

Indole-3-Carbinol Inhibits Laryngeal Cancer Growth Through Cell Cycle Arrest

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Abstract: The growth of a variety of tumors are inhibited by indole-3-carbinol (I3C) obviously. But, its role in laryngeal cancer is not clear. The goal of this study was to research the probable roles that laryngeal cancer cell apoptosis and proliferation Hep-2 was influenced by I3C. I3C dose-dependently therapy obviously inhibited Hep-2 cell proliferation, and, I3C promoted apoptosis and induced cell morphological changes at 100, 200, 300, 400 μ M doses. We discovered that I3C shows anticancer effect through various signal pathways after Hep-2 cells I3C therapy. In Hep-2 laryngeal cancer cell line, through decreasing cell cycle-related proteins that include cyclin D1, CDK6, CDK4, and pRb, G1 arrest was induced by I3C. Apart from this, BALB/c nude mice constructed tumor-bearing mouse models. BALB/c nude mice were divided into three groups: treated with I3C, untreated control group and pretreated with I3C. After 8 weeks treatment, the untreated control group developed bigger tumors compared to mice treated or pretreated with I3C, and in the tumors such as cyclin D1, CDK6, CDK4 and pRb cell cycle-related proteins were obviously decreased. Further, the study result showed there was no harmful side effect in the heart, liver and kidney of the I3C-treated nude mice. In conclusion, both in vivo and in vitro I3C inhibited proliferation and induced the Hep-2 cells apoptosis, and showed low toxicity to normal cells. By suppressing the expression of cyclin families and CDK, we deduce that I3C can inhibit the Hep-2 cells growth in vitro. On normal organs and tissues, the I3C had no toxic effects and was safe.

Keywords: Laryngeal Cancer, Indole-3-carbinol, Cell Cycle, Apoptosis

1. Introduction

In the head and neck, in highly developed countries, laryngeal cancer which is one of the leading malignant tumors is reported to have a higher incidence [1]. There are many choices including the traditional ways of chemotherapy, radiotherapy and surgery that can be used to treat laryngeal cancer. But, it is known to all that the curative rate and effect is poor and demand obvious increasement. For treating laryngeal cancer, a less toxic and highly effective way is lacking, although for the cancer treatment, many chemotherapeutic drugs are available.

The beneficial effects of intake of fruits and vegetables have

been shown in lowering the incidence of cancers in recent dietary and epidemiological studies. Indole-3-carbinol (I3C), which is a natural phytochemical the vegetables of the cruciferous family ingredient, by inducing apoptosis displays anticancer activity [2, 3] and cell cycle arrest [3, 5], showing antimetastatic properties [2, 6] and suppressing angiogenesis gene products [7]. In some signal transduction pathways that were associated with the cell growth inhibition, the anticancer activity of I3C is also reflected. the inhibition effects of I3C have been evaluated by previous studies on the PI3K/Akt pathway [2, 7, 8] and nuclear factor-kB [9] signal transduction pathways. And mitogen-activated protein kinases (MAPK) expression was also been down-regulated by I3C

[10].

The study showed the probable effects of I3C on cell cycle arrest induction in vitro in Hep-2 laryngeal cancer cells, and characterize involved in vitro the proteins. Furthermore, the therapeutic and preventive effects of I3C were evaluated by us in laryngeal cancer.

2. Materials and Methods

2.1. Reagents

From the Cancer Research Institute of Sun Yatsen University (Guangzhou, China), the Hep-2 laryngeal cancer cell line and 16HBE human normal bronchial epithelial cell line were purchased [11-13] and in our laboratory conserved and in liquid nitrogen stored. Fetal bovine serum (HyClone, USA), RPMI 1640 medium, cell counting kit-8 (Dongji, Japan), Cyclin CDK4, CDK6, pRb, D1, Rb antibody and GAPDH antibody (Cell Signaling Technology, USA), 0.25% trypsin solution (Invitrogen, USA) were purchased from mentioned above companies.

2.2. Cell Grouping and Culture

The 16HBE human normal bronchial epithelial cell line and Hep-2 laryngeal cancer cell line were cultured in RPMI 1640, with 20 mg/ml ampicillin and kanamycin and 10% fetal bovine serum supplemented. In an incubator with 5% CO₂ at 37°C, all the cells were maintained. In logarithmic growth phase, in the experiment cells were used. In DMSO, in the Hep-2 and 16HBE group, I3C diluted with 0 mM, 100 mM, 200 mM, 300 mM, 400 mM final concentration was added, and control group which uses the same vehicle.

2.3. Cell Proliferation Assay

In 96 well plates, 16HBE and Hep-2 cells were seeded with per well for normal culture 1×10^3 cells. With a final concentration mentioned above in the experimental groups I3C were added. In each group the experiments were done in duplicate for 3 times, and also a blank control was used. each well added into 10 ml of CCK-8 and incubated at 37°C for 1 hour after 0 h, 24 h, 48 h and 72 h culture, and at 450 nm the absorbance value was detected.

2.4. For Detection of Cell-cycle Distribution Propidium Iodide Single Staining

16HBE and Hep-2 cells were harvested by trypsinization and in each group the cells were washed twice with cold phosphate-buffered saline after with different concentrations of I3C mentioned above treatment for 48 h. For overnight fixation 70% ethanol was used; in 500 μ l PBS cells were resuspended after centrifugation. Then 20 ml of 1 mg/ml of RNase and 0.5% TritonX-100 were added. 5 ml of 1 mg/ml propidium iodide (PI) solution was added after standing for 30 min at room temperature and at room for 30 min temperature incubated. On a FACStar (Becton-Dickinson, Mountain View, California, USA), then Flow cytometry

analysis was performed to determine the cell-cycle distribution. For each sample approximately 10,000 cells were examined, and with CELLQuest software (BD Biosciences, USA) the data were analyzed.

2.5. Western Blot

In the buffer which contains 1% Nonidet-P40, protease inhibitor 'cocktail' (Roche) and 2 mM dithiothreitol, the Hep-2 cells and in nude mice tumor xenograft were lysed and harvested. by 12% SDS-PAGE lysates were resolved, transferred to nitrocellulose membranes, and with primary antibodies against cyclin CDK4, CDK6, D1, Rb, pRb and GAPDH immunoblotted., proteins were detected with enhanced chemiluminescence (ECL) reagent, after immunoblotting with secondary antibodies.

2.6. Animal Grouping and Feeding

Beijing HuaFukang Biological Technology Co. Ltd. (HFK Bioscience, Beijing, China) purchased female BALB/C nude mice that were 4~6 weeks old and prior to the experiment underwent adaptive feeding one week. Contained 0.5% I3C feed nude mice and Beijing HuaFukang Biological Technology Co. Ltd manufactured the conventional nude mice feed. As previously described, animal welfare and xenograft tumor inoculation were performed [14, 15]. The nude mice was divided into three groups and each group eight nude mice, which include the preventive treatment group (before the inoculation of laryngeal cancer Hep-2 cells with feed containing 0.5% I3C for two weeks nude mice fed), the treatment group (when laryngeal cancer cells were inoculated feed containing 0.5% I3C) and the control group (with laryngeal cancer cells fed with inoculated and regular feed). The experiment duration was 8 weeks. After the inoculation of laryngeal cancer Hep-2 cells, for volume calculation and statistical analysis the xenograft tumors long and short diameter in 2, 4, 6, 8 Week were measured. The following equation estimated tumor volume: volume = $1/2 * L * W^2$, where W is width and L is length [16]. After inoculation eight weeks, sacrificed the animals and for further evaluation the xenograft tumors, the liver, heart and kidney were preserved. Ahe Animal Ethics Committee of Renmin Hospital of Wuhan University approved the study.

2.7. Hematoxylin and Eosin Staining

For 24 hours, formalin fixed the heart, liver and kidney that were the xenograft tumor specimens, which were for 3 hours respectively dehydrated by 70%, 80%, 90% ethanol, and then for 1 hours 100% ethanol I, for 2 hours 100% ethanol II and for 20 minutes vitrified by xylene I and xylene II. for 40 minutes in paraffin I and II after immersing, the specimens were sliced and embedded. As follows staining was performed: for 15 minutes hematoxylin staining, for 35 seconds decoloring hydrochloric acid alcohol solution, for 10 minutes eosin staining and for 40 seconds decoloring 90% ethanol. Then for mounting neutral balsam was used and under the microscope the section was observed and

photographed.

2.8. Statistical Analysis

All values were expressed as mean \pm SEM. SPSS16.0 one-way ANOVA using the SPSS statistical software carried out statistical analyses. As statistically significant probability values (P -value) <0.05 were considered.

3. Results

3.1. The Growth of Laryngeal Cancer Cells Were Inhibited by I3C

Used CCK-8 method the experiment studied whether I3C could effectively inhibit the carcinoma cells proliferation. with I3C significantly treatment inhibited the Hep-2 cells proliferation (Figure 1A) in a time- and dose-dependent manner. After 72 h treatment when the final concentration of I3C reached 300 mM, both exceeded 60% the inhibition efficiency in Hep-2 cell line additionally; and when the concentration was 400 mM it reached to about 90%. Specially, the same experiments were performed in human bronchial epithelial cells 16HBE, in order to explore I3C the effects on the division and proliferation of normal human cells and tissues, (Figure 1B). The study indicated that compared with laryngeal cancer cell line Hep-2on 16HBE cell I3C400 mM had much less inhibitory effect proliferation.

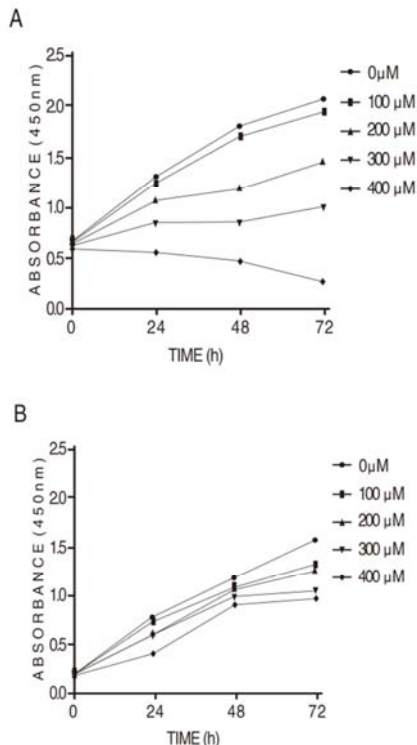


Figure 1. The viability of cells after I3C treatment. The viability of nasopharyngeal carcinoma cells Hep-2 and human bronchial epithelial cells 16HBE were detected by CCK-8 assay. (A) The proliferation of Hep-2 cells were inhibited by I3C in a dose- and time-dependent manner. (B) The proliferation of 16HBE cells were not inhibited by I3C.

3.2. In Laryngeal Cancer Cells I3C Induced Cell-Cycle Arrest

To determine on cell cycle the I3C effect, after 48 hours treatment of I3C with different concentrations using PI staining, the study evaluated the Hep-2 cells cell cycle distribution. The results indicated that after the treatment the laryngeal cancer cells cell cycle re-distributed. Significantly proportion of G0/G1 phase cells increased, significantly proportion of G2/M phase cells reduced and proportion of S phase cells had no significant change with the increase of I3C concentration. From 43.5% to 64.7% G0/G1 phase cells increased, from 33.5% to 15.3% G2/M phase cells decreased, from 18.7% to 19.3% S phase cells differed in Hep-2 cells. (Figure 2A). But, in 16HBE cell line significantly each phase cells did not elevate when from 0 to 400 mM the concentration of I3C changed (Figure 2B).

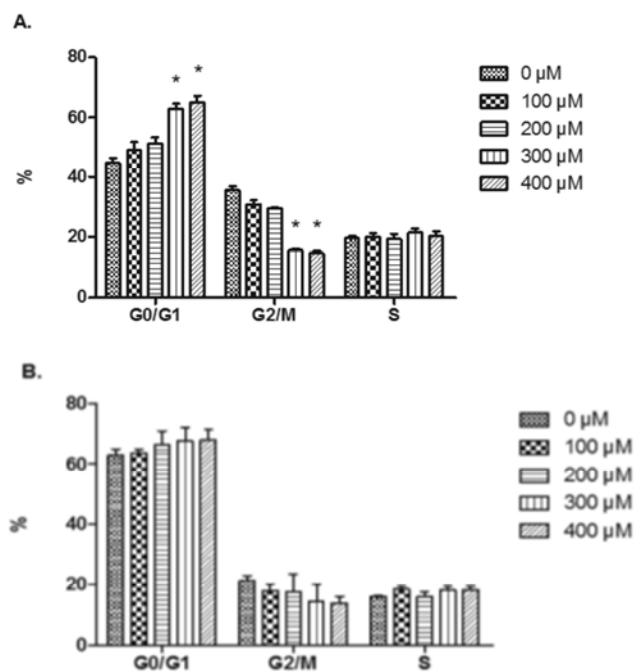


Figure 2. The result of cell cycle distribution. The cell cycle distribution was detected by flow cytometry. (A) The proportion of G0/G1 phase cells increased and G2/M phase cells decreased in Hep-2 cell lines as I3C concentration increased. (B) The proportion of all phase cells barely elevated in 16HBE cell line as I3C concentration increased. The data are presented as the mean \pm SEM. * $p < 0.05$, compared to the proportion of each phase cells in 0 mM I3C treatment.

3.3. After Treatment in Vivo and in Vitro I3C Changed the Expression of Cell Cycle Related Proteins

In the cell cycle of laryngeal cancer cell lines, the study indicated above implicate that I3C had a significant regulatory role. So, the study evaluated the several key proteins expression involved in laryngeal cancer cells cell cycle regulation. We observed that cyclin CDK4, CDK6, D1 and pRb the protein expression levels were down-regulated as I3C concentration increased in Hep-2 cells (Figure 3). Additionally, in the nude mice Hep-2 xenograft tumors these

proteins expression as compared to the control group from I3C treatment and I3C preventive groups decreased. (Figure 4)

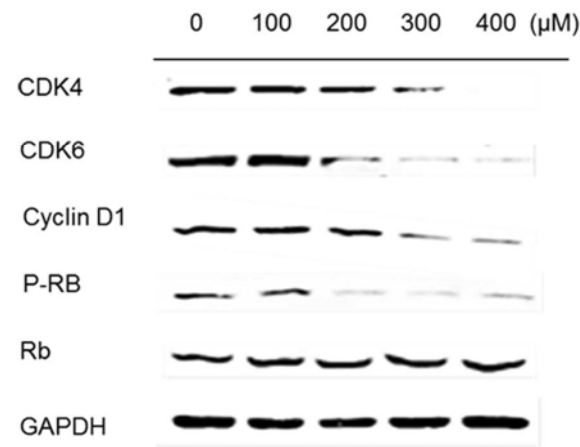


Figure 3. The expression of cell cycle related proteins in vitro. Key proteins related to cycle regulation were detected by WB. As I3C concentration increased, cyclin D1, CDK4, CDK6 and pRb protein expression in Hep-2 cell line were down-regulated. The data are presented as the mean±SEM.

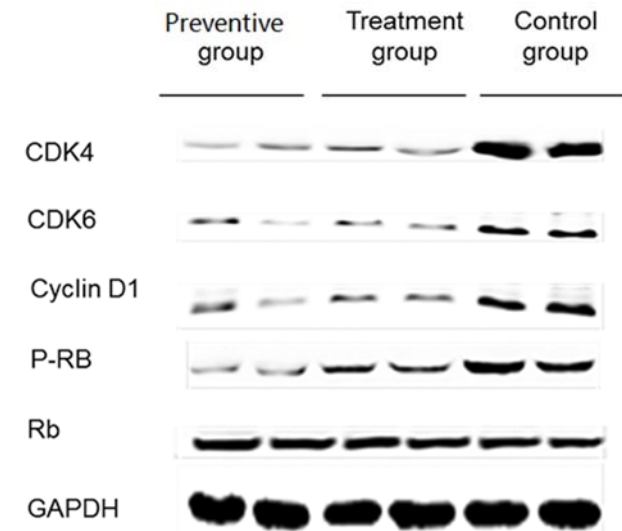


Figure 4. The expression of cell cycle related proteins in vivo. Cyclin D1, CDK4, CDK6 and pRb protein expression were down-regulated in the xenograft tumors of Hep-2 in I3C preventive and I3C treatment groups compared to the control group. The data are presented as the mean±SEM.

3.4. After Treatment in Vivo and in Vitro I3C Changed the Expression of NF-kB and MAPK Signaling

Cells and xenograft tumors detected the NF-kB and MAPK proteins expression. when the I3C concentration increased, the Hep-2 cells down-regulated P-JNK, P-P38/ERK1/2, and NF-kB (p65) (Figure 5). As compared to the control group, from I3C preventive and I3C treatment groups, in the nude mice xenograft tumors of Hep-2, these proteins expression also decreased (Figure 6).

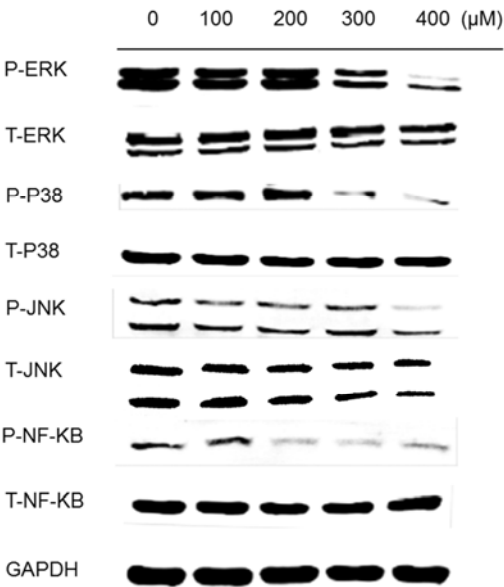


Figure 5. The expression of MAPK and NF-kB proteins in vitro. The expression of MAPK and NF-kB signaling decreased as I3C concentration increased in Hep-2. The data are presented as the mean±SEM.

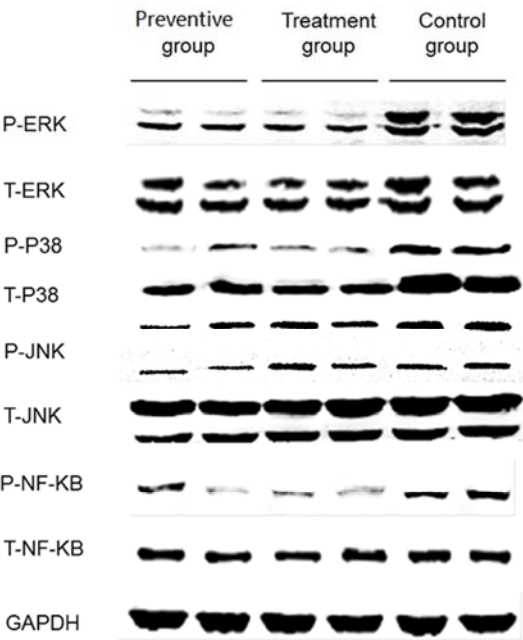


Figure 6. The expression of MAPK and NF-kB proteins in vivo. MAPK and NF-kB proteins expression were down-regulated in the xenograft tumors of Hep-2 in I3C preventive and I3C treatment groups compared to the control group. The data are presented as the mean±SEM.

3.5. In Nude Mice Anti-tumor Efficacy of I3C

In vivo animal experiments, the I3C anti-tumor effects were studied. Rapidly in the control group the tumor volumes increased, and the control group was given only conventional nude mice feed. In the experiment 8th week, the xenograft tumor mean volume in Hep-2 cells of the nude mice control group, I3C treatment group and I3C preventive treatment group were (2596±478.37) mm³, (1482±249.12) mm³ and (802±225.32) mm³ (Figure 7).

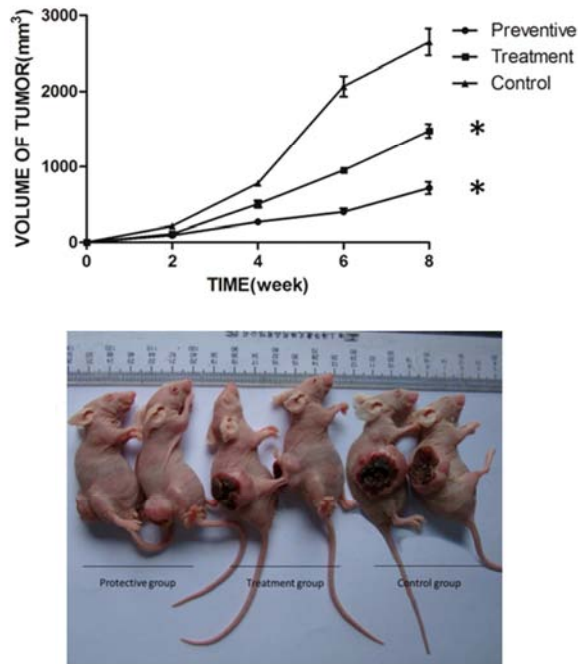


Figure 7. Anti-tumor efficacy of I3C in nude mice. The effect of I3C on tumor growth of Hep-2 in xenograft nude mice. The data are presented as the mean \pm SEM. * $p < 0.05$, compared to control group.

3.6. As an Anti-tumor Agent Safety of I3C

During treatments in order to evaluate whether I3C causes pathological damages to the experimental animals vital organs, the sections of the nude mice heart, liver and kidney routine HE staining in different groups was performed. In the heart, liver and kidney in all groups no necrosis, degeneration or structural disorders was observed, showing that the containing 0.5% I3C feed had no side or toxic effects (Figure 8).

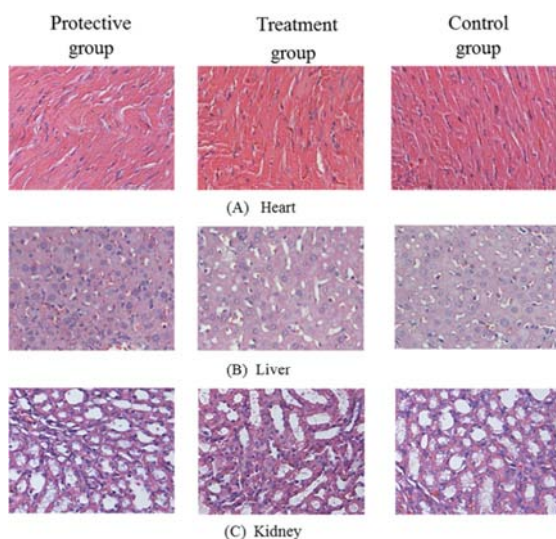


Figure 8. The result of HE staining of main organs of nude mice after I3C treatment. (A) HE staining of heart, (B) HE staining of liver, (C) HE staining of kidney.

4. Discussion

In the laryngeal cancer treatment chemotherapy plays an important role. The high toxicity of the drugs being used is the current chemotherapy major problem [17]. So, laryngeal cancer chemotherapy needs drugs with improved efficacy and safety.

An active ingredient is I3C which is extracted from natural cruciferous plants. In various tumor cells anti-tumor effect is showed by I3C significantly. In colon, prostate, lung and breast cancer cells cell cycle arrest is induced effectively. In laryngeal cancer the I3C studies were not sufficient. *in vitro* and *in vivo* in laryngeal cancer the study indicated the I3C effect in apoptosis [18, 19]. In this study, the Hep-2 human laryngeal cancer cell lines proliferation can be inhibited by I3C, but the 16HBE human normal bronchialepithelial cell line can not be inhibited by I3C. While exerting little effect on normal cells, this phenomenon indicated that I3C suppressed the tumor cells growth *in vitro*. The study showed that I3C could specially inhibit the carcinoma cells proliferation and to normal cells I3C might be safe. Thus, as an anti-tumor drug I3C which was used is promising.

From the previous studies [20-21], the study results showed that in the Hep-2 cell line G0/G1 cell cycle arrest was induced by I3C and *in vitro* and *in vivo* the cyclin CDK4, CDK6, D1 and pRb downregulation in Hep-2 cells accompanied the arrest. The cell cycle regulation mechanism is the cyclins, CDKs, and CDKs reciprocity. In cell cycle progression CDKs are the core effectors. CDK activity is regulated by inhibiting and activating the phosphorylation events and CDKs as well as through the association with their cyclin partners. CDK4/6 phosphorylate Rb protein and cyclin D associated with CDK4/6. At the restriction point in G1 phase, they initiate progression. In mid-late G1 phase cyclin D/CDK4/6 activity occurs, and for the retinoblastoma gene product pRb hyperphosphorylation it is required. At the late G1 restriction point the protein Rb controls progression and the protein is the G1/S transition major regulator. Such as overexpression of related CDKs cyclin D, or mutations to CDKs, that affect p16 binding, any component of this pathway alterations will lead to Rb phosphorylation and progression from phase G1 to S subsequently [22]. In many human tumors these alterations have been showed, which suggested that in their pathogenesis the cell cycle pathway inactivation can play an important role.

ERK1/2, JNK and p38 MAPK mediate MAPK signaling, and they are important in the cell differentiation, transformation and proliferation control. Due to its transcriptional regulation, a transcriptional factor, NF- κ B is critically involved in tumor progression [23]. Further, block apoptosis showed signaling pathway NF- κ B, which by death receptors induced and promote cancer cells proliferation. In various tumor cells, NF- κ B constitutive activation has been detected [24]. The study showed that through a MAPK pathway by cell cycle proteins transcriptional downregulation the cell cycle arrest observed may be regulated. Western blotting indicated that I3C expression down-regulated the

MAP kinase and NF- κ B pathway several components in xenograft tumors and laryngeal cancer cells. Also I3C inhibits from G1 to S phase in vitro cell cycle progression and laryngeal cancer cell growth, and in vivo suppresses tumor formation of laryngeal cancer cell. The most likely mechanism that is underlying the I3C induced growth arrest involves NF- κ B and MAP kinases pathway down-regulation, which leads to in G1-related CDKs reduction, such as p-RB and CDK4, and in CDK4 and cyclin D1 expression decrease [24].

In the experiment, in the preventive and treatment group containing 0.5% I3C normal diet was given. After the inoculation of tumor cells eight weeks, the tumor volume was the biggest in control group, moderate in treatment group and the smallest in I3C preventive treatment group. Thereby converting into several condensation products, studies indicated that I3C is unstable in such as gastric juice acidic milieu. I3C exhibits such as anti-proliferative and pro-apoptotic against tumor or endothelial cells biological activities, but the effective concentrations of I3C used in vitro in those experiments were relatively high (in most cases) >100 mM [25]. A previous study [26] detected I3C with 250 mg/kg I3C using an HPLC method following the dosing of mice in tissues. At 15 min the 28 mM I3C maximum level was observed. In mice and humans pharmacokinetic studies showed that in plasma the I3C concentration within 1 h fell below the detection limit [27]. In the mice in preventive and treatment group the I3C average amount intake was close to 250 mg/kg. It showed that in plasma the I3C concentration was approximate 28 mM. In much less than 300 mM concentrations, I3C could inhibit in those two groups the xenograft tumor growth. It indicates that in vivo I3C could inhibit the tumor growth effectively. The possibility is that metabolites are more active than against laryngeal cancer cells I3C in vivo. The studies have established that 400 mg I3C ingestion twice daily is tolerated well [28]. In the study, mice were with I3C orally treated at about a 250 mg/kg per day dosage, which is equivalent to in an average adult human a 20.3 mg/kg dose [29]. Within the range in clinical trials the dose is that has been used. Moreover, since in this experiment the inoculated laryngeal cancer cells amount was large (approximately 2×10^6), it was not impossible to be prevented by I3C completely. Due to mutation the tumor cells initial number in the human body was I3C small and stable blood plasma concentrations could destroy the mutated tumor cells majority, which might play an important role in the laryngeal cancer tumor prevention. In the prevention of laryngeal cancer tumor I3C could destroy the mutated tumor cells majority, which might play an important role.

5. Conclusion

The study indicated that I3C inhibited laryngeal cancer cell growth effectively. As a natural ingredient, to normal cells and tissues I3C caused little damage, and to use was relatively safe. The animal experiments results showed that I3C had certain tumor preventive effect and inhibited the tumors formation

and development. In the future, it is important for us to focus on in recurrent laryngeal cancer the I3C therapeutic effect.

Acknowledgements

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