Trop J Nat Prod Res, November 2023; 7(11):5250-5255
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Ursolic Acid-Loaded Chitosan Nanoparticles Modulate the Expression Pattern of Apoptotic Markers Towards Oral Tumour Inhibition in Golden Syrian Hamsters

Mohan Karthik[1], Ellapan Paari[1], Shanmugam M Sivasankaran[1], Chakaravarthy Elanchezhiyan[2], Shanmugam Manoharan[1]

[1]*Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar- 608002, Tamilnadu, India.*
[2]*Department of Zoology, Annamalai University, Annamalainagar-608002, Tamilnadu, India*

ARTICLE INFO

Article history:
Received 30 September 2023
Revised 27 October 2023
Accepted 13 November 2023
Published online 01 December 2023

ABSTRACT

Apoptosis, a systematic and harmonized sequential process, eliminates cells that contain potentially dangerous mutations. Cancer cells evade apoptosis by interfering with the functions of pro-apoptotic and anti-apoptotic proteins. The efficiency of ursolic acid-loaded chitosan nanoparticles (UACNP) in modulating pro-apoptotic and anti-apoptotic markers' expression towards tumor inhibition was assessed using immunohistochemistry in 7,12-dimethylbenz(a)anthracene (DMBA)-induced oral carcinoma in golden Syrian hamsters. An oral tumor was developed in the buccal pouch of hamsters by painting with DMBA, three times per week for 14 weeks. While buccal mucosa mutant p53 and Bcl-2 proteins were overexpressed, Bax, Bid, Bad, caspase-3 and 9 were downregulated in tumor-bearing hamsters. UACNP administration (12.5 mg/Kg b.w) to hamsters, on alternative days to DMBA exposure reverted the immunoexpression pattern of apoptotic markers towards the inhibition of tumor formation. The present investigation also observed that UACNP can improve apoptotic marker expression in the chemotherapeutic phase (DMBA → UACNP) as evidenced by the reduction in the number of tumors as well as tumor size. The observed findings thus highlight the apoptotic efficiency of UACNP, which could probably be attributed to its tumor-preventing efficacy in DMBA-induced oral carcinogenesis.

Keywords: Oral cancer, DMBA, Ursolic acid loaded-chitosan nanoparticles, Apoptosis

Introduction

Oral cancer is an aggressive life-threatening cancer that causes a severe health burden and high mortality around the globe. Oral cancer affects approximately 0.4 million people every year globally. Tobacco and alcohol abuse are pointed out as potent oral carcinoma risk factors.[1] Oral carcinoma frequently arises on lips, gums, tongue, palates and salivary glands. The treatment of oral carcinoma still faces significant difficulties because of its rapid progression, resistance to drugs, and lack of response, despite advancements in oral cancer treatments such as targeted therapy, gene therapy, and therapy with potent chemotherapeutic drugs. Early detection, prevention and early treatment can significantly improve oral cancer patients' life quality.[2,3]

Cells with potentially harmful mutations are eliminated by apoptosis, an ordered and coordinated sequential process. Cancer cells avoid apoptosis by interfering with the actions of pro-apoptotic and anti-apoptotic proteins. A defect in the apoptotic mechanism would result in uncontrollable and abnormal growth of cells that ultimately leads to the formation of a tumor. Cellular apoptosis has been aberrantly dysregulated at various stages of oral squamous cell carcinoma.[4,5]

*Corresponding author. E mail: sakshiman@rediffmail.com
Tel: +91 94425 48117

Citation: Karthik M, Paari E, Sivasankaran SM, Elanchezhiyan C, Manoharan S. Ursolic Acid-Loaded Chitosan Nanoparticles Modulate the Expression Pattern of Apoptotic Markers Towards Oral Tumour Inhibition in Golden Syrian Hamsters. Trop J Nat Prod Res. 2023; 7(11):5250-5255. http://www.doi.org/10.26538/tjnpr/v7i11.33

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Defects in apoptotic pathways as well as the caspase cascade were documented in oral carcinoma. Studies utilizing natural products against oral carcinoma revealed promising results for the induction of apoptosis in oral malignant cells. Apoptosis-mediated cancer cell death is characterized by apoptotic body formation, cellular shrinkage, chromatin condensation, DNA fragmentation and cell blebbing. Apoptotic induction in eliminating the targeted oral cancer cells has been pointed out as a valid or effective strategy to treat or combat oral cancer.[6,7]

Animal models could help to study the sequential precancerous to cancerous alterations occurring in carcinogenesis and serve as ideal preclinical models to test the efficiency of anticancer drugs before entering into human clinical trials. Golden Syrian hamsters serve as a suitable model to study the chemopreventive/chemotherapeutic efficacy of natural products. Oral carcinoma induced by painting with DMBA in the buccal mucosa has been widely studied for assessing histological, genetic, and biomolecular alterations occurring in oral cancer as DMBA-induced abnormalities mimic human oral carcinogenesis.[8-10]

Several signaling pathways involved in the development of cancer can be inhibited by natural products because of their ability to act on multiple targets. Traditional medicines play a vital role in the treatment and prevention of several diseases including cancer. Ursolic acid, a plant metabolite, has been extensively utilized by researchers to explore its diverse pharmacological effects including cancer. Ursolic acid exhibited antidiabetic, hepatoprotective, cardioprotective, antioxidant, anti-inflammatory and anticancer properties.[11-13] Moreover, due to its low bioavailability, nowadays ursolic acid-loaded chitosan nanoparticles (UACNP) are synthesized to enhance their therapeutic properties. Studies on ursolic acid nanoparticles against experimental carcinogenesis have not been documented well. Recently, the chemopreventive efficacy of UACNP has been explored in our laboratory in hamster buccal pouch carcinogenesis[14] and this study was designed to evaluate the molecular mechanism behind the anticancer potential of UACNP against DMBA-induced oral

carcinogenesis by analyzing the immunoexpression pattern of pro-apoptotic and anti-apoptotic markers.

Materials and Methods

Most of the biochemicals and Analar grade chemicals used for the study were procured from Sigma Aldrich Pvt, Ltd, India. The antibodies (primary and secondary) were procured from Santa Cruz Biotechnology, USA and Bio Genex, USA.

Animals

Golden Syrian hamsters were purchased from the National Institute of Nutrition, India. Hamsters were cared for by institutional ethical committee guidelines (Approval number - IAEC/1249/7/19) at Annamalai University's central animal house, Annamlainagar, Tamilnadu.

Experimental protocol

Thirty hamsters were equally divided into five groups. Group I animals served as vehicle-treated control and received liquid paraffin alone topically on the buccal pouches, three times a week for 14 weeks. Group II hamsters were used to induce tumors through topical application of 0.5% DMBA in liquid paraffin on the buccal pouches, three times a week for 14 weeks and served as tumor-bearing animals in the study. Group III hamsters received DMBA topically, three times a week, and UACNP administration (12.5 mg/Kg b.w orally), three times a week on alternative days of DMBA application, for 14 weeks. Hamsters that received DMBA topically on the buccal pouches (three times a week) for 10 weeks followed by oral administration of UACNP (12.5 mg/Kg b.w, three times a week) for 8 weeks served as group IV. Group V hamsters were treated with UACNP alone (12.5 mg/Kg b.w, three times a week) for 14 weeks.

Synthesis and characterization of UACNP

The synthesis and characterization of UACNP have been described in detail in our recent publication.[14] Briefly, the UACNP were synthesized using the ionic gelation method according to the procedure of Calvo et al.[15] The scanning electron microscope (SEM) and Fourier-Transform Infrared Spectroscopy (FTIR) were used to characterize the synthesized nanoparticles. The size and zeta potential of the nanoparticles were found as 144.0 nm and 30.5 mV respectively. The SEM image of UACNP revealed a spherical shape with a smooth surface.[14]

Immunohistochemical studies

Immunohistochemical analyses were done to analyze the status of pro-apoptotic (mutant p53, Bax, Bid, Bad, Caspases 3 and 9) and anti-apoptotic (Bcl-2) markers in the experimental hamsters' buccal mucosa tissues. In brief, the antigen-retrieved paraffin-embedded slides were treated with monoclonal antibodies corresponding to pro-apoptotic and anti-apoptotic markers. Following incubation, the slides were treated with secondary antibodies labeled with horseradish peroxidase. To visualize antigen-antibody complex, the slides were treated with chromogen, diaminobenzidine, and subsequently counterstained with hematoxylin. The slides were then examined for the immunoexpression pattern of the pro-apoptotic and anti-apoptotic markers under the microscope.

Results and Discussion

Histological studies revealed keratin pearl formation, severe hyperplasia and dysplasia in tumor-bearing hamsters. The severity of the above-said histological abnormalities was drastically and moderately reduced in DMBA + CACNP and DMBA → CACNP treated hamsters respectively.[14] Immunoexpression patterns of pro-apoptotic and anti-apoptotic markers observed among various experimental groups are depicted in Figures 1 to 7. The immunoexpression patterns of mutant p53 and Bcl-2 were overexpressed in the buccal pouches of hamsters bearing oral tumors. On the other hand, the expression patterns of Bax, Bad, Bid and caspases 3 and 9 were downregulated in the buccal pouches of tumor-bearing hamsters. Oral administration of UACNP significantly reverted the expression status of the above-mentioned apoptotic markers in the group III hamsters (DMBA + UACNP) to near normal expression pattern. However, in the group IV hamsters (DMBA → UACNP), the treatment slightly improved the expression patterns of the studied apoptotic markers. The expression status of apoptotic markers was similar in animals treated with liquid paraffin alone (Group I) and in animals treated with UACNP alone (Group V).

Oral cancer causes a significant health burden on the patients and a severe economic burden on their families. The 5-year survival outcome and life quality of these patients are found to be very poor despite advancements in treatment strategies. Therefore searching for natural products or synthetic entities with fewer side effects and potent anticancer efficacy could enhance the overall quality of life and survival outcome of the patients. Any defect in apoptotic cascade signals could thus favor tumor aggressiveness and metastasis. Thus, in this study, the pro-apoptotic and anti-apoptotic protein expression patterns were determined to assess the effectiveness of UACNP in inducing apoptosis in hamster buccal pouch carcinoma.

A spectrum of genetic and epigenetic alterations in the apoptotic signaling pathways have been documented well in oral carcinoma.[16] The defect and deregulations in the extrinsic apoptotic pathway have been documented in oral carcinogenesis.[16] The p53 is crucial for preventing the etiopathogenesis of several carcinogenesis. Daneste et al.[17] reported abnormal mutant p53 expression patterns in oral carcinoma. The p53 mutation has been noticed in over 50% of human cancers.[18] Kamat et al.[19] suggested that p53 expression status in oral carcinoma can be used to identify the chances of developing local recurrence as well as to predict survival outcomes. Novack et al.[20] reported that p53 expression was abnormal in oral epithelial dysplasia. The p53 mutation is associated with decreased survival and resistance to chemotherapy in oral carcinoma patients.[21,22] Abnormal expression of p53 has been well documented in experimental oral carcinoma as well.[23-25]

The B-cell lymphoma-2 (Bcl-2) gene plays a crucial role in the inhibition of apoptosis by favoring the survival of cancer cells. Elevated Bcl-2 expression contributes to the poor prognosis of several cancers.[26] Enhanced Bcl-2 expression pattern was noticed from precancerous to cancerous lesions.[27] Abnormal expression of Bcl-2 in oral tumor tissues was well documented.[28,29]

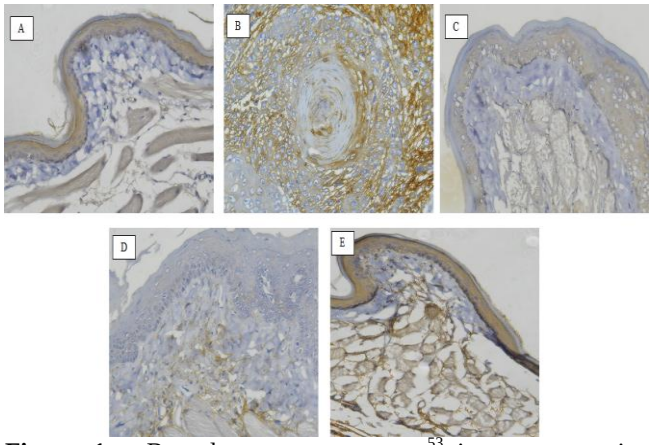

Figure 1: Buccal mucosa mutant p^{53} immunoexpression pattern in experimental hamsters

A. There was no mutant p53 expression in the basal and parabasal cells of control hamsters.

B. The expression of mutant p53 was greater in the keratin pearls surrounded by dysplastic epithelial cells of tumor bearing hamsters.

C. In basal cells of DMBA + UACNP treated hamsters, little mutant p53 expression was visible.

D. mutant p53 expression in the basal cells was low in buccal mucosa tissues from DMBA → UACNP treated hamsters.

E. The basal and parabasal cells of hamsters treated only with UACNP did not show the expression of mutant p53 gene.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Overexpression of Bcl-2 was noticed in the early phases of carcinogenesis.[30] Bax, a proapoptotic gene, competes with Bcl-2 in the process of programmed cell death. The fate of cell survival or cell death is decided by the Bcl-2/Bax ratio and is altered in several cancers.[31,32] Higher Bcl-2/Bax ratio was reported in OSCC.[33] Bid and Bad, the two important pro-apoptotic proteins, play a vital role in the process of programmed cell death. Bid and Bad regulated the process of apoptosis by binding with Bcl-2 protein.[34]

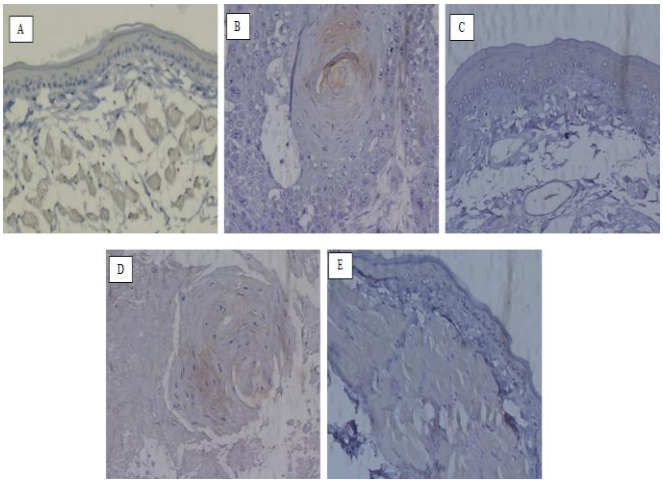

Figure 2: Buccal mucosa Bcl-2 immunoexpression pattern in experimental hamsters
Bcl-2 was not expressed in the epithelium of control hamsters.
Bcl-2 overexpression was noticed in the buccal mucosa of DMBA alone treated hamsters.
Hamsters treated with DMBA +UACNP showed mild expression of Bcl-2.
Basal and parabasal cells of DMBA →UACNP treated hamsters showed moderate Bcl-2 expression.
Hamster buccal mucosa received UACNP treatment alone revealed no Bcl-2 expression in the basal or parabasal cells.

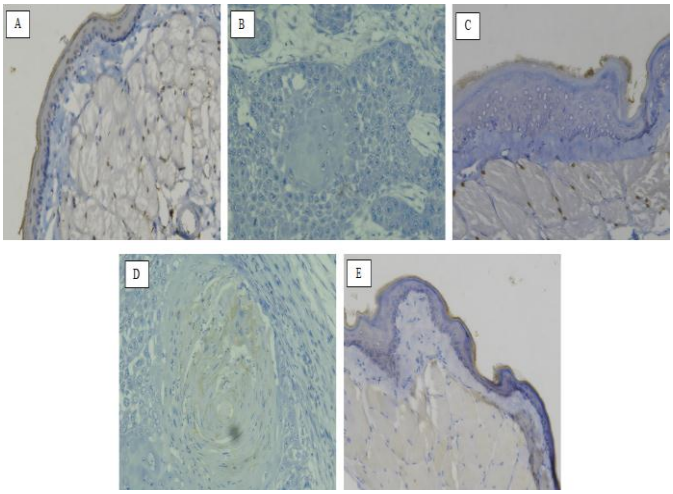

Figure 3: Buccal mucosa Bax immunoexpression pattern in experimental hamsters
Control hamsters exhibited basal cell expression of Bax.
B. Hamster buccal mucosa treated with DMBA alone showed no Bax expression.
C. Bax expression was mild to moderate in the basal cells of DMBA + UACNP treated hamsters.
D. Bax expression in the basal cells was low in hamsters treated with DMBA →UACNP.
E. Bax expression was noticed in the basal cells of hamsters that were given only UACNP.

Sinicrope *et al*.[35] reported that bad and bid proteins can be utilized as prognostic markers in cancer. It has also been reported that Bid is involved in the process of DNA repair via programmed cell death.[36] Bad has a crucial role in downregulating the expression of Bcl-2.[37] Abnormal expression of Bid and Bad has been reported in oral carcinogenesis.[38]

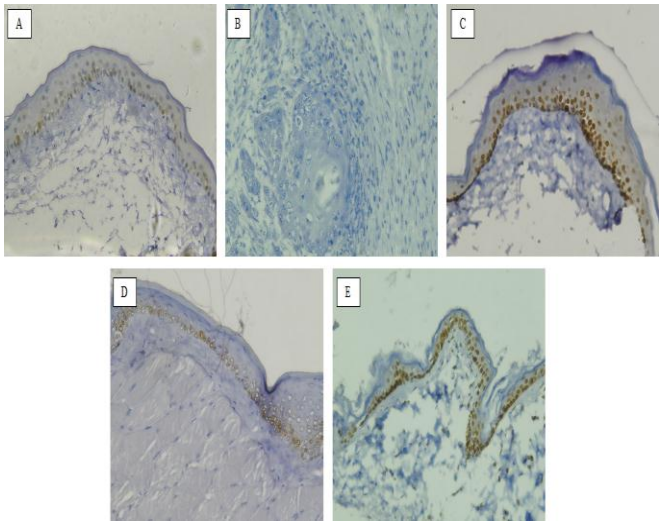

Figure 4: Buccal mucosa Bid immunoexpression pattern in experimental hamsters
A. Control hamsters displayed Bid expression throughout the epithelial layer.
B. Hamster treated with DMBA alone had no evidence of Bid expression.
C. Bid expression was observed throughout basal and parabasal cells of DMBA + UACNP treated hamsters.
D. Hamsters given DMBA → UACNP exhibited mild to moderate Bid expression.
E. Bid was expressed across the epithelial layer in buccal mucosa tissues from hamsters that had only received UACNP treatment.

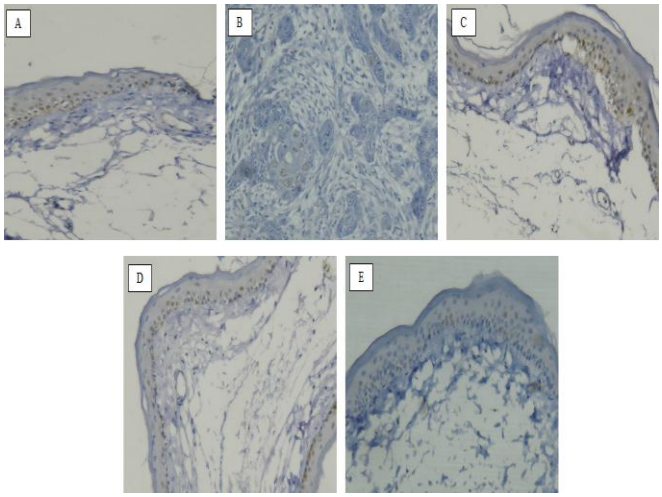

Figure 5: Buccal mucosa Bad immunoexpression pattern in experimental hamsters
A. Control hamster buccal mucosa tissues displayed Bad expression was noticed throughout the epithelial layer, of control hamsters.
B. Hamster bearing tumors showed low Bad expression.
C. Buccal mucosa of DMBA + UACNP treated hamsters showed expression of Bad throughout the epithelial layer.
D. Buccal mucosa from DMBA →UACNP treated hamsters exhibited a mild Bad expression in the basal cells.
E. Bad expression was low throughout the buccal mucosa from hamsters that had only received UACNP treatment.

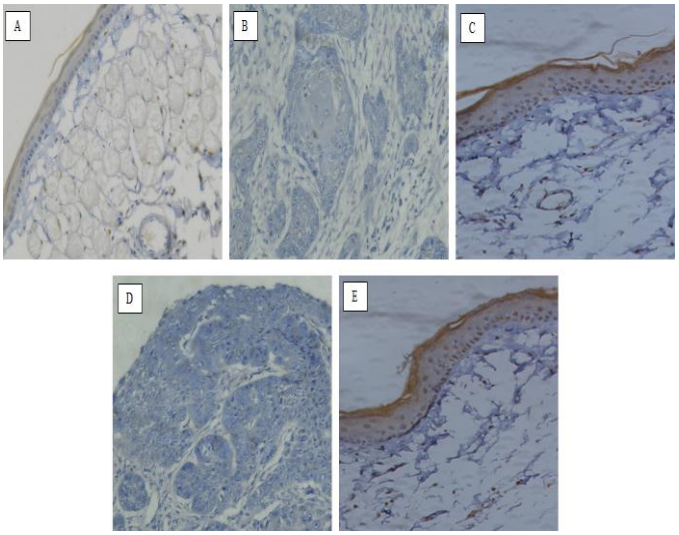

Figure 6: Buccal mucosa caspase-3 immunoexpression pattern in experimental hamsters
A. Caspase-3 was expressed in the basal cells of buccal mucosa from control hamsters.
B. Caspase-3 expression was very low in the buccal mucosa of hamsters received only DMBA treatment.
C. Caspase-3 was expressed in the basal cells of buccal mucosa from hamsters that had been treated with DMBA + UACNP.
D. DMBA → UACNP treated hamsters exhibited very mild to moderate caspase-3 expression.
E. The basal and parabasal cells expressed caspase-3 in the buccal mucosa of hamsters that had received only UACNP treatment

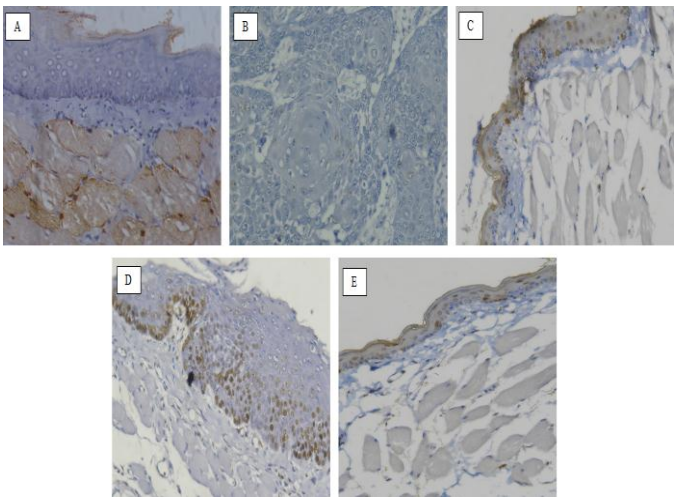

Figure 7: Buccal mucosa caspase-9 immunoexpression pattern in experimental hamsters
A. In the basal cells of control hamsters, caspase-9 expression was observed.
B. Caspase-9 expression was low in hamsters received DMBA alone.
C. Caspase-9 expression was noticed in the basal cells of DMBA + UACNP treated hamsters.
D. In DMBA→ UACNP treated hamsters, Caspase-9 expression was mild to moderate in the basal and parabasal cells.
E. Caspase-9 expression was observed in the basal cells of hamsters that were given only UACNP.

Apoptotic initiation and execution are regulated by caspases, a vital apoptotic signaling molecule.[39] Apoptotic initiation is triggered by caspases 8 and 9 and the apoptotic execution is achieved by caspases 3, 6 and 7. Caspase 3 downregulation was noticed in various cancers. The expression pattern of caspase-9 in experimental oral cancer was found to be downregulated.[40] In the current investigation, overexpression of mutant p53 accompanied by enhanced Bcl-2 expression and downregulation of Bax, Bid, and Bad expression revealed the deregulations of apoptotic cascade in tumor-bearing hamsters. Ursolic acid-induced p53 and caspase expression and downregulated Bcl-2 expression in melanoma cells.[41] Ursolic acid inhibited hepatocarcinoma cell proliferation through apoptotic induction by activating the p53 pathway.[42] Wang et al.[43] reported that ursolic acid stimulated the expression of caspases 3 and 9 in both *in vitro* and *in vivo* conditions. Cervical cancer cell growth was inhibited through apoptotic induction by ursolic acid nanoparticles.[44] Shan et al.[45] reported that ursolic acid can upregulate caspases 3 and 9 and downregulate Bcl-2 in colorectal carcinogenesis. The present results of the study corroborate these findings. The present study noticed an upregulation of Bax, Bid, Bad, caspases 3 and 9 and downregulation of mutant p53 and Bcl-2 in the chemopreventive phase (DMBA+UACNP) and improvement in the expression of the above markers in the chemotherapeutic phase (DMBA →UACNP). Oral administration of ursolic acid nanoparticles modulated the expression of pro-apoptotic and anti-apoptotic markers towards tumor inhibition in the chemopreventive phase (group III) and towards reduction in the tumor numbers and size in the chemotherapeutic phase (group IV) that explains the apoptotic efficiency of UACNP in DMBA induced oral carcinogenesis. Thus, the observed findings postulate that UACNP has either activated or stimulated the apoptotic cascade in tumor cells. The above findings were further confirmed with the decreased caspases-3 and 9 expression in tumor-bearing hamsters.

Conclusion

The present study revealed that UACNP can modulate the expression pattern of apoptotic markers towards inhibition of oral tumor formation in golden Syrian hamsters since UACNP modulated both pro-apoptotic and anti-apoptotic proteins towards inhibition of tumor formation in DMBA-induced oral cancer.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

Post-metric Research increment to Mr.M.Karthik from the Government of Tamilnadu is gratefully acknowledged

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-249.
2. Dhanuthai K, Rojanawatsirivej S, Thosaporn W, Kintarak S, Subarnbhesaj A, Darling M, Kryshtalskyj E, Chiang CP, Shin HI, Choi SY, Lee SS, Aminishakib P. Oral cancer: A multicenter study. Med Oral Patol Oral Cir Bucal. 2018;23(1):e23-e29.
3. Li H, Zhang Y, Xu M, Yang D. Current trends of targeted therapy for oral squamous cell carcinoma. J Cancer Res Clin Oncol. 2022;148(9):2169-2186.
4. Kashyap D, Garg VK, Goel N. Intrinsic and extrinsic pathways of apoptosis: Role in cancer development and prognosis. Adv Protein Chem Struct Biol. 2021;125:73-120.
5. Polverini PJ, Nör JE. Apoptosis and predisposition to oral cancer. Crit Rev Oral Biol Med. 1999;10(2):139-152.

6. Silva FFVE, Padín-Iruegas ME, Caponio VCA, Lorenzo-Pouso AI, Saavedra-Nieves P, Chamorro-Petronacci CM, Suaréz-Peñaranda J, Pérez-Sayáns M. Caspase 3 and Cleaved Caspase 3 Expression in Tumorogenesis and Its Correlations with Prognosis in Head and Neck Cancer: A Systematic Review and Meta-Analysis. Int J Mol Sci. 2022;23(19):11937.
7. Häcker G. The morphology of apoptosis. Cell Tissue Res. 2000;301(1):5-17.
8. Buddhan R, Manoharan S Elanchezhiyan C, Muralinaidu R, Karthik M. Myrtenal modulates the immunoexpression of cell proliferative, angiogenic and invasive markers in dmba-induced hamster oral carcinogenesis. Trop J. Pharm Res. (2020): 550-557.
9. Manimaran A, Manoharan, S. Tumor Preventive Efficacy of Emodin in 7,12-Dimethylbenz[a]Anthracene-Induced Oral Carcinogenesis: a Histopathological and Biochemical Approach. Pathol Oncol Res. 2018 24(1), 19–29.
10. Naseer I, Elanchezhiyan C, Manoharan S, Hassan T, Neelakandan M. Vanillin Modulates the Status of Glycoconjugates in Favouur of Tumor Suppression in 7, 12dimethylbenz [a] anthracene Induced Oral Carcinoma in Golden Syrian Hamster. Trop J. Pharm Res. 2021. 5(4), 617-620.
11. Mlala S, Oyedeji AO, Gondwe M, Oyedeji OO. Ursolic Acid and Its Derivatives as Bioactive Agents. Molecules. 2019;24(15):2751.
12. Kornel A, Nadile M, Retsidou MI, Sakellakis M, Gioti K, Beloukas A, Sze NSK, Klentrou P, Tsiani E. Ursolic Acid against Prostate and Urogenital Cancers: A Review of in vitro and in vivo Studies. Int J Mol Sci. 2023;24(8):7414.
13. Sandhu SS, Rouz SK, Kumar S, Swamy N, Deshmukh L, Hussain A, Haque S, Tuli HS. Ursolic acid: a pentacyclic triterpenoid that exhibits anticancer therapeutic potential by modulating multiple oncogenic targets [published online ahead of print, 2023 Jan 4]. Biotechnol Genet Eng Rev. 2023;1-31.
14. Karthik M, Manoharan S, Muralinaidu R. Ursolic acid-loaded chitosan nanoparticles suppress 7,12-dimethylbenz(a)anthracene-induced oral tumor formation through their antilipidperoxidative potential in golden Syrian hamsters [published online ahead of print, 2023 May 10]. Naunyn Schmiedebergs Arch Pharmacol. 2023;10.1007/s00210-023-02509-2.
15. Calvo P, Remuñan-López C, Vila-Jato JL, Alonso MJ. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm Res. 1997; 14(10):1431-6. doi: 10.1023/a:1012128907225
16. He S, Chakraborty R, Ranganathan S. Proliferation and Apoptosis Pathways and Factors in Oral Squamous Cell Carcinoma. Int J Mol Sci. 2022;23(3):1562.
17. Daneste H, Sadeghzadeh A, Mokhtari M, Mohammadkhani H, Lavaee F, Moayedi J. Immunoexpression of p53 mutant-type in Iranian patients with primary and recurrence oral squamous cell carcinoma. Eur J Transl Myol. 2022;33(1):10.4081/ejtm.2022.10847.
18. Hu J, Cao J, Topatana W, Juengpanich S, Li S, Zhang B, Shen J, Cai L, Cai X, Chen M. Targeting mutant p53 for cancer therapy: direct and indirect strategies. J Hematol Oncol. 2021;14(1):157.
19. Kamat MS, Puranik RS, Das Rai AB, Patil BR, Patil S. Correction to: Assessing immuno-expression of p53 protein and TP 53 gene amplification in histologically negative surgical margins of oral squamous cell carcinoma patients and normal oral mucosa. Clin Oral Investig. 2023;27(1):431. Erratum for: Clin Oral Investig. 2022;26(10):6235-6243.
20. Novack R, Zhang L, Hoang LN, Kadhim M, Ng TL, Poh CF, Kevin Ko YC. Abnormal p53 Immunohistochemical Patterns Shed Light on the Aggressiveness of Oral Epithelial Dysplasia. Mod Pathol. 2023;36(7):100153.
21. Sinevici N, O'sullivan J. Oral cancer: Deregulated molecular events and their use as biomarkers. Oral Oncol. 2016;61:12-18.
22. Patel KR, Vajaria BN, Singh RD, Begum R, Patel PS. Clinical implications of p53 alterations in oral cancer progression: a review from India. Exp Oncol. 2018;40(1):10-18.
23. Vinothkumar, V., Manoharan, S., Sindhu, G., Nirmal, M. R., &Vetrichelvi, V. Geraniol modulates cell proliferation, apoptosis, inflammation, and angiogenesis during 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis. Mol Cell Biochem. 2012; 369(1-2): 17–25.
24. Manoharan S, Palanimuthu D, Baskaran N, Silvan S. Modulating effect of lupeol on the expression pattern of apoptotic markers in 7, 12-dimethylbenz(a)anthracene induced oral carcinogenesis. Asian Pac J Cancer Prev. 2012;13(11):5753-5757.
25. Manoharan S, Rajasekaran D, Prabhakar MM, Karthikeyan S, Manimaran A. Modulating Effect of Enicostemmalittorale on the Expression Pattern of Apoptotic, Cell Proliferative, Inflammatory and Angiogenic Markers During 7, 12-Dimethylbenz (a) Anthracene Induced Hamster Buccal Pouch Carcinogenesis. Toxicol Int. 2015;22(1):130-140.
26. Bhutani N, Poswal P, Moga S, Arora S. Immunohistochemical expression of bcl-2; an apoptosis regulatory protein in squamous cell carcinoma of oropharynx: A diagnostic cross-sectional study. Ann Med Surg (Lond). 2021;67:102480.
27. Dwivedi R, Chandra S, Mehrotra D, Raj V, Pandey R. Predicting transition from oral pre-malignancy to malignancy via Bcl-2 immuno-expression: Evidence and lacunae. J Oral BiolCraniofac Res. 2020;10(4):397-403.
28. Popović B, Jekić B, Novaković I, Luković LJ, Tepavcević Z, Jurisić V, Vukadinović M, Milasin J. Bcl-2 expression in oral squamous cell carcinoma. Ann N Y Acad Sci. 2007;1095:19-25.
29. Chamorro-Petronacci CM, Lafuente-Ibanez De Mendoza I, Suarez-Peñaranda JM, Padin-Iruegas E, Blanco-Carrion A, Lorenzo-Pouso AI, Ortega KL, Pérez-Sayáns M. Immunohistochemical Characterization of Bcl-2 in Oral Potentially Malignant Disorders. Appl Immunohistochem Mol Morphol. 2021;29(9):706-712.
30. Segura IG, Secchi DG, Galíndez MF, Carrica A, Bologna-Molina R, Brunotto M, Centeno VA. Connexin 43, Bcl-2, Bax, Ki67, and E-cadherin patterns in oral squamous cell carcinoma and its relationship with GJA1 rs12197797 C/G. Med Oral Patol Oral Cir Bucal. 2022;27(4):e366-e374.
31. Chen Y, Kayano T, Takagi M. Dysregulated expression of bcl-2 and bax in oral carcinomas: evidence of post-transcriptional control. J Oral Pathol Med. 2000;29(2):63-69.
32. Khodapasand E, Jafarzadeh N, Farrokhi F, Kamalidehghan B, Houshmand M. Is Bax/Bcl-2 ratio considered as a prognostic marker with age and tumor location in colorectal cancer?. Iran Biomed J. 2015;19(2):69-75.
33. Escobar E, Gómez-Valenzuela F, Peñafiel C, Hormazábal-Hevia A, Herrera-Fuentes C, Mori-Aliaga D. Immunohistochemical expression of COX-2, Ki-67, Bcl-2, Bax, VEGF and CD105 according to histological grading in oral squamous cell carcinoma. Rev Esp Patol. 2023;56(3):147-157.
34. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.
35. Sinicrope FA, Rego RL, Foster NR, Thibodeau SN, Alberts SR, Windschitl HE, Sargent DJ. Proapoptotic Bad and Bid protein expression predict survival in stages II and III colon cancers. Clin Cancer Res. 2008;14(13):4128-4133.

36. Zinkel SS. Investigation of the proapoptotic BCL-2 family member bid on the crossroad of the DNA damage response and apoptosis. Methods Enzymol. 2008;442:231-250.
37. Hattori T, Ookawa N, Fujita R, Fukuchi K. Heterodimerization of Bcl-2 and Bcl-X(L) with Bax and Bad in colorectal cancer. Acta Oncol. 2000;39(4):495-500.
38. Coutinho-Camillo CM, Lourenço SV, Nishimoto IN, Kowalski LP, Soares FA. Expression of Bcl-2 family proteins and association with clinicopathological characteristics of oral squamous cell carcinoma. Histopathology. 2010;57(2):304-316.
39. Kumar S, Dorstyn L, Lim Y. The role of caspases as executioners of apoptosis. Biochem Soc Trans. 2022;50(1):33-45.
40. Manoharan S, Sindhu G, Nirmal MR, Vetrichelvi V, Balakrishnan S. Protective effect of berberine on expression pattern of apoptotic, cell proliferative, inflammatory and angiogenic markers during 7,12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis. Pak J Biol Sci. 2011;14(20):918-932.
41. Manu KA, Kuttan G. Ursolic acid induces apoptosis by activating p53 and caspase-3 gene expressions and suppressing NF-kappaB mediated activation of bcl-2 in B16F-10 melanoma cells. Int Immunopharmacol. 2008;8(7):974-981.
42. Yu YX, Gu ZL, Yin JL, Chou WH, Kwok CY, Qin ZH, Liang ZQ. Ursolic acid induces human hepatoma cell line SMMC-7721 apoptosis via p53-dependent pathway. Chin Med J (Engl). 2010;123(14):1915-1923.
43. Wang X, Zhang F, Yang L, Mei Y, Long H, Zhang X, Zhang J, Qimuge-Suyila, Su X. Ursolic acid inhibits proliferation and induces apoptosis of cancer cells in vitro and in vivo. J Biomed Biotechnol. 2011;2011:419343.
44. Wang S, Meng X, Dong Y. Ursolic acid nanoparticles inhibit cervical cancer growth in vitro and in vivo via apoptosis induction. Int J Oncol. 2017;50(4):1330-1340.
45. Shan J, Xuan Y, Zhang Q, Zhu C, Liu Z, Zhang S. Ursolic acid synergistically enhances the therapeutic effects of oxaliplatin in colorectal cancer. Protein Cell. 2016;7(8):571-585.