SIRT2 Expression Is Higher in Uveal Melanoma than In Ocular Melanocytes

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Key Words

Uveal melanoma · Sirtuin 2 · Uveal melanoma progression

Abstract

Purpose: Sirtuins (SIRT2s) are the family of proteins associated with the cell cycle and that correlate with cancer development and progression. SIRT2s have never been studied in uveal melanocytes. The aim of this study is to characterize the expression of SIRT2 in uveal melanoma (UM) cases and compare it with the expression of SIRT2 in melanocytes of the uveal tract of normal human eyes (NHE). **Methods:** Twenty-one formalin-fixed, paraffin-embedded human UM cases were immunostained for SIRT2, along with 15 NHE obtained from the Eye Bank of Canada. **Results:** SIRT2 expression was higher in melanomas than in normal melanocytes of both tumor and donor eyes ($p < 0.0001$). No significant difference in SIRT2 expression was found when comparing normal melanocytes in UM and NHE cases. **Conclusions:** SIRT2 expression is significantly stronger in UM cells than in normal ocular melanocytes. This finding may indicate an important role of SIRT2 as a prognostic marker in UM progression. SIRT2 should also be investigated as a possible therapeutic target.

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Introduction

Ocular melanoma is the second-most common melanoma in adults after skin melanoma, corresponding to 5% of all melanomas. It has mortality rates up to 40% after 20 years of follow-up [1, 2]. The most frequent ocular melanoma is the uveal melanoma (UM) that comprises 82.5% [3, 4] of all cases.

Although skin melanomas and UMs show different behaviors, both arise from melanocytes. As skin melanomas, UMs occur predominantly in Caucasians. Several risk factors are associated with an increased incidence of UM, such as the presence of congenital ocular and ocular dermal melanocytosis (nevus of Ota). Shields et al. [5] describe the ABCDEF guide to demonstrate the increased risk of malignant transformation of the iris nevus. The guide is set up as follows: A for age (<40 years); B for blood in the anterior chamber; C for clock hour mass inferiorly; D for diffuse configuration; E for ectropion, and F for feathery margins. The factors to predict the transformation of a choroidal nevus into a melanoma include tumor proximity to the optic disc, subretinal fluid symptoms, orange pigment, absence of drusen and thickness >2 mm [5–7].

The prognosis of UM is related to morphological factors such as (a) the size of the tumor: large tumors have an increased risk of metastasis [8]; (b) cell type: mixed tumors have an increasing risk of metastasis, and (c) extraocular extension: extraocular spread is related to a higher risk of metastasis. Genetic studies have also shown that the loss of chromosome 3 is associated with poor outcome. Furthermore, molecular studies have shown that cluster differentiation could be made, classifying tumors according to their low (class 1) and high risk of metastasis (class 2) [9]. Metastasis spreading occurs by hematogenous dissemination to the liver (93 %), followed by the lung (24%) and bones (16%) [10].

Recent studies have correlated the expression of proteins in UMs with prognostic factors and malignant transformation. A recent study compared the expression of forkhead transcription factors class 1 (FOXO1) in eyes with UM to normal ocular melanocytes. FOXO1, as Sirtuins (SIRT), are proteins related to the cell cycle. FOXO1 is a protein crucial to the normal development of the vascular system. The expression of FOXO1 decreases with age; however, in UM eyes, this does not happen, indicating that this protein may stimulate tumor growth [11].

SIRTs are a family of NAD⁺-dependent protein-modifying enzymes involved in many cellular processes. To date, seven distinct SIRT have been identified in humans, named SIRT1–7 [12, 13], and they have different subcellular localizations and functions. In many types of cancers, the expression of SIRTs is also altered. SIRT2 is found primarily in the cytoplasm of normal and malignant cells [14, 15]. It is involved in the cell cycle progression and functions through deacetylation of multiple target proteins such as alpha tubulin [14], histone 4 at lysine 16 [16] and p53 [17], all of which are important for cellular homeostasis. In addition, SIRT2 may also regulate the cell cycle via interaction with FOXOs [18], specifically FOXO1 being an important substrate [19].

At the same time, SIRT2 has been implicated in the regulation of mitosis. There has been evidence of SIRT2 involvement in the neoplastic progression of different human cancers, but its role seems to be context dependent. In some cases, it acts as a tumor suppressor, while in other cases, it acts as an oncogene [20–23]. For example, the expression of SIRT2 is downregulated in gliomas, ductal breast cancers, squamous cell carcinoma and esophageal adenocarcinoma. On the other hand, SIRT2 may be upregulated in acute myeloid leukemia and neuroblastoma [24].

Recent studies have shown that inhibitors of SIRT1/2 slowed the growth of malignant skin melanoma cell lines [20]. Moreover, it has been shown that SIRT2 loss confers

drug resistance to skin melanoma cell lines against tyrosine kinase inhibitors [24].

As has been consistently reported, UM is very aggressive, and often patients die of metastatic disease. Since SIRT2 has shown to be involved in the progression of multiple cancers, including skin melanoma, the objective of this study is to evaluate SIRT2 expression in UM cells and in normal melanocytes.

Materials and Methods

UM and Normal Eye Cases

Formalin-fixed, paraffin-embedded blocks of 21 cases of human UM with clinical information from the Henry C. Witelson Ocular Pathology Laboratory and 15 normal human eyes (NHE) obtained from the Eye Bank of Canada were investigated for SIRT2 expression. In all cases, the uveal tract was analyzed, while tumor cell staining was only evaluated in the UM cases. In UM cases, the choroid was analyzed in 13/21 eyes, and the iris was analyzed in 20/21 eyes due to the absence of these structures. In all NHE, the iris was not evaluated due to its absence. Clinical information was retrieved from the patients' files, including age, gender location in uveal tract, larger tumor dimension (LTD), cell type and a group of other prognostic factors including close vascular loops, lymphocytic infiltration and extraocular extension. All data accumulation was in accordance with Canada and Province of Quebec legislation and the tenets of the Declaration of Helsinki.

Immunohistochemistry

Immunostaining for SIRT2 was performed using the Ventana BenchMark fully automated slide stainer according to the protocols and instructions provided by the manufacturer (Ventana Medical Systems Inc., Oro Valley, Ariz., USA). The slides were incubated with a monoclonal anti-SIRT2 antibody (sc28298; Santa Cruz Biotech) at a dilution of 1:200. Due to the highly pigmented melanoma cells, Fast Red was used as chromogenic substrate. Tonsil slides were used as positive controls, and negative controls were performed omitting the primary antibody.

Immunohistochemical SIRT2 expression was evaluated independently by two researchers assigning a score to each case according to their relative staining intensity, as previously described [25, 26]. Briefly, intensity was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). Extent was scored as 0 (negative), 1 (<50% positive cells), 2 (50–80% positive cells) or 3 (>80%) positive cells. Immunostaining was then converted into an immunoreactive score (IRS), calculated by multiplying the intensity and extent of staining, which results in a score range of 0–9. High IRS tumors had a score of 6–9, while low IRS tumors had a score of <6.

Data Management and Statistical Analysis

Data are presented as means $\pm$ standard error, and n represents the number of cases. Means for each group were compared using one-way ANOVA and Student's t test. Differences were considered significant at $p < 0.05$. A Kaplan-Meier curve was used to evaluate differences in metastasis between high and low IRS tumors using clinical information. Statistics were performed using the Microsoft Excel and the GraphPad Prism 5.0 software.

Fig. 1. a UM with a high SIRT2 IRS (IRS = 9), with score 3 in intensity and extension. **b** UM with a low SIRT2 IRS (IRS = 2), with score 1 in intensity and 2 in extension.

Fig. 2. a IRS in melanoma and melanocytes. **b** SIRT2 IRS of different cell types of UM. **c** Kaplan-Meier curve of metastasis and IRS score (high vs. low). M = Melanocytes; n.s. = not significant.

Results

All UMs were positive for SIRT2. When specimens were classified as having a low or a high expression, 42.9% (n = 9) of the UMs had a high IRS (fig. 1a) and 57.1% (n = 12) had a low IRS (fig. 1b). However, all normal melanocytes, whether from normal eyes or tumor eyes, had a low IRS.

The mean SIRT2 IRS ($\pm$ standard deviation) for each benign and malignant melanocytic cell group was as follows: 4.4 ± 2.9 for UM cells, 0.9 ± 0.7 for choroidal melanocytes in eyes with UM and 1.0 ± 1.7 for iris melanocytes in eyes with UM. For NHE, the IRS of choroidal melano-

cytes was 0.5 ± 0.6 . The IRS of the UMs was higher than that of normal melanocytes in tumor eyes ($p < 0.0001$) and melanocytes in normal eyes ($p < 0.0001$) (fig. 2a). There were no differences in IRS for normal melanocytes between normal eyes and tumor eyes ($p > 0.05$) (fig. 2a).

The average age of the patients was 76.1 ± 5.6 years (range 52–85, n = 17). As SIRT2s have been associated with the aging process, the association between SIRT2 IRS and age was evaluated. Pearson's correlation test showed no correlation ($p > 0.05$). In addition, 52.6% of the patients were female (n = 18), and there were 8 (47.1) UMs from the choroid and 9 (52.9%) from the choroidal and ciliary

body. SIRT2 IRS showed no differences with regard to the tumor localization. According to cell type, 23.8% of the cases were epithelioid, 71.4% of a mixed cell type and 4.8% were of a spindle cell type. SIRT2 IRS showed no differences according to the cell type of the tumor (fig. 2b). The LTD was considered large in 7 cases and medium in another 7 cases. SIRT2 IRS showed no differences between the two sizes. There were no small tumors in our study. Factors of bad prognosis were found in 10 cases (58.8%). Kaplan-Meier curve analysis of SIRT2 IRS and metastasis showed no differences between tumors with a high and those with a low IRS (n = 17; fig. 2c).

Discussion

To the best of our knowledge, this is the first study of SIRT2 in UM. Recent studies of SIRT2 involvement in cancer have shown conflicting results. While some have shown a potential oncogenic effect, as in skin melanoma [20, 23], others have shown a potential tumor-suppressive effect, as in gliomas [21], breast cancers [22] and non-melanoma skin cancers [22].

The increase in SIRT2 expression in UM cells as compared to normal melanocytes suggests a potential oncogenic function, as seen in skin melanoma [20, 24]. However, further studies are needed in order to confirm this hypothesis, since the molecular basis of skin melanoma is different from that of UM. Moreover, they may not share the same molecular hallmarks and pathways. The negative correlation in Pearson's test showed that the SIRT2 IRS of melanocytic cells is an independent factor from the tumor and is not only related to the age of the study group.

No relationship between SIRT2 IRS with metastasis, LTD, tumor cell type and other prognostic factors was

found in this particular study. Therefore, the IRS of SIRT2 does not seem to have any prognostic significance. The importance of SIRT2 in UM may rely on the oncogenesis of the disease rather than on its progression. Further in vitro experiments should be conducted to determine the oncogenic function of SIRT2 in UM. SIRT2 expression must be evaluated in several UM cell lines, and knock-down experiments must be performed in both low- and high-grade UM cell lines to evaluate their proliferation rate.

A high SIRT2 expression may not be limited to UM in the eye. Therefore, other ocular melanomas should be evaluated with respect to SIRT2 expression, such as conjunctival and orbital melanomas.

Compared to other types of proteins, SIRT2 seems to have different roles in tumorigenesis, depending on the cellular context.

In conclusion, SIRT2 is more highly expressed in UM cells as compared to uveal melanocytes in both normal eyes and eyes containing UM. This result suggests that SIRT2 may have an oncogenic role in UM. Further studies are needed to determine how SIRT2 is implicated in the tumorigenesis of UM.

Statement of Ethics

This study was conducted in accordance with Canada and Province of Quebec legislation and the tenets of the Declaration of Helsinki.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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