAMs) have been shown to be linked to poorer prognosis, both regarding their number or distribution, in a number of human malignancies. COX-2 expression in TAMs has been investigated in very few studies, however, evidence of paracrine interactions between tumour cells and macrophages, has been found, with COX-2 being expressed in both. Bamba, Ota *et al*. and Chapple, Cartwright *et al.* showed TAMs to be a predominant source of COX-2 expression in experimental models of colorectal carcinogenesis and colon adenomas. In fact, in 2004, 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. They were able to conclude that COX-2 expressing TAMs were more abundant than COX-2 expressing tumour cells in uveal melanoma.[13]

Also regarding uveal melanoma, COX-2 expression has been widely described, although its biological role has not been totally elucidated. Cryan et al.[12] showed moderate or intensive positive immunoreactivity for COX-2 in 90.6% of uveal melanoma specimens on their series and the group of Figueiredo et al. described COX-2 expression in 58% of their immunostained cases. Accordingly, our series showed positive expression of COX-2 in 17 out of 18 melanoma specimens (94.4%).

The work of Figueiredo et al. showed that choroid and ciliary body melanomas express COX-2 and its expressions correlated with histologic poor prognostic factors as cell type (epithelioid tumours), mean diameter of the 10 largest nuclei (MTLN), presence of lymphocytic infiltration and vascular closed loops, thus reflecting a more malignant phenotype. Cryan et al. 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.[12,14]

Marshall J.C. et al. have recently studied COX-2 expression and inhibition on human uveal melanoma cell proliferation and macrophage nitric oxide production, 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. Furthermore, Amfenac significantly reduced proliferation rates in all cell lines both transfected and not transfected to express COX-2 and was even able to partially overcome the inhibition of macrophage nitric oxide production by a melanoma conditioned medium.[15]

As for in vivo studies, Marshall J.C et al. were able to show that the use of topical Nepafenac delayed progression of intraocular tumours and the development of distant metastasis in a xenograft animal model of human uveal melanoma. This group studied COX-2 expression and inhibition in an ocular and metastatic animal model of uveal melanoma during twelve weeks by evaluating the influence of Nepafenac, on rabbits' survival rate, weight, general condition, intraocular tumour growth, malignant cell dissemination by the levels of circulating malignant cells (CMCs), and metastasis formation. They were able to conclude that: 1) all veterinary standards of animal well-being were significantly worse on the control group; 2) there were more detectable tumours on the control groups; 3) the development of metastasis in the lung was considerably delayed in the experimental group; 4) the cumulative

incidence of micrometastasis in the experimental group was lower and delayed and 5) CMCs were detected earlier in the control group.[10]

Based on previous reports we proposed to evaluate the immunoexpression of COX-2 in conjunctival melanomas and nevi lesions. To the best of our knowledge this was the first study to evaluate such expression in both conjunctival specimens.

However, in our study we were not able to establish a statistically significant difference in COX-2 expression - intensity of staining and percentage of cells with positive reactivity – between conjunctival nevi and melanomas. Unlike Chwirot and Kuzbicki who were able to differentiate benign from malignant lesions, based on a threshold percentage value (the melanoma indicator) of 20% COX-2 immunostained cells, we were not able to find such a difference in conjunctival melanocytic lesions.

It is extremely important to understand whether other factors may be responsible for the results obtained. As a main limitation of the study we point out the small number of specimens submitted to staining. In addition to this, 4 conjunctival nevi and 2 melanomas were not able to be evaluated.

Accordingly, we cannot yet consider COX-2 expression exclusive of conjunctival melanomas, nor can the COX-2 expression score be used as a differential diagnosis tool for the more challenging cases, as we proposed. However, considering the small cohort in this study, and the technical difficulties in assessing expression in 6 of our 40 specimens, we cannot exclude the possibility that this result may change in larger studies.

In fact, although both tumours expressed COX-2 in all ranges of intensity, it seems that in the conjunctival melanoma group there were lesser cases of lower intensity, with scores of 0 and 1, and more cases of higher COX-2 expression, with scores of 2 and 4 (Fig.1).

This observation raises other questions, and it would take a larger study to address them. Future directions were pointed out by the questions we raised. 1) Given that both tumours seem to express COX-2, is it possible that quantitative measures may differentiate them? 2) Would a better threshold for COX-2 expression give us the significance we are looking for? 3) Can conjunctival nevi be proved to express lower levels of COX-2 compared to melanomas? 4) Would clinical and histological properties that constitute risk factors for malignancy, like the ones discussed before in this section, somehow correlate with higher intensities of expression?

In conclusion, this study has opened a new door for research in conjunctival tumours. We have shown that COX-2 has a role in the development of both benign and malignant tumours of the conjunctiva and we hope that further studies can address the potential use of an anti-COX-2 drug as therapeutic adjuvant or even a palliative option for conjunctival melanoma.

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