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Inhibition of bladder cancer cell proliferation by allyl isothiocyanate (mustard essential oil)

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ABSTRACT

Natural compounds hold great promise for combating antibiotic resistance, the failure to control some diseases, the emergence of new diseases and the toxicity of some contemporary medical products. Allyl isothiocyanate (AITC), which is abundant in cruciferous vegetables and mustard seeds and is commonly referred to as mustard essential oil, exhibits promising antineoplastic activity against bladder cancer, although its mechanism of action is not fully understood. Therefore, the aim of this study was to investigate the effects of AITC activity on bladder cancer cell lines carrying a wild type (*wt*; RT4) or mutated (T24) *TP53* gene. Morphological changes, cell cycle kinetics and *CDK1*, *SMAD4*, *BAX*, *BCL2*, *ANLN* and *S100P* gene expression were evaluated. In both cell lines, treatment with AITC inhibited cell proliferation (at 62.5, 72.5, 82.5 and 92.5 μM AITC) and induced morphological changes, including scattered and elongated cells and cellular debris. Gene expression profiles revealed increased *S100P* and *BAX* and decreased *BCL2* expression in RT4 cells following AITC treatment. T24 cells displayed increased *BCL2*, *BAX* and *ANLN* and decreased *S100P* expression. No changes in *SMAD4* and *CDK1* expression were observed in either cell line. In conclusion, AITC inhibits cell proliferation independent of *TP53* status. However, the mechanism of action of AITC differed in the two cell lines; in RT4 cells, it mainly acted via the classical *BAX/BCL2* pathway, while in T24 cells, AITC modulated the activities of *ANLN* (related to cytokinesis) and *S100P*. These data confirm the role of AITC as a potential antiproliferative compound that modulates gene expression according to the tumor cell *TP53* genotype.

1. Introduction

It has been estimated that up to 33% of cancer cases are preventable by changes in diet and associated factors [1]. Nutritional interventions may not only prevent disease in healthy populations but may also confer therapeutic effects in affected individuals [2,3]. For example, *Ficus racemosa* bark, a medicinal herb, enhances the effects of hypoglycemic drugs, leading to decreased glucose levels in diabetic patients without side effects [4]. Anti-atherosclerotic effects of garlic-based drugs were observed in 196 asymptomatic men aged 40–74 [5]. Recently, Loganathan et al. [6] demonstrated that the *Ganoderma lucidum* mushroom inhibits breast-to-lung cancer metastasis in mice by downregulating pro-invasive genes such as *HRAS*, *VIL2*, *S100A4*, *MCAM*, *I2PP2A* and *FN1*. Furthermore, decreased *BCL2* and increased *BAX* gene expression have been previously reported to occur in association with increased apoptosis rates in human pharyngeal squamous carcinoma cells treated with capsaicin derived from hot peppers [7]. Substances derived from fruits and vegetables and essential oils have been extensively investigated, and some possess the ability to induce apoptosis in addition to being associated with significant antiproliferative activities [8,9]. Furthermore, these natural compounds can potentially reduce or eliminate undesirable effects of typical therapies [10]. For example, an epidemiological study revealed an inverse relationship between the ingestion of crude broccoli and the risk of bladder cancer [11]. Because of its high bioavailability in urine, allyl isothiocyanate (AITC) is considered a promising agent for the treatment and prevention of bladder cancer [12,13]. Cell cycle arrest at G2/M phase due to decreased activity of the CDK1/cyclin B complex has been observed in human glioma cells (GBM 8401) treated with AITC [14].

AITC is an aliphatic isothiocyanate that is derived from sinigrin and is abundant in cruciferous vegetables and mustard seeds [15]. It has been proposed that AITC would be selective for tumor cells because low levels of this compound have been detected

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in normal epithelial cells [16]. However, its mechanism of action has not yet been fully elucidated. Kumar et al. [17] reported that AITC induces apoptosis in Ehrlich ascites tumors (EAT) by modulating the expression of the *BCL-2* and *BAX* genes. Recently, Wang et al. [18] demonstrated the therapeutic benefits of AITC in lung diseases via its promotion of increased expression of *MRP1*, a gene involved in protection against oxidative stress and xenobiotics.

Globally, bladder cancer is the seventh most common neoplasia in men and the 17th in women, and specifically, urothelial cell carcinoma (UCC) is responsible for approximately 90% of these malignant tumors [19,20]. Urothelial cancer frequently involves mutations in the *TP53* gene [21], and high rates of recurrence and progression are observed [22]. Cyclophosphamide, arylamines and cigarette smoking are the main risk factors associated with the etiology of UCC [23]. The most used and successful chemotherapies for UCC include combinations of methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) or a combination of gemcitabine and cisplatin [24,25]. However, these protocols are associated with adverse effects, such as high systemic toxicity, a lack of selectivity and tumor resistance following prolonged treatment [26]. Furthermore, genes related to the sensitivity of tumors to chemotherapy may play critical roles in the selection of preferential treatments [27].

Therefore, the aim of this study was to investigate the effects of AITC on cellular responses and gene (*ANLN*, *S100P*, *SMAD4*, *BCL2*, *BAX* and *CDK1*) expression in UCC cell lines with a wild type (*wt*) or mutated *TP53* gene. Our results will help to clarify the mechanism of action of AITC.

2. Materials and methods

2.1. Cell lines and test compound

The human urothelial carcinoma cell lines RT4 (from a low grade tumor with a *wt TP53* gene) and T24 (from an invasive tumor with the *TP53* allele, encoding an in-frame deletion of tyrosine 126) were obtained from the Cell Bank of the Federal University of Rio de Janeiro, Brazil, and maintained as previously described by da Silva et al. [28]. AITC was purchased from Sigma–Aldrich (USA) and diluted into 2% Tween-20 prior to use. All AITC treatments were performed for 3 h, as suggested by Zhang et al. [29], and the concentrations used were defined based on prior study [37]. The IC50 values of AITC for the RT4 and T24 cell lines were 310 and 350 μM, respectively.

2.2. Cell proliferation and cell viability

Cells were seeded into 12-well plates at a density of 2×10^4 cells/well to evaluate cell proliferation. Twenty-four hours later, the cells were treated with AITC (5.0, 62.5, 72.5, 82.5 and 92.5 μM) for 3 h. The cells were then washed with Hank's solution, and fresh medium was added, followed by incubation at 37 °C for 21, 45 or 69 h. The cells were detached using trypsin–EDTA and counted using an automated cell counter. Cell viability was evaluated 21 h after treatment with 5.0, 62.5, 72.5 or 82.5 μM AITC using trypan blue staining. Briefly, 10 μL of 0.4% trypan blue was added to 10 μL of cell suspension, and the cells were incubated for five minutes. Viability was analyzed using the automated cell counter. Assays were performed in triplicate.

2.3. Cell morphology

Initially, 2×10^5 cells were seeded into culture plates and incubated at 37 °C with 5% CO_2. Twenty-four hours later, the cells were treated with 5.0, 62.5, 72.5 or 82.5 μM AITC for 3 h. Next, the cells were washed with Hank's solution (0.4 g KCl, 0.06 g KH_2PO_4, 0.04 g Na_2HPO_4, 0.35 g $NaHCO_3$, 1 g glucose and 8 g NaCl in 1 L H_2O), and fresh medium was added for an additional incubation period of 21, 45 or 69 h. Morphological changes were evaluated by phase-contrast microscopy before and after AITC treatment. Cultures and treatments were performed in triplicate. The scale bars were constructed using *AxioVision* (version 4.8).

2.4. Quantitative real-time polymerase chain reaction (RT-PCR) and evaluation of ANLN, S100P, SMAD4, BCL2, BAX and CDK1 gene expression

Cells were seeded into plates at a density of 1×10^5 cells/plate. Twenty-four hours later, the cells were treated with AITC (5.0, 62.5, 72.5 or 82.5 μM) for 3 h. Afterwards, the cells were washed with Hank's solution, and fresh medium was added. The cells were collected for RNA extraction after an additional 21 h at 37 °C.

Total RNA was isolated using the RNeasy Mini Kit® (Qiagen) according to the manufacturer's protocol. RNA concentrations and purities were determined using a NanoVue spectrophotometer (GE Healthcare). Complementary DNA was synthesized using the High Capacity Kit (Applied Biosystems, USA) according to the manufacturer's instructions. *ANLN* (*Hs01122612_m1*), *S100P* (*Hs00195584_m1*), *BCL2* (*Hs00608023_m1*), *SMAD4* (*Hs00929647_m1*), *BAX* (*Hs00414514_m1*) and *CDK1* (*Hs00364293_m1*) gene expression levels were assayed using the TaqMan system (Applied Biosystems, Foster City, CA, USA). Each tube contained 2 μL of cDNA template, 5 μL of TaqMan 2× Master Mix (Applied Biosystems), 2.5 μL of water and 0.5 μL of 20× primers/probes (Assays-on-Demand gene expression products, Applied Biosystems). β-Actin was used as a housekeeping gene. The reaction was performed using the following thermal cycling conditions: 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 1 min. Fluorescence data were collected during each annealing/extension step. The reactions were performed in triplicate at 21 h after AITC treatment using the 7500 FAST Real-Time PCR System (Applied Biosystems) and SDS software, version 1.2.3 (Sequence Detection Systems 1.2.3, 7500 Real-Time PCR Systems, Applied Biosystems). The relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method [30]. A gene interaction network was created using String 9.05 software (http://string-db.org/newstring_cgi/show_input_page.pl) and MCL clustering algorithms.

2.5. Statistical analyses

Statistical analyses were performed using SigmaStat 3.5 and SAS software, version 9.2 (Statistical Analysis System, SAS Institute, Cary, NC, USA). Gene expression data, cell viability and cell cycle kinetics were analyzed using a one-way ANOVA followed by Tukey's test. The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Cell proliferation and cell viability

The numbers of cells in the AITC-treated RT4 and T24 cultures were generally lower than those in the controls (Figs. 1A and 2A). Inhibition of RT4 proliferation was observed at 45 and 69 h following treatment with 62.5, 72.5, 82.5 and 92.5 μM AITC. No changes in T24 cells were observed at 21 h, but decreased cell numbers were detected at 45 (62.5, 72.5, 82.5 and 92.5 μM AITC) and 69 h (5.0, 62.5, 72.5, 82.5 and 92.5 μM AITC). No differences in RT4 and T24

Table 1
Percentage of viable cells (RT4 and T24 cell lines) at 21 h after treatment with allyl isothiocyanate (AITC).

Cell line	Negative control[a]	Control Tween[b]	AITC (μM)			
			5	62.5	72.5	82.5
RT4	89.6 ± 5.8	88.4 ± 6.0	87.9 ± 20.2	87.8 ± 4.3	78.3 ± 2.2	77.7 ± 1.6
T24	75.2 ± 5.3	87.3 ± 12.2	89.8 ± 7.0	87.1 ± 3.4	90.4 ± 5.6	89.3 ± 9.0

[a] No treatment.
[b] Cells treated with 2% Tween-20 (vehicle control); data are presented as the mean ± SD.

cell viability were observed at 21 h following AITC treatment in the trypan blue assay (Table 1).

3.2. Cell morphology

Microscopic evaluations indicated that the RT4 and T24 cells exhibited morphological alterations (elongated cells) following AITC treatment (Figs. 1B and 2B). Scattered cells, as well as cellular debris, were observed in the AITC-treated cultures 21, 45 and 69 h after treatment.

3.3. ANLN, S100P, SMAD4, BCL2, BAX and CDK1 gene expression

ANLN, *BCL2* and *BAX* were upregulated and *S100P* was downregulated in T24 cells after treatment with 62.5, 72.5 and 82.5 μM AITC. By contrast, *BAX* (62.5, 72.5 and 82.5 μM AITC) and *S100P* (82.5 μM AITC) were upregulated and *BCL2* was downregulated following AITC exposure (62.5, 72.5 and 82.5 μM) in RT4 cells (Figs. 3 and 4). Fig. 5 depicts the interaction network involving the investigated genes, which were clustered in the apoptosis pathway

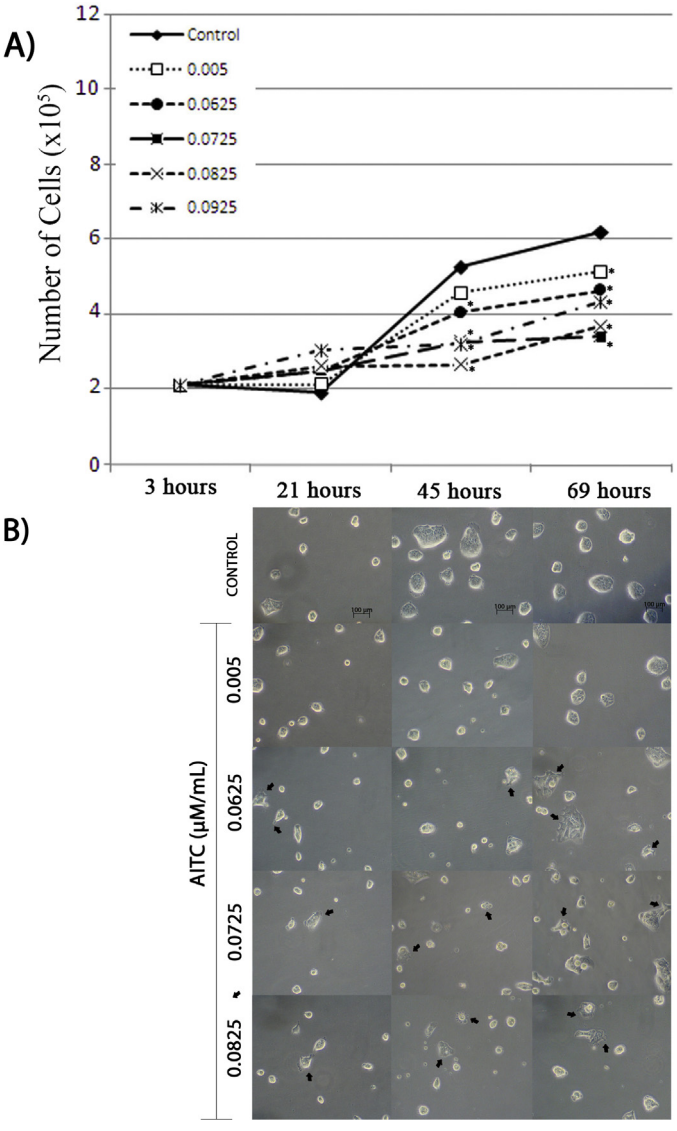

Fig. 1. (A) RT4 cell proliferation after treatment with allyl isothiocyanate (AITC). Each point represents the mean value obtained from three independent experiments. *$p < 0.05$ (compared with Tween control). (B) Photomicrograph of RT4 (wild type *TP53*) cells before and after treatment with AITC (400× magnification). Black arrows indicate morphological alterations after AITC treatment.

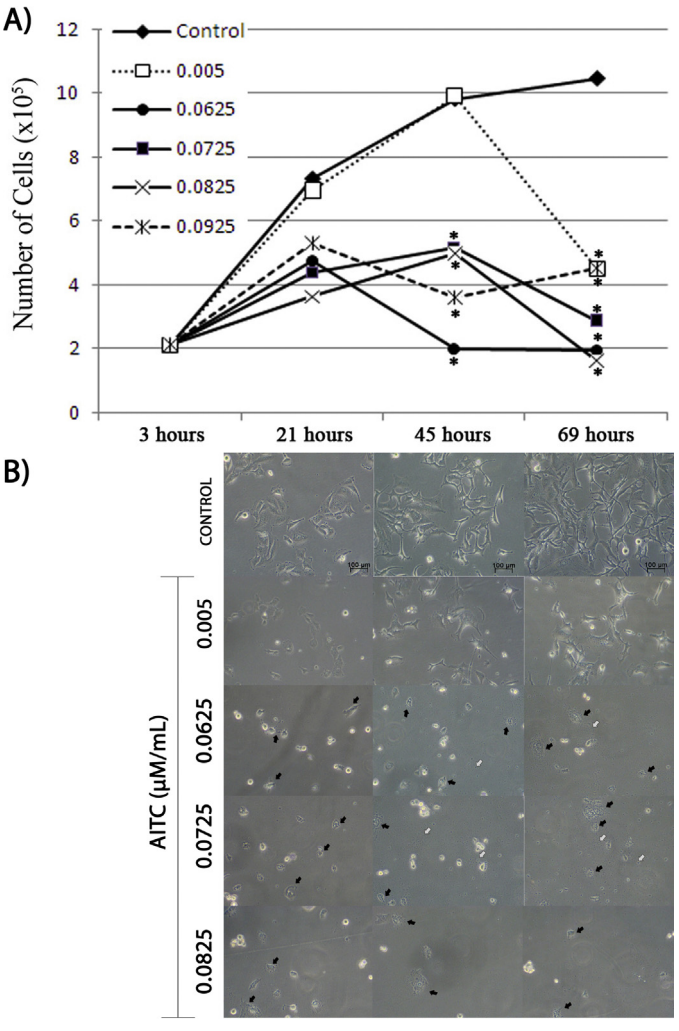

Fig. 2. (A) T24 cell proliferation after treatment with allyl isothiocyanate (AITC). Each point represents the mean value obtained from three independent experiments. *$p < 0.05$ (compared with Tween control). (B) Photomicrograph of T24 (mutant *TP53*) cells before and after treatment with AITC (400× magnification). Black arrows indicate morphological alterations and white arrows indicate cellular debris after AITC treatment.

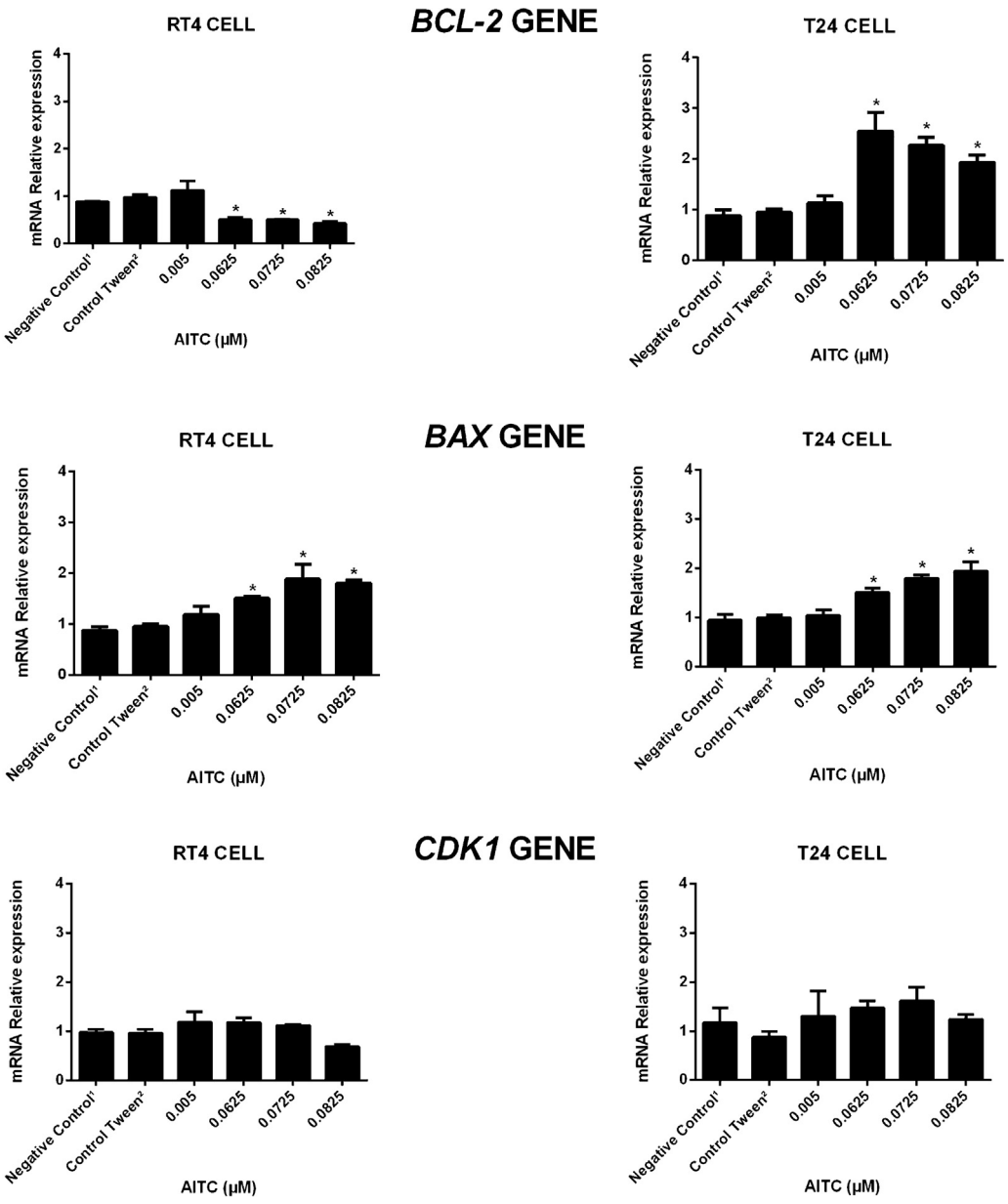

Fig. 3. Relative mRNA levels of *BAX*, *BCL2* and *CDK1* in RT4 and T24 cells after treatment with allyl isothiocyanate (AITC). Negative control – no treatment; control Tween – treated with 2% Tween-20 (AITC vehicle control); β-actin (endogenous RNA control). $^{*}p < 0.05$ (compared with Tween control). Cell cultures were performed in triplicate.

(*SMAD*, *BAX*, *BCL2* and *TP53*) and the cell cycle pathway (*CDK1* and *ANLN*).

4. Discussion

Medicinal plants may be more compatible with the human body and have lower rates of side effects than typical chemotherapeutic drugs [31]. Furthermore, some plant-derived compounds not only possess antiproliferative activities but also might preferentially destroy malignant cells with low levels of toxicity to non-neoplastic cells. For example, Bhattacharya et al. [32] demonstrated that the delivery of AITC through urinary excretion to bladder tissues in a rat orthotopic model inhibited cancer development and muscle invasion. Other *in vitro* and *in vivo* studies have also demonstrated the anticarcinogenic potential of some isothiocyanates (ITC) [17,33,34].

In this study, we investigated the activity of AITC in urothelial carcinoma cell lines carrying a wild type (RT4) or mutated (T24) *TP53* gene. The microscopic analyses revealed morphological alterations 21, 45 and 69 h after AITC treatment, but no changes with respect to viability were detected in either cell line after 21 h of treatment. The induction of morphological alterations by AITC has also been observed in glioma cells (GBM-8401) [14]. Gonçalves et al. [35] also reported morphological changes in Vero cells following treatment with cisplatin and suggested that irregular cell morphologies might indicate compromised polymerization of the actin cytoskeleton and/or activities of actin-binding proteins. However, Janson et al. [36] suggested that early-phase morphological changes were not representative of the cytotoxic and apoptosis-inducing effects of a compound. Nevertheless, in addition to morphological alterations, our data indicated scattered and decreased numbers of RT4 and T24 cells following AITC treatment, suggesting that AITC possesses antiproliferative effects that are independent of *TP53* status. These findings are in agreement with our previous observation of increased apoptosis rates and G2 phase cell cycle arrest in AITC-treated RT4 and T24 cells, respectively [37].

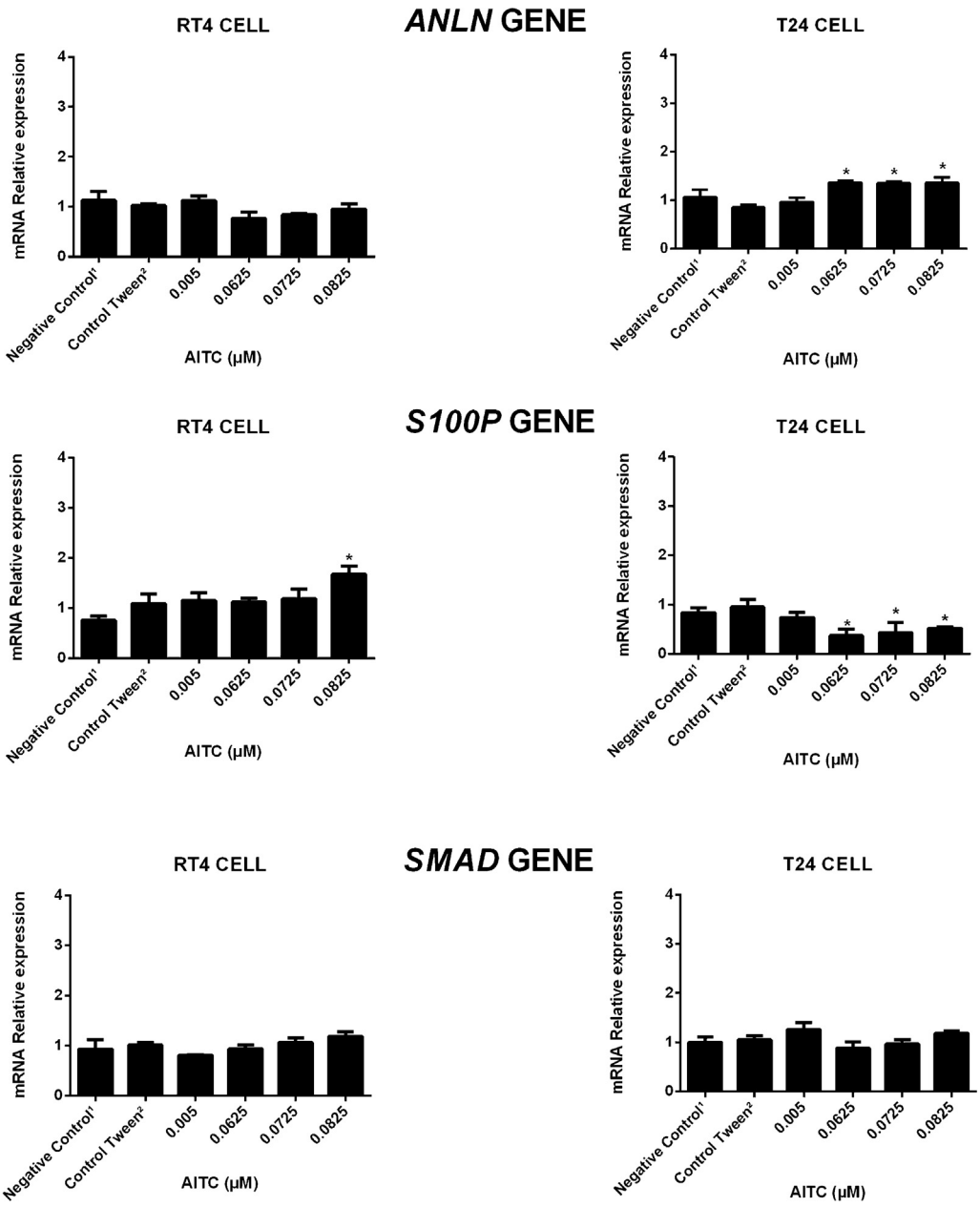

Fig. 4. Relative mRNA levels of *ANLN*, *S100P*, and *SMAD4* in RT4 and T24 cells after treatment with allyl isothiocyanate (AITC). Negative control – no treatment; control Tween – treated with 2% Tween-20 (AITC vehicle control); β-actin (endogenous RNA control). $^{*}p < 0.05$ (compared with the respective Tween controls). Cell cultures were performed in triplicate.

To better understand the mechanisms of action of AITC, we also evaluated its effects on the gene expression profile. Overexpression of *BAX* (a proapoptotic gene) and decreased expression of *BCL2* (an anti-apoptotic gene) were observed in RT4 cells. The balance between *BCL-2* and *BAX* expression is typically regulated by the *TP53* tumor suppressor gene [38,39]. Our results for RT4 (wt *TP53*) cells support these findings. However, we detected not only *BAX* but also *BCL2* overexpression in T24 cells, although inhibited cell proliferation was also observed. Teijido et al. [40] reported that the upregulation of Bcl2 stabilizes the loose binding of Bax to mitochondrial membranes, thereby inhibiting cytochrome c release and, consequently, inhibiting apoptosis. Thus, we suggest that early apoptosis is the main mechanism underlying the inhibition of cell proliferation in T24 cells. These findings corroborate with and expand upon our previous observation of increased apoptosis in RT4 cells and G2/M cycle arrest in T24 cells [37].

We observed the upregulation of *S100P* in RT4 cells and downregulation in T24 cells following AITC treatment (82.5 μM; the highest concentration used). *S100P*, a signal-dependent Ca^{2+} mediator gene, plays important roles in several intra- and extra-cellular processes, including the regulation of protein phosphorylation, enzyme activity, apoptosis, gene transcription and cell proliferation and differentiation [41,42]. Because the decreased numbers of mutated *TP53* cells that were observed by microscopy following AITC treatment cannot be explained by the classical *BAX*/*BCL-2* mechanism, we suggest that the downregulation of *S100P* may play a role. Notably, significant inhibition of cell growth has also been detected in *S100P*-knockdown colon cancer cells [43]. According to Dairkee et al. [44], the upregulation of *S100P* is likely associated with drug resistance in cell lines.

SMAD4 is a tumor suppressor gene that encodes a protein that plays important roles in the transduction of signals from

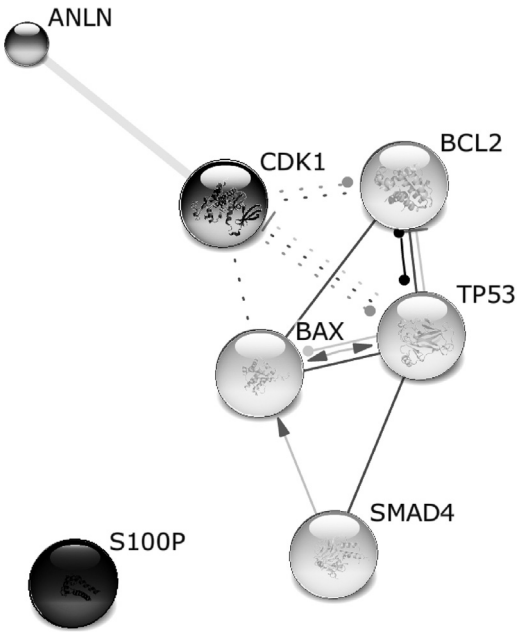

Fig. 5. Gene interaction network created using String 9.05 software and the MCL clustering algorithm. White balls indicate genes that cluster exclusively with apoptotic processes; gray balls depict genes that cluster with cell cycle processes. The black ball indicates genes that are related to a variety of cell processes. Continuous lines represent interactions. The symbol –| indicates gene inhibition, and arrows depict gene activation.

transforming growth factor-β (TGF-β). TGF-β signaling pathways are involved in the regulation of numerous cellular processes, including growth, development, death, oncogenesis and tumor progression [45]. Therefore, the antitumor effects of *SMAD4* depend on its potential to mediate the growth inhibition induced by TGF-β. Reports in the literature have indicated that *SMAD4* modulation is associated with tumor cell proliferation and contributes to invasiveness and metastatic phenotypes in a rat prostate cancer model [46,47]. However, we did not detect any changes in *SMAD4* expression in either cell line following treatment with AITC, suggesting that this gene is not directly related to the main mechanisms by which AITC inhibits cell proliferation.

Because we previously observed G2/M cell cycle arrest in T24 cells after AITC treatment [37], we also chose to investigate the expression of *ANLN*, given that the actin-binding protein anillin plays a special role in cell division [48]. We observed the upregulation of *ANLN* in T24 cells (but not in wt RT4 cells), suggesting AITC might indirectly affect cell division. Ronkainen et al. [49] recently reported that cytoplasmic anillin expression might be a marker of favorable prognosis in renal cell carcinoma patients. The disruption of mitotic microtubules in BALB cells following treatment with sulforaphanes, another type of isothiocyanate compound, has also been described [50], indicating an additional manner in which this class of compounds may affect the cell cycle (via the blocking of cell division prior to the initiation of metaphase).

The protein kinase encoded by *CDK1* is essential for the G1/S and G2/M phase transitions in the eukaryotic cell cycle. Chen et al. [14] described an association between decreased CDK-1 protein levels and G2/M phase arrest in GBM-8401 cells (human brain malignant glioma cells) after treatment with AITC. In contrast, we did not detect any changes in *CDK1* expression in either RT4 or T24 cells, suggesting that MPF is not involved in the inhibition of cell proliferation by AITC in bladder cancer cells.

In conclusion, the inhibition of cell proliferation by AITC is independent of *TP53* status. However, the molecular targets of AITC appear to differ. For example, while AITC acts via *BAX/BCL2* in RT4 cells, suggesting cell death by apoptosis, altered expression of *ANLN* and *S100P* were detected in T24 cells, suggesting the inhibition of proliferation due to cell cycle arrest.

Author contributions

All authors reviewed the manuscript. ALVS performed the experiments, interpreted the data and wrote the preliminary version of the manuscript. GNS contributed to the experimental design, data interpretation and the writing of the manuscript. DMFS contributed to the experimental design, data interpretation and the writing of the manuscript.

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Conflict of interest statement

The authors declare that they have no competing interests.

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