Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

# COX-2 as a determinant of lower disease-free survival for patients affected by ameloblastoma

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ARTICLE INFO

*Keywords:*
Ameloblastoma
Odontogenic tumors
COX-2
Cyclooxigenase-2
CD34

ABSTRACT

Ameloblastoma is a locally aggressive neoplasm with a poorly understood pathogenesis. Therefore, the aim of this study is to investigate whether COX-2 expression is associated with ameloblastoma microvascular density (MVD) and with tumor aggressiveness. Sixty-three cases of primary ameloblastomas arranged in tissue microarray were submitted to immunohistochemistry against cyclooxigenase-2 (COX-2) and CD34. Clinicopathological parameters regarding sex, age, tumour size, tumour duration, tumour location, treatment, recurrences, radiographic features, vestibular/lingual and basal cortical disruption and follow-up data were obtained from patients' medical records and correlated with the proteins expression. The results on BRAF-V600E expression were obtained from our previous study and correlated with COX-2 and CD34 expressions. Log-rank univariate analysis and multivariate Cox regression model were done to investigate the prognostic potential of the molecular markers. Twenty-eight cases (44.4%) exhibited cytoplasmic positivity for COX-2, predominantly in the columnar peripheral cells, with a mean MVD of 2.2 vessels/mm$^2$. COX-2 was significantly associated with recurrences ($p < 0.001$) and BRAF-V600E expression ($p < 0.001$), whereas lower MVD was associated with the use of conservative therapy ($p = 0.004$). Using univariate and multivariate analyses, COX-2 was significantly associated with a lower 5-year disease-free survival (DFS) rate ($p < 0.001$ and $p = 0.012$, respectively), but not with a higher MVD ($p = 0.68$). In conclusion, COX-2 expression in ameloblastomas is not associated with MVD, but it is significantly associated with recurrences and with a lower DFS.

## 1. Introduction

Odontogenic tumors is a heterogeneous group of neoplasms that account for less than 5% of all cases diagnosed in specialized oral pathology centers, and ameloblastoma represents the most common subtype in many epidemiological studies [1,2].

Given its locally aggressive clinical behavior that is associated with frequent recurrences and a high morbidity, ameloblastoma is considered the most important entity in this group of neoplasms [2,3].

The molecular basis of ameloblastoma has been intensely investigated in the last decades, but more important findings were obtained recently with the detection of BRAF-V600E mutation in approximately 60% of the cases, which has been considered an important event for the development of the neoplasm and was shown by our group to carry a prognostic role for the affected patients [4,5]. However, more studies are still necessary to better comprehend the pathogenesis of ameloblastoma and the investigation of molecules already known to be involved with different biological processes in other human neoplasms remain to be fully addressed in the context of ameloblastoma.

Angiogenesis, frequently measured by CD34 expression [6], is considered of great importance for tumor maintenance, growth and spread. It has been investigated in a large number of human neoplasms, and several authors have already attempted to demonstrate the importance of angiogenesis to the pathogenesis of ameloblastoma [7–9]. Cyclooxygenase (COX) is one of the molecules with known angiogenic potential in different human tumors. It is a key regulatory enzyme in

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https://doi.org/10.1016/j.prp.2018.03.014
Received 24 January 2018; Received in revised form 27 February 2018; Accepted 13 March 2018

the conversion of arachidonic acid into prostaglandins that have important functions in almost every system and regulate different physiological processes such as immunity, cell reproduction, maintenance of vascular integrity and tone [10].

There are three known isoforms of cyclooxygenase, COX-1, COX-2 and the recently described COX-3 [11]. COX-2 is an enzyme that is not found in normal conditions, but is induced in a variety of pathological conditions [10,12]. Different studies have showed a strong association between COX-2 expression and an increased number of blood vessels in various human cancers, as well as with a higher expression of the angiogenic marker VEGF [13,14]. However, although VEGF was shown to be over-expressed in ameloblastomas [15,16], the importance of COX-2 for angiogenesis in ameloblastoma has not been investigated. Moreover, to date, the few studies addressing COX-2 expression pattern in this neoplasm does not consider its clinical importance [17–19].

Therefore, in order to test the hypothesis that COX-2 would be involved with the angiogenic process of ameloblastomas and that this protein is a determinant of a worse prognosis for the affected patients, using a large sample of primary ameloblastomas we aim in this study to determine whether the expression of COX-2 enzyme is associated with the ameloblastoma vascular density measured by CD34 expression and whether the enzyme is associated with clinicopathological parameters consistent with tumor aggressiveness.

## 2. Material and methods

### 2.1. Samples

Formalin-fixed, paraffin-embedded, non-decalcified tissue blocks of 63 solid ameloblastomas from the Department of Otolaryngology and Head and Neck Surgery of the A. C. Camargo Cancer Center with available clinical and follow-up informations were retrospectively retrieved. Haematoxylin and eosin (H&E) stained slides were used to confirm the original diagnoses. Clinical data regarding age, sex, tumor size, tumor location, tumor duration according to the patient, treatment, presence of recurrences, microscopic subtype, radiographic pattern, vestibular/lingual/palatine bone cortical disruption, basal bone cortical disruption, and follow-up data were obtained from patients' medical records. Moreover, none of the patients included in this study was using non-steroidal anti-inflamatory drugs. The disease-free survival (DFS) was obtained by calculating the time between the treatment date and the date of recurrence or the last follow-up date obtained. This study was approved by the institutional research ethics committee (A. C. Camargo Cancer Center, Protocol No. 669/05), the informed consent was obtained from patients and it was performed in accordance with the Declaration of Helsinki.

### 2.2. Tissue microarray

The construction of tissue microarray (TMA) block was previously described and already used for other studies of our group. Briefly, after the microscopic review of the cases retrieved, areas of interest in each case were selected from their respective H&E stained sections and a TMA block was constructed (Beecher Instruments, Silver Springs, USA). For this study morphologically representative areas in the center of the tumor containing as much neoplastic cells and surrounding stroma as possible were selected for all cases. The areas identified in the donor paraffin block were punctured twice with a 1.0 mm needle, and the two cylinders obtained were transferred to the receptor paraffin block. Sequential 3 µm-thick sections were cut and collected on adhesive slides. A map specifying the exact position of each core was made and a cylinder of normal liver tissue was included in the lower left corner of the TMA block for orientation.

### 2.3. Immunohistochemistry

Immunohistochemical reactions were done in 3 µm histological sections of all cases. Samples were dewaxed with xylene and then hydrated in an ethanol series. Antigen retrieval was performed using citrate solution (pH 6.0) in a pressure cooker for 3 min, and the endogenous peroxidase activity was blocked using 10% hydrogen peroxide. After washing in PBS buffer (pH 7.4), slides were incubated overnight with primary antibodies against COX-2 (clone CX-294, 1:200 dilution; Dako, Hamburg, Germany) and CD34 (QBEnd10; 1:50 dilution; Dako, Carpinteria, CA, USA). All slides were subsequently exposed to super-sensitive, non-biotin based, immunohistochemical visualization system (Advance Kit-DakoCytomation), and diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA), and subsequently counter-stained with Carazzi haematoxylin. Positive controls were used for each antibody, including breast cancer and Kaposi's sarcoma sections, respectively. Negative controls were obtained by omitting the primary antibodies.

### 2.4. Immunohistochemistry quantification

TMA glass slide containing the immunohistochemical reactions against CD34 was scanned into high-resolution image using the Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc, Vista, CA) and the digital image obtained in .svs format was visualized using the ImageScope software (Aperio Technologies Inc., Vista, CA). CD34 staining was analyzed using the Microvessel Analysis V1 algorithm (Aperio Technologies Inc.) and the microvascular density (MVD), defined as the number of vessels/mm$^2$, was collected [20,21]. COX2 immunoexpression was semi-quantified by two observers independently, blinded to clinicopathological and CD34 data, which classified each case as negative (no expression), weak positive (presence of 1–30% of tumor cells positivity) or strong positive (presence of > 30% of tumor cells positivity). Disagreements were jointly settled.

### 2.5. Statistical analysis

Quantitative results obtained with CD34 expression for MVD were categorized as above or below the mean value. To investigate the association of COX-2 and CD34 with clinicopathological parameters the two-tailed Fisher's exact test and the chi-square test were used. Survival analysis was calculated using the Kaplan–Meier method and the Log-rank univariate test was used to compare the survival curves. COX proportional hazard model was used as multivariate analysis and two different models were created. First, all parameters that achieved a $p$-value < 0.25 were included in the COX model. Second, all clinicopathological parameters, including COX2, MVD and BRAF-V600E expression, which were believed to carry an important biological potential do predict ameloblastoma aggressiveness no matter what $p$-value they have achieved in the univariate analysis, were included in the model. Lastly, we have also performed the univariate and multivariate survival analyses restricted to those cases with at least 24 months of follow-up. Original data on BRAF-V600E was obtained from our previous study using the same studied population [4]. IBM SPSS version 20 (Chicago, IL, USA) software was used for statistical analyses and a $p$-value ≤ 0.05 using a 95% confidence interval was considered statistically significant.

## 3. Results

### 3.1. Clinicopathological and follow-up features

In this study, we investigated COX-2 immunoexpression in 63 cases of ameloblastoma that remained available in our original TMA block. Detailed clinicopathological data of the current sample is presented in Table 1. Briefly, there was a similar gender distribution (M:F ratio of