



## Research Article

# EZH2 gene and CA-IX prompt relation: An effective therapeutic target approach for melanoma progression

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### Abstract

**Objectives:** Melanoma is a common type of skin cancer originating from melanocytes. Its standard treatments include surgery, chemotherapy, radiotherapy, and targeted therapy. However, due to limitations in drug treatments, new targets must be identified. One important enzyme in carcinogenesis, CA-IX, creates an acidic and hypoxic niche within tumor cells. Additionally, EZH2, a gene that encodes a histone-lysine N-methyltransferase, is involved in DNA methylation and plays a crucial role in cancer development by modulating epigenetic changes. In this study, our goal is to elucidate the effect of CA-IX inhibition on the EZH2 gene in melanoma.

**Methods:** We evaluated the effects of CA-IX inhibition with AZA on EZH2 gene expression levels in the A375 human melanoma cell line. Cell culture, ELISA, and qPCR experiments were conducted. The cytotoxic activities of AZA were assessed using the WST-1 assay. CA-IX protein levels were measured using a Human Carbonic Anhydrase IX ELISA Kit. qPCR was performed using the QuantiNova LNA Probe PCR assay.

**Results:** An  $IC_{50}$  value was observed at a concentration of 10.7  $\mu$ M for AZA in the WST-1 assay. Decreased CA-IX protein levels were observed following AZA treatment ( $p < 0.0001$ ). Additionally, EZH2 mRNA levels were significantly reduced when CA-IX protein was inhibited by AZA ( $p < 0.05$ ).

**Conclusion:** Inhibition of CA-IX and the consequent changes in the acidity of the tumor microenvironment may modulate EZH2 levels. CA-IX could be a promising target for the epigenetic treatment of melanoma.

**Keywords:** Acetazolamide, carbonic anhydrase-IX, carbonic anhydrase inhibitor, enhancer of zeste homolog 2, melanoma

**How to cite this article:** Ozensoy Guler O, Oz Bedir BE, Terzi E. EZH2 gene and CA-IX prompt relation: An effective therapeutic target approach for melanoma progression. Int J Med Biochem 2024;7(1):13–17.

Cancer is a disease caused by the accumulation of genetic and epigenetic alterations [1, 2]. In addition to genetic alterations, epigenetic regulations that occur during transcription are also important factors in carcinogenesis [3]. Melanoma is the most lethal and aggressive form of skin cancer and is one of the types with high malignancy [4, 5]. Although melanoma can be detected at an early stage, metastatic melanoma has a poor prognosis. As the drugs used in the treatment of melanoma are limited, new strategies to combat melanoma have been investigated [6].

The genes of the Polycomb group are a group of important epigenetic regulators that repress transcription [3]. One of the Polycomb genes, Enhancer of Zeste Homolog 2 (EZH2),

encodes a histone-lysine N-methyltransferase that is involved in DNA methylation and also methylates the H3 lysine at position 27 [7]. EZH2 is the enzymatic catalytic subunit of Polycomb repressive complex 2 (PRC2). The catalysis of EZH2 is associated with the repression of gene expression and is considered a critical epigenetic event during tissue development. EZH2 acts as a regulator in the cell cycle, autophagy, and apoptosis processes and prevents cellular senescence by supporting DNA repair. EZH2 is involved in many biological processes, for example, in oncogenic metabolic pathways [3]. There are mutations and high expression of EZH2 in various cancers, suggesting that EZH2 plays a critical role in the development and progression of carcinogenesis [8]. EZH2 inhibitors

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**Submitted:** August 24, 2023 **Accepted:** November 28, 2023 **Available Online:** January 23, 2024

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have been investigated and developed as an important new therapeutic target. One of these inhibitors, tazemetostat, has been approved for the treatment of epithelioid sarcomas. The development of highly effective EZH2 inhibitors is of crucial importance for the treatment of cancer epigenetics [3]. EZH2 expression is also controlled by hypoxia via HIF response elements (HRE) in the EZH2 gene promoter. The expression of EZH2 increases, and thus the proliferation of cancer cells is promoted in a hypoxic environment [9, 10]. A prominent enzyme associated with hypoxia is carbonic anhydrase IX (CA-IX), a fundamental enzyme for all metabolic processes related to acid-base balance, and a transmembrane protein associated with carcinogenesis [11]. Since CA-IX is localized in the cell membrane, it increases extracellular acidosis and causes angiogenesis, epithelial-mesenchymal transformation, invasion, hypoxia, and signal transduction [12]. Because of all these functions, CA-IX inhibitors have become attractive for cancer treatment [13]. Acetazolamide (AZA), the most potent CA inhibitor, has been used clinically for many years and has also been used for CA-IX inhibition *in vivo* tumor models [14].

Due to its crucial role in carcinogenesis, gene expression, and developmental processes, CA-IX also activates the epigenetic program of the epithelial-mesenchymal transition in cancer cells, thus contributing to cancer cell survival [15, 16]. Since melanomas exhibit a higher expression level of CA-IX, epigenetic changes may play a crucial function in the development of melanomas. The role of EZH2 in this process, known to be associated with epigenetic regulation, is not yet clear. Therefore, in our study to clarify the relationship between EZH2 and the tumor-associated enzyme carbonic anhydrase CA-IX, we tested the hypothesis that inhibition of CA-IX is associated with EZH2 in melanoma.

## Materials and Methods

### Cell culture

The A375 human melanoma cell line was obtained from Hacettepe University Pharmacy Faculty Biochemistry Department, Ankara, Türkiye. All cells were cultured in DMEM (Capricorn, DMEM/HPA) including 10% Fetal Bovine Serum (FBS) (Capricorn, FBS-HI-11A), 1% Streptomycin/Penicillin (Capricorn, PS-B) at optimum conditions (37°C, 5% CO<sub>2</sub>). Acetazolamide (AZA) was purchased from Sigma (BCBZ9159). It was prepared as a stock solution by dissolving it in DMSO at a concentration of 300 µM.

### Cytotoxicity test

Using 96-well plates, all cells were cultured with DMEM supplemented with 10% FBS. The cells were treated with 0, 2.5, 5, 10, 25, 50, 100 µmol/L concentrations of AZA for 24 h and 48 h, in triplicate, in a humidified 5% CO<sub>2</sub> atmosphere. WST-1 reagent was added to the wells and incubated at 37°C for 2 h. The plates were then read at 450 nm on an Epoch™ Microplate Spectrophotometer (BioTek, USA). The IC<sub>50</sub> value of AZA was determined as 10.7 µmol/L using a WST-1 assay (Cayman Chemical).

While calculating IC<sub>50</sub> values:

% Viability = (Average absorbance of inhibitor × 100) / (Average absorbance of control)

% Inhibition = 100 – ((Average absorbance of inhibitor × 100) / (Average absorbance of control))

Then, the inhibitor concentration values were entered into the GraphPad Prism 9.1.0 program, converted into a graph, and the inhibitor dose (IC<sub>50</sub>) value that inhibited 50% of the cells was calculated.

### ELISA

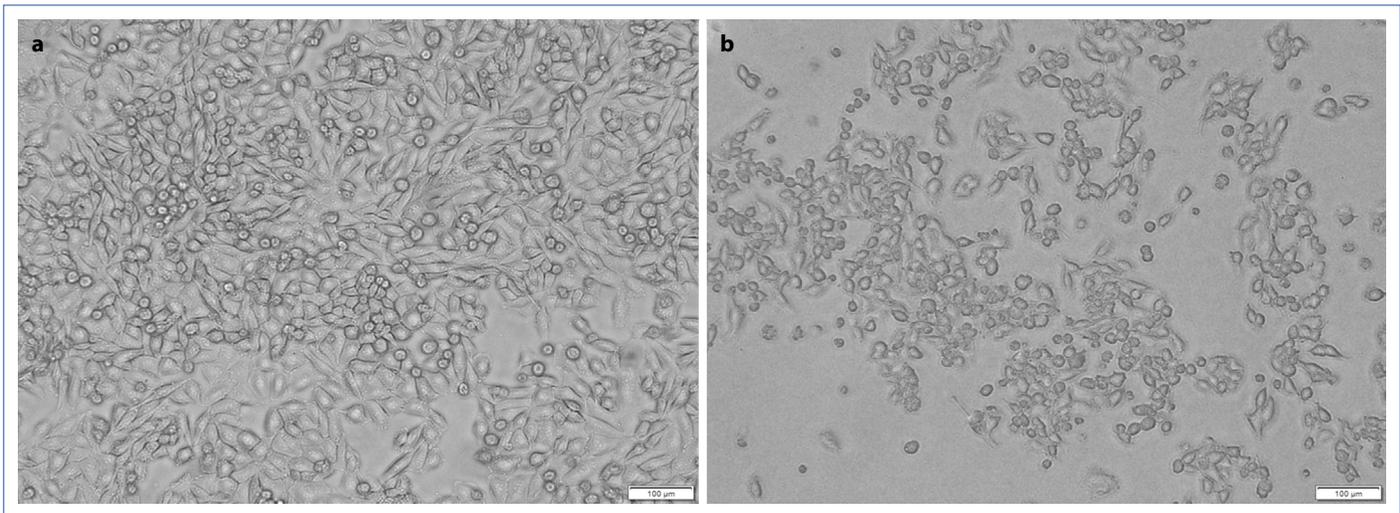
CA-IX levels were measured using the Human Carbonic Anhydrase IX ELISA Kit BT Lab (Zhejiang, China). Firstly, the collected cells were diluted in DPBS to reach a concentration of one million/mL. The damaged cells were lysed with prepared freeze-thaw cycles. After centrifugation (4°C, 1500 g, 10 min), the supernatants were collected carefully. Briefly, 40 µL of supernatant, 10 µL of primary antibody, and 50 µL of Streptavidin-Horseradish Peroxidase were mixed to reach a final volume of 100 µL at 37°C for 60 min. After incubation, chromogen solutions A and B were added to the wells. Following a 10 min incubation at 37°C, a stop solution was used to end the reaction. All experiments were performed in triplicate (n=3) and read at 450 nm by an Epoch™ Microplate Spectrophotometer (BioTek, USA).

### RNA isolation

The cells were trypsinized and washed with DPBS for RNA isolation. The miRNeasy Kit and QIACube Connect (QIAGEN) were used for total RNA isolation according to the manufacturer's instructions (QIAGEN). Total RNAs were quantified with the QIAxpert Instrument. cDNA synthesis was performed with the QuantiNova Kit.

### qPCR

qPCR was performed using the QuantiNova LNA Probe PCR assay. PCRs were conducted at a final volume of 50 µL. All PCR reagents were purchased from QIAGEN. Each reaction mixture contained 10 µL of 2× QuantiNova Probe PCR Master Mix, 2 µL of 10× Probe PCR Assay, and 3 µL of RNase-free water. The reactions were run for 45 cycles on a Rotor Gene Q device (QIAGEN), with denaturation at 3 min at 25°C, annealing at 10 min at 45°C, and extension at 5 min at 85°C. Primer sequences' catalog numbers are UPFH0128337 for EZH2 and UPFH1132903 for CA9. The relative gene expression was calculated with the 2<sup>-ΔΔCT</sup> method. Relative mRNA amounts were calculated using the CT method.  $\Delta CT = CT_{\text{target}} - CT_{\text{GAPDH}}$ ,  $\Delta\Delta CT = CT_{\text{treatment}} - CT_{\text{calibrator}}$  where the calibrator was the no-treatment group. CT was then converted to fold change using the formula 2<sup>-ΔΔCT</sup> [17]. The GAPDH gene was selected as an internal control to normalize the relative expression levels of EZH2 and CA9. The comparative quantification of the individual target genes was based on the cycle threshold (CT), which was normalized to GAPDH using the ΔΔCT method. GAPDH is constitutively expressed at high levels in almost all tissues. For this reason, we use GAPDH



**Figure 1.** (a) Images of A375 melanoma cells without treatment. (b) 24 hours after AZA treatment.

AZA: Acetazolamide.

as the housekeeping gene for qPCR. In addition, according to the literature, the GAPDH gene was the most stable gene, so we used this gene as the housekeeping gene [18, 19].

### Statistical analysis

Statistical analysis for ELISA was performed with Student's t-test in the GraphPad Prism 5.0 program. The relative gene expression was calculated with the  $2^{-\Delta\Delta Ct}$  method. When analyzing qPCR data, we first calculate Delta Ct ( $\Delta Ct$ ), obtained by subtracting the reference gene Ct from the target gene Ct. Ct was then converted to fold change using the formula  $2^{-\Delta\Delta Ct}$ . The qPCR results were analyzed with the GeneGlobe program. All significance levels were accepted as  $p < 0.05$ .

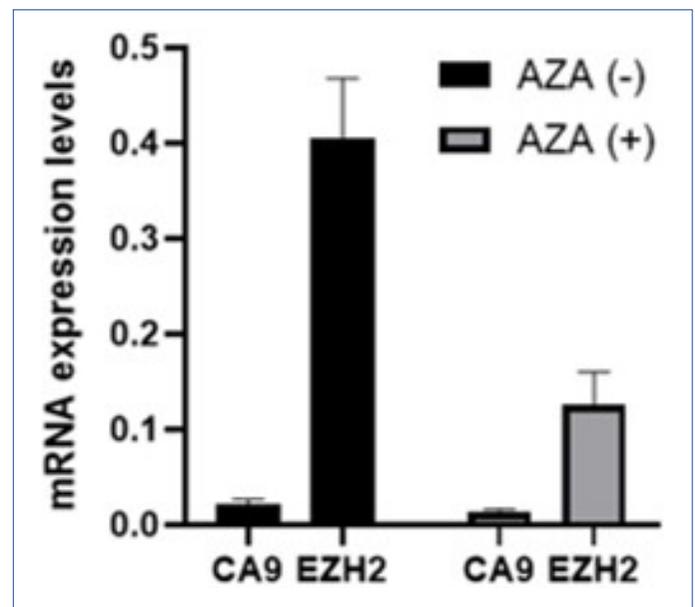
## Results

### Acetazolamide reduces the viability of A375 melanoma cells

A375 melanoma cells were treated with AZA for 24h and 48h (Fig. 1). After treatment with 0, 2.5, 5, 10, 25, 50, 100  $\mu M$  AZA concentrations for 24h, the viability of A375 melanoma cells were 100%, 100%, 70.2%, 50.3%, 29.6%, 28.6%, and 27.3%, respectively. Additionally, after 48h with the same AZA concentrations, the viabilities were 100%, 100%, 52.6%, 14.3%, 13.3%, 12.5%, and 12.1%, respectively. Based on these results, the dose of AZA for A375 melanoma cells was determined to be 10.7  $\mu M$  at 24h.

### CA-IX levels decreased in A375 melanoma cells treated with AZA

The CA-IX levels in A375 melanoma cells were 5.31 ng/mL in the AZA non-treated group, while it was 1.81 ng/mL in the AZA-treated group. CA-IX levels decreased in cells treated with AZA ( $p < 0.0001$ ). The decrease indicates that AZA can inhibit the CA-IX enzyme in melanoma cells. Moreover, AZA is not only a classical CA inhibitor but also effectively inhibits the CA-IX isoenzyme, which plays a role in tumorigenesis (Fig. 2).

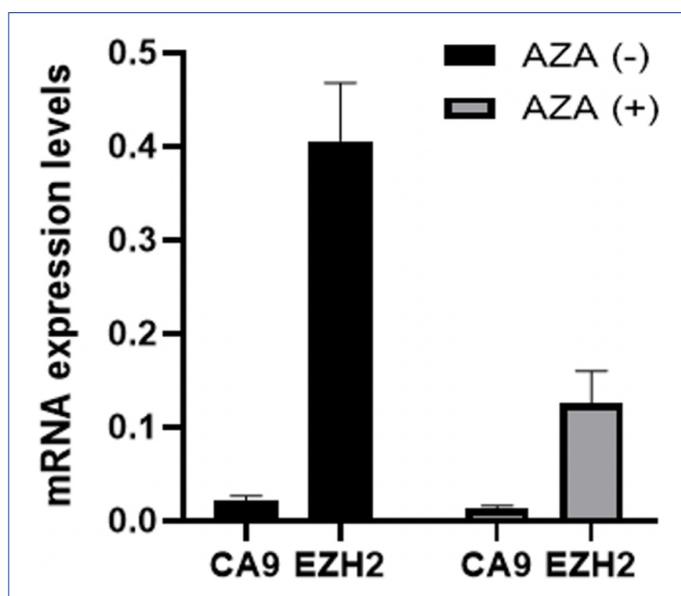


**Figure 2.**  $2^{-\Delta\Delta Ct}$  values of GAPDH-normalized CA9 and EZH2 genes in A375 melanoma cells treated with 10.7  $\mu M$  AZA ( $p = 0.4136$ , CA9;  $p < 0.05$ , EZH2).

EZH2: Enhancer of Zeste Homolog 2.

### EZH2 mRNA levels decreased in A375 melanoma cells treated with AZA

To assess the mRNA expression of EZH2 and CA9 in A375 melanoma cells, qPCR was performed. The results indicated that while the mRNA expression of CA9 decreased after AZA treatment, the change was not statistically significant ( $p = 0.4136$ ). However, EZH2 mRNA expression decreased significantly ( $p < 0.05$ ) (Fig. 3). AZA treatment resulted in a 1.67-fold decrease in CA9 and a 3.57-fold decrease in EZH2 gene expression (Fig. 4). The substantial reduction in EZH2 suggests that AZA could be used to inhibit this critical Polycomb protein.



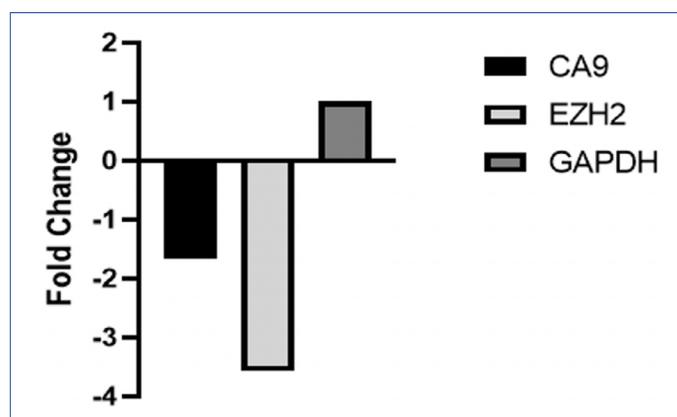
**Figure 3.** mRNA expression levels of CA9 and EZH2 genes in A375 melanoma cells treated with 10.7  $\mu\text{mol/L}$  AZA ( $p=0.4136$ , CA9;  $p<0.05$ , EZH2).

## Discussion

CA-IX is a transmembrane isoform of CAs and regulates acidity in cancer cells [20, 21]. Additionally, acidosis is thought to be responsible for epigenetic changes in cancer [22]. In this study, we aimed to investigate the effect of CA-IX inhibition on the EZH2 polycomb protein, which is a key epigenetic modulator.

CA-IX is an important regulator of many processes involved in carcinogenesis and is highly expressed in melanomas [23]. Andreucci et al. [21] demonstrated that melanoma, breast, and colon cancer cells exposed to acid significantly increase CA-IX expression. Federici et al. [23] observed that CA-IX was expressed by malignant human melanoma cells and that specific CA-IX inhibitors suppressed the growth of melanoma cells. Chafe et al. [24] discovered that CA9 expression was associated with decreased immune activity in tumors from patients with metastatic melanoma and that the CA-IX inhibitor SLC-0111 may represent a significant therapeutic option. In summary, literature indicates that an aggressive melanoma phenotype with high CA-IX expression and the application of inhibitors to this protein should improve prognosis. In our study, we observed a notable decrease in CA-IX protein levels in melanoma cells post-AZA treatment. Given the role of CA-IX in melanoma, its inhibition may be a promising strategy [23, 25].

EZH2 is an epigenetic regulator that plays a crucial role in DNA methylation and melanoma development [26]. High expression of EZH2 has been shown to be associated with malignant forms of melanoma [27, 28]. Mahmoud et al. [29] highlighted a close association between EZH2 and metastatic melanoma, suggesting that EZH2 facilitates melanoma progression by activating oncogenic signaling pathways and deactivating tumor suppressive signaling pathways. Consid-



**Figure 4.** The fold increase rates of the mRNA expressions of CA9 and EZH2 genes in A375 melanoma cells treated with 10.7  $\mu\text{mol/L}$  AZA after normalization with GAPDH compared to the control group (-1.67 fold change, CA9; -3.57 fold change, EZH2).

ering that epigenetic changes are known to be induced by acidosis, we postulate that CA-IX inhibition impacts EZH2, a key regulator of epigenetics. Huang et al. [16] observed increased expression of EZH2 and CA-IX under hypoxic conditions. Our study aimed to demonstrate the effect of CA-IX inhibition on EZH2 levels through qPCR experiments. Our results indicate that inhibiting CA-IX leads to a marked reduction in EZH2 levels. Our study suggests that CA-IX inhibition can modulate epigenetic alterations by lowering EZH2 levels, proposing an alternative method to modulate epigenetics by targeting CA-IX in melanoma (Fig. 1).

## Conclusion

In summary, various innovative strategies for melanoma treatment have been explored over the years. One such strategy involves the pivotal modulator EZH2, which is essential for the survival of melanoma cells by regulating gene expression. Our preliminary data suggest that EZH2 gene levels decrease upon CA-IX inhibition, which is associated with poor prognosis in melanoma cells, making CA-IX inhibition a potentially valuable approach for advancing melanoma treatment strategies. Additionally, our research acts as a link between past and prospective studies on melanoma treatment approaches.

**Conflict of Interest:** The authors declare that there is no conflict of interest.

**Financial Disclosure:** The authors declared that this study has received no financial support.

**Peer-review:** Externally peer-reviewed.

**Authorship Contributions:** Concept – O.O.G.; Design – O.O.G.; Supervision – O.O.G.; Funding – O.O.G.; Data collection &/or processing – B.E.O.B., E.T.; Analysis and/or interpretation – O.O.G., B.E.O.B., E.T.; Literature search – O.O.G., B.E.O.B., E.T.; Writing – O.O.G., B.E.O.B., E.T.; Critical review – O.O.G.

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