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ORIGINAL PAPER

# Inhibition of 7,12-dimethylbenzanthracene-induced skin tumors by a nutrient mixture

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**Abstract** The annual incidence of all forms of skin cancer, the most common of all human cancers, is increasing yearly. A unique nutrient mixture (NM) was shown to exhibit anticancer activity in vivo and in vitro. We examined the effect of NM on the development of skin cancer induced by 7,12-dimethylbezanthracene (DMBA) in female SENCAR mice by a complete carcinogenesis protocol. Mice ($n = 55$) were divided into four groups and carefully shaved on dorsum. After 2 days, the mice in Groups 1 ($n = 10$), 3 ($n = 20$), and 4 ($n = 20$) were treated topically with 100 nM DMBA in 0.2 ml of acetone twice a week for 4 weeks; Group 2 ($n = 5$), the control group, was treated with acetone 0.2 ml. Groups 1 and 2 were fed the regular diet. Group 3A ($n = 10$) was fed a diet containing 0.5% NM from the day of DMBA treatment and 3B ($n = 10$) the regular diet and received NM (75 mg in 0.4 ml of 1:1 acetone/water) topically to the shaved area 15 min before DMBA application twice a week for 4 weeks. Group 4 mice were fed a diet containing 0.5% NM for 2 weeks prior to the application of DMBA and then divided into two groups: 4A ($n = 10$) was fed the 0.5% NM diet as in 3A, and 4B ($n = 10$) the regular diet as described for 3B. Body weight and diet consumption of the mice were monitored and the skin tumors (papillomas) were counted and recorded. Ten weeks thereafter the mice were euthanized, skinned, and tumors were processed for histology. NM significantly ($P < 0.0001$) inhibited DMBA-induced skin tumor multiplicity by 59, 62, 69, and 86% in NM-treated Groups 3A, 3B, 4A, and 4B, respectively. These results suggest that NM has strong potential as a useful therapeutic regimen for skin cancer by significantly inhibiting the incidence and tumor multiplicity of DMBA-induced skin tumors.

**Keywords** Papillomas · DMBA · Skin cancer · Squamous cell carcinoma · Nutrients · SENCAR mice

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## Introduction

Skin cancer, the most common of all human cancers, accounts for half of all cancers diagnosed and is reported to be increasing annually. Squamous cell carcinoma (SCC) is the second most common form of skin cancer with 1,000,000 new cases estimated in 2007 in the USA and closely associated with sun exposure and chemical carcinogens [1]. It is widely accepted that SCCs, which are responsible for the majority of non-melanoma skin cancer-related deaths, result from the accumulation of genetic alterations [2].

Skin tumors can be effectively induced in mice by the repetitive application of a carcinogen. Slaga et al. [3] proposed that the skin tumor initiation by chemical carcinogens, such as 7,12-dimethylbenz[a]-anthracene (DMBA), is probably an irreversible stage that involves a somatic mutation, mainly in the Ha-*ras* oncogene. In contrast to the initiation stage, the promotion stage is reversible and requires a series of applications to induce benign skin tumors. The progression stage appears to involve the accumulation of additional genetic changes in cells, which form skin papillomas that can be considered as a clone of initiated cells.

SENCAR mice were reported to be the most sensitive mouse group to carcinogens tested by Slaga et al. [4]. Skin tumor promotion and progression stages are characterized by selective inflammation and sustained

hyperplasia, differentiation, alterations, and genetic instability of initiated cells developing into papillomas and carcinomas [3, 5]. Mice treated topically with tumor promoters have demonstrated that the histologically sustained cellular hyperplasia plays a critical role in tumor promotion [6].

Rath and Pauling [7] defined common pathomechanisms for all cancers, the destruction of extracellular matrix (ECM) as a precondition for cancer cell invasion, growth, and metastasis and suggested intervention through natural inhibitors of plasmin-induced proteolysis, such as lysine and its analogs. Cancer cells form tumors and spread by degrading the ECM through various matrix metalloproteinases (MMPs). The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of the cancer. Rath and Pauling postulated that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM proteolysis and, as such, have the potential to modulate tumor growth and expansion. These nutrients can exercise their antitumor effect through the inhibition of MMPs, and in addition, by strengthening of connective tissue surrounding cancer cells through their effect on collagen synthesis. These two processes are essential for a tumor encapsulating effect. MMPs and constituents of ECM play a critical role in neoplastic invasion and metastasis.

We have developed strategies to inhibit cancer development and its spread using naturally occurring nutrients such as lysine, proline, ascorbic acid, and green tea extract (nutrient mixture, NM), which have exhibited synergestic anticancer activity in vivo and in vitro in a number of cancer cell lines, including human and murine melanoma cells lines [8, 9], inhibiting cancer cell growth, MMP secretion, invasion, metastasis, and angiogenesis. The present study examines the in vivo effect of NM on the development of skin cancer induced by DMBA in female SENCAR mice by a complete carcinogenesis protocol.

## Methods and materials

### Materials

The DMBA obtained from Sigma Chemical Co. (St. Louis, MO, USA) was dissolved in acetone to 100 nM in 0.2 ml acetone. Stock solution of the NM was composed of the following in the ratio indicated: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1,000 mg; L-proline 750 mg; L-arginine 500 mg; *N*-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1,000 mg; selenium 30 μg; copper 2 mg; manganese 1 mg.

### Animals

Female SENCAR mice, ~6 weeks of age on arrival, were purchased from National Cancer Institute (Frederick, MD, USA) and maintained in microisolator cages under pathogen-free conditions on a 12-h light/12-h dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals.

### Experimental design

We studied the development of skin cancer induced by DMBA in female SENCAR mice using a complete carcinogenesis protocol based on work by Hanausek et al. [10]. After 1-week of isolation, the dorsal area of the 6-week-old female SENCAR mice ($n = 55$) was carefully shaved with an electric clipper to avoid cuts and bruises. The mice were then divided into groups: 1–4. After 2 days, the mice in Groups 1 ($n = 10$), 3 ($n = 20$), and 4 ($n = 20$) were treated topically with 100 nM DMBA in 0.2 ml of acetone twice a week for 4 weeks, while Group 2 ($n = 5$) mice received only acetone 0.2 ml topical treatments. Groups 1 and 2 mice were fed the regular diet. Group 3 mice were further divided into two sub-groups: 3A and 3B. Group 3A ($n = 10$) mice were fed a regular diet containing 0.5% NM from the day of DMBA treatment and Group 3B ($n = 10$) mice were fed the regular diet and received NM (75 mg in 0.4 ml of 1:1 acetone/water) topically to the shaved area 15 min before DMBA application twice a week for 4 weeks. Group 4 mice were fed a regular diet supplemented with 0.5% NM for 2 weeks prior to the application of DMBA. At this time, the mice were further divided into two groups. Group 4A ($n = 10$) was fed the 0.5% NM diet as in 3A, and 4B ($n = 10$) the regular diet as described for 3B (see Table 1 for a summary of the interventions). Body weight and diet consumption of the mice were monitored and the skin tumors (papillomas) were counted and recorded. Ten weeks thereafter the mice were euthanized, skinned, and tumors were processed for histology.

### Histology

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4–5 μm. Sections were deparaffinized through xylene and

**Table 1** Summary of interventions

| Groups | Topical DMBA 100 nM in 0.2 acetone 2×/week × 4 weeks | Pretreatment 0.5% NM in diet 2 weeks prior to DMBA | Diet—regular or 0.5% NM supplemented | Topical NM 75 mg/0.4 ml prior to DMBA treatment |
|---|---|---|---|---|
| 1 ($n = 10$) | DMBA | None | Regular | None |
| 2 ($n = 5$) | None | None | Regular | None |
| 3A ($n = 10$) | DMBA | None | 0.5% NM | None |
| 3B ($n = 10$) | DMBA | None | Regular | Topical NM |
| 4A ($n = 10$) | DMBA | Pre-treatment | 0.5% NM | None |
| 4B ($n = 10$) | DMBA | Pre-treatment | Regular | Topical NM |

graduated alcohol series to water and stained with hematoxylin and eosin for evaluation using a standard light microscope.

### Statistical analysis

The results were expressed as means ± SD for the groups. Data were analyzed by independent sample "$t$" test.

## Results

### Tumor incidence and multiplicity

Nutrient mixture treatment significantly ($P < 0.0001$) inhibited DMBA-induced skin tumor multiplicity (number of tumors per mouse) over the control group (Group 1) by 59, 62, 69, and 86% in Groups 3A, 3B, 4A, and 4B, respectively, as shown in Fig. 1. Gross photographs of mice with papillomas representative of each group are shown in Fig. 2. Tumor incidence (number of mice with tumors) was decreased over the control by 20% in Groups 3A, 3B, and 4A and by 50% in Group 4B. Two-week pretreatment with a diet enriched by 0.5% NM was the most effective treatment in inhibiting tumors, followed by topical NM treatment prior to DMBA application, and with NM 0.5% supplemented diet during the study. The number of DMBA-induced papillomas in female SENCAR mice increased from 5 to 8 weeks and was significantly higher for the control group than the NM-treated groups, as shown in Fig. 3.

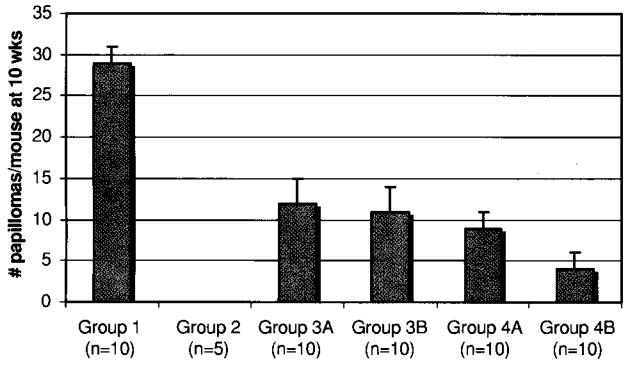


**Fig. 1** Effect of NM on multiplicity of DMBA-induced papillomas in female SENCAR mice at 10 weeks. NM significantly ($P < 0.0001$) inhibited DMBA-induced skin tumor multiplicity by 59, 62, 69, and 86% in Groups 3A, 3B, 4A, and 4B, respectively. Topical NM treatment prior to DMBA application was the most effective treatment in inhibiting tumors, followed by 2-week pretreatment with diet enriched by 0.5% NM and treatment with NM 0.5% during the study

### Histopathology

Skin lesions included papillary proliferative lesions, both papillomas and keratocanthomas, with associated varying degrees of epidermal hyperplasia and generally severe dermal thickening with fibrosis and mild to moderate infiltration with inflammatory cells (Fig. 4). In the control group of mice (Group 1), not only were there significantly more papillomas, but also the incidence of early to well-developed SCCs with areas of ulceration was high, as shown in Fig. 4b–f. In mice receiving dietary NM only post DMBA application (Groups 3A and 3B), the papilloma incidence was reduced over the control, but histologically showed inflammation, infiltration, and SCC, as shown in Fig. 4g–k. Among the DMBA-treated mice, the mice that underwent dietary pretreatment with NM combined with topical treatment with NM (Group 4B) or dietary NM during the study (Group 4A), demonstrated similar pathological changes as Group 3, with hyperplastic papillomatous lesions without the evidence of infiltration and keratoacanthomas, as seen in Fig. 4l.

Mean body weights of SENCAR mice in different groups did not differ significantly neither at the beginning nor at the termination of the study, ranging at termination between 36.1 and 39.8 g. Consumption of food and water did not differ among the groups.

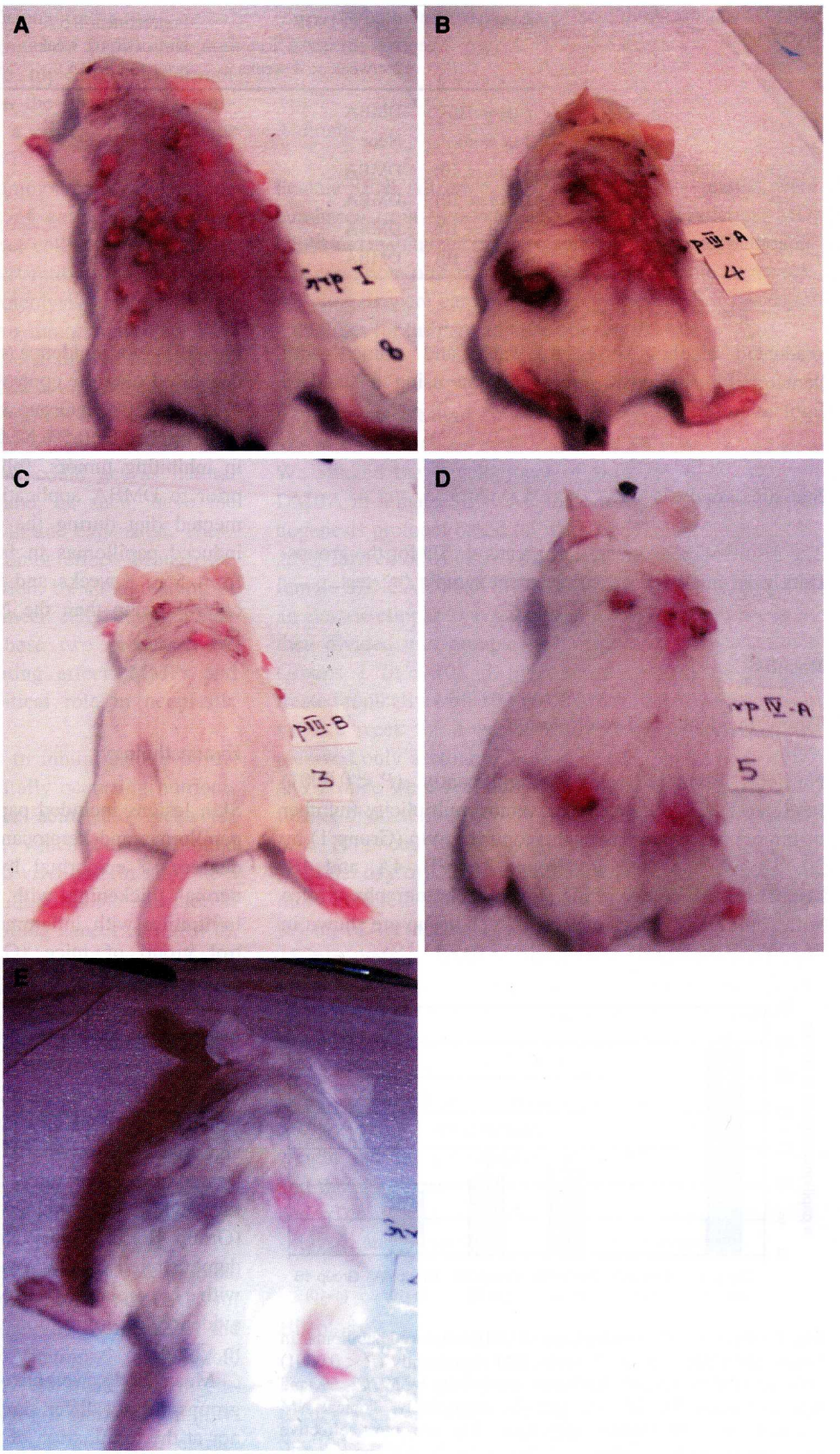


**Fig. 2** Effect of NM on inhibition of papillomas in female SENCAR mice—photographs of representative mice in each group. (**a**) Group 1 (#8); (**b**) Group IIIA (#4); (**c**) Group IIIB (#3); (**d**) Group IVA (#5); (**e**) Group IVB (#4). NM significantly inhibited DMBA-induced skin tumor multiplicity by 59, 62, 69, and 86% in Groups 3A, 3B, 4A, and 4B, respectively

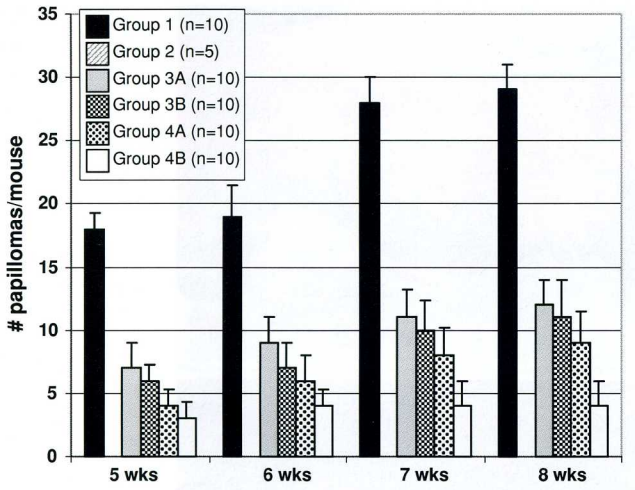


**Fig. 3** Effect of NM on mean number of DMBA-induced papillomas in female SENCAR mice from 5 to 8 weeks. The number of DMBA-induced papillomas in female SENCAR mice increased from 5 to 8 weeks and was significantly higher for the control group than the NM-treated groups

## Discussion

Dietary and topical treatment of SENCAR mice with the NM significantly inhibited DMBA-induced skin tumor incidence and multiplicity. Two-week pretreatment with a diet enriched by 0.5% NM was the most effective treatment in inhibiting tumors, followed by topical NM application 15 min prior to DMBA application. The combination of pretreatment with dietary NM and topical NM application yielded the lowest mean number of papillomas. In addition, histologically, the mice that underwent dietary pretreatment with NM combined with topical treatment with NM (Group 4B) or dietary NM with DMBA application, demonstrated hyperplastic papillomatous lesions without the evidence of infiltration or cytological atypia. In contrast, the control group of mice not only exhibited significantly more papillomas, but also the incidence of early to well-developed SCCs with areas of ulceration was high. In mice receiving dietary NM only post DMBA application, the papilloma incidence was reduced over the control, but histologically showed inflammation, infiltration, and SCC. Thus, NM had beneficial effects on DMBA-induced tumor initiation and tumor promotion/progression.

Initiation involves mutation of cellular DNA, resulting in activation of oncogenes and inactivation of tumor suppressor genes. Quintanilla [11] demonstrated the presence of an activated c-Ha-*ras* gene in mouse skin papillomas and carcinomas induced by DMBA. Most tumor-initiating agents either generate or are metabolically converted to electrophilic reactants, which bind covalently to cellular DNA [3, 5]. Free radicals and the modified DNA bases by these radicals have also been strongly implicated in carcinogenesis in general [12–14].

Antioxidants have been shown to inhibit the induction of cancer by a wide variety of chemical carcinogens and radiation at many target sides in mice, rats, hampsters, and man [15]. Both carcinogens and tumor promoters have been shown to decrease the cellular activity of superoxide dismutase and catalase. Vitamins E and C were found to inhibit DMBA skin initiation as well as skin tumor promotion [15]. Epigallocatechin gallate [16] and selenium [17] have been reported to inhibit carcinogen activation and to scavenge electophiles.

The ECM also plays a key role in the development of cancer. We have previously shown that the ECM synthesized by normal fibroblasts treated with NM demonstrated increased stability and significantly reduced osteosarcoma cell growth rate, invasive activity (MMP-2 and MMP-9 secretion and Matrigel invasion), and adhesion to collagen I and other substrates, suppressing tumor growth independent of the immune system function and inhibiting critical steps in cancer metastasis [18]. D'Armiento et al. [19] showed that overexpression of the highly specific proteolytic enzyme collagenase in transgenic mice augmented skin sensitivity to carcinogenesis when treated with DMBA and a promoter.

The NM was designed by defining critical physiological targets in cancer progression and metastasis. These include ECM integrity and MMP activity. ECM formation and structure is dependent upon adequate supplies of ascorbic acid and the amino acids lysine and proline, which insure proper synthesis and hydroxylation of collagen fibers. Manganese and copper are also essential for collagen formation. Lysine is a natural inhibitor of plasmin-induced proteolysis and, as such plays an important role in ECM stability [7, 20]. Green tea extract has shown to be a promising agent in controlling cancer cell growth, metastasis, angiogenesis, and other aspects of cancer progression [21–25]. *N*-acetyl cysteine has been observed to inhibit MMP-9 activity [26] and invasive activities of tumor cells [27]. Selenium has been shown to interfere with MMP secretion and tumor invasion [28], as well as migration of endothelial cells through ECM [27]. In addition to addressing ECM properties, some nutrients are critical in inducing cancer cell death. A recent study confirmed that ascorbic acid inhibits cell division and growth through production of hydrogen peroxide [29]. Since arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in breast cancer cells [30].

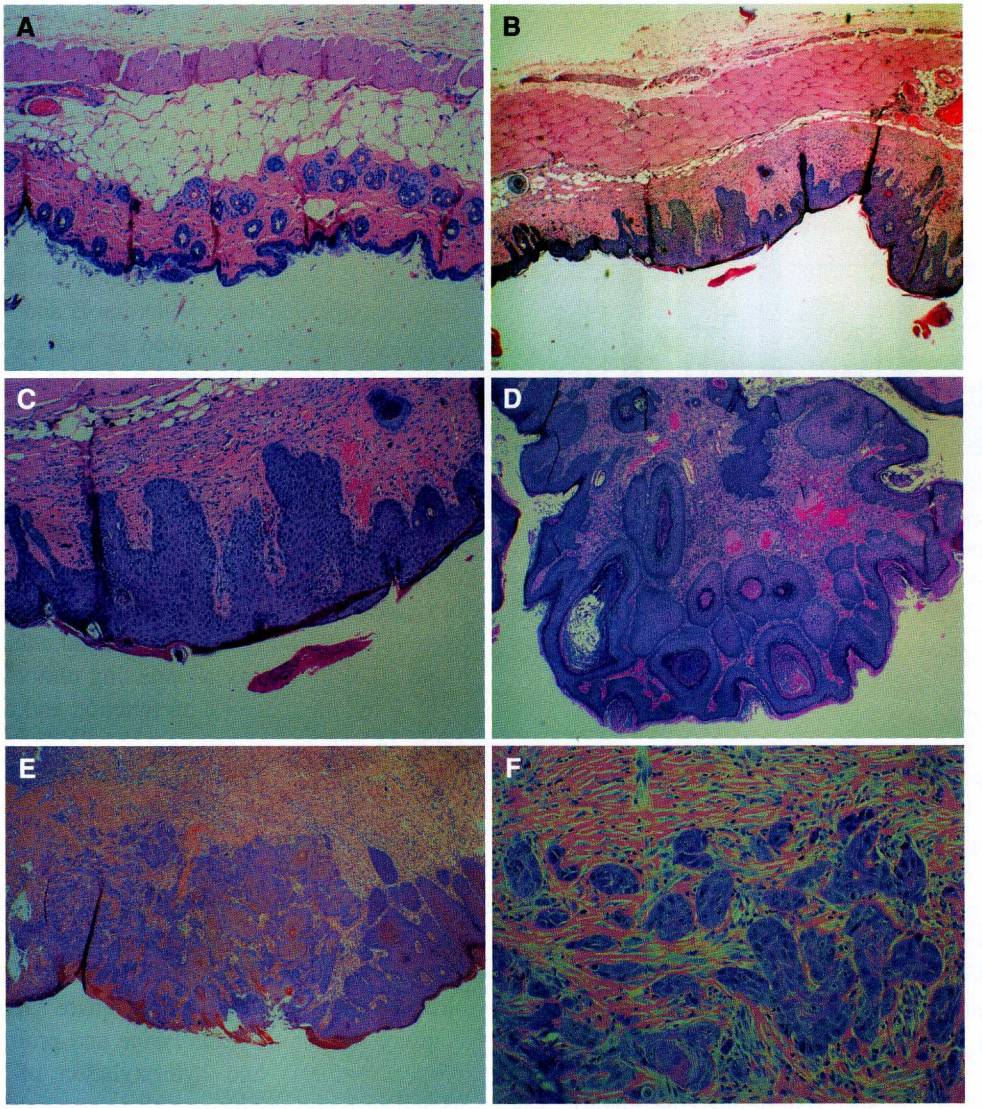


**Fig. 4** Effect of NM on histopathology of DMBA-induced papillomas in SENCAR mice. Among the DMBA treated mice, the mice that underwent dietary pretreatment with NM combined with topical treatment with NM (Group 4B) or dietary NM during the study (Group 4A), demonstrated hyperplastic papillomatous lesions without evidence of infiltration or cytological atypia. In contrast, in the control group of mice (Group 1) not only were there significantly more papillomas, but also the incidence of early to well-developed SCCs with areas of ulceration was high. In mice receiving dietary NM only post DMBA application (Groups 3A and 3B), the papilloma incidence was reduced over the control, but histologically showed inflammation, infiltration, and SCC. (**a**) 100× Group 2—normal skin, (**b**) 40× Group I—severe diffuse epidermal hyperplasia and dermal fibrosis, (**c**) 100× Group 1—severe epidermal hyperplasia, (**d**) 40× Group 1—papilloma, (**e**) 40× Group 1—area of ulceration with invasive SCC, (**f**) 200× Group I—invasive SCC, (**g**) 40× Group 3A—large papilloma, (**h**) 40× Group 3A—severe ulceration with dermal fibrosis and superficial inflammatory cell infiltration, (**i**) 200× Group 3A—nests of SCC invading fibrosed dermis, (**j**) 200× Group 3B—granulomatous inflammation associated with keratin in dermis, (**k**) 400× Group 3B—early SCC invasion, (**l**) 40× Group 4B—keratocanthomas

## Conclusions

The results of the present study show that the NM was effective in inhibiting initiation as well as the promotion/progression of DMBA-induced papillomas in SENCAR mice, especially when the mice were provided a diet supplemented with 0.5% NM for 2 weeks prior to topical application of DMBA. These findings together with our earlier results clearly indicate anticancer potential of NM. Furthermore, use of the NM would not pose any toxic effect clinically, especially in the relevant doses, as in vivo safety studies demonstrate. During an in vivo study on possible toxicity from NM, we found that NM had neither adverse effect on vital organs (heart, liver,

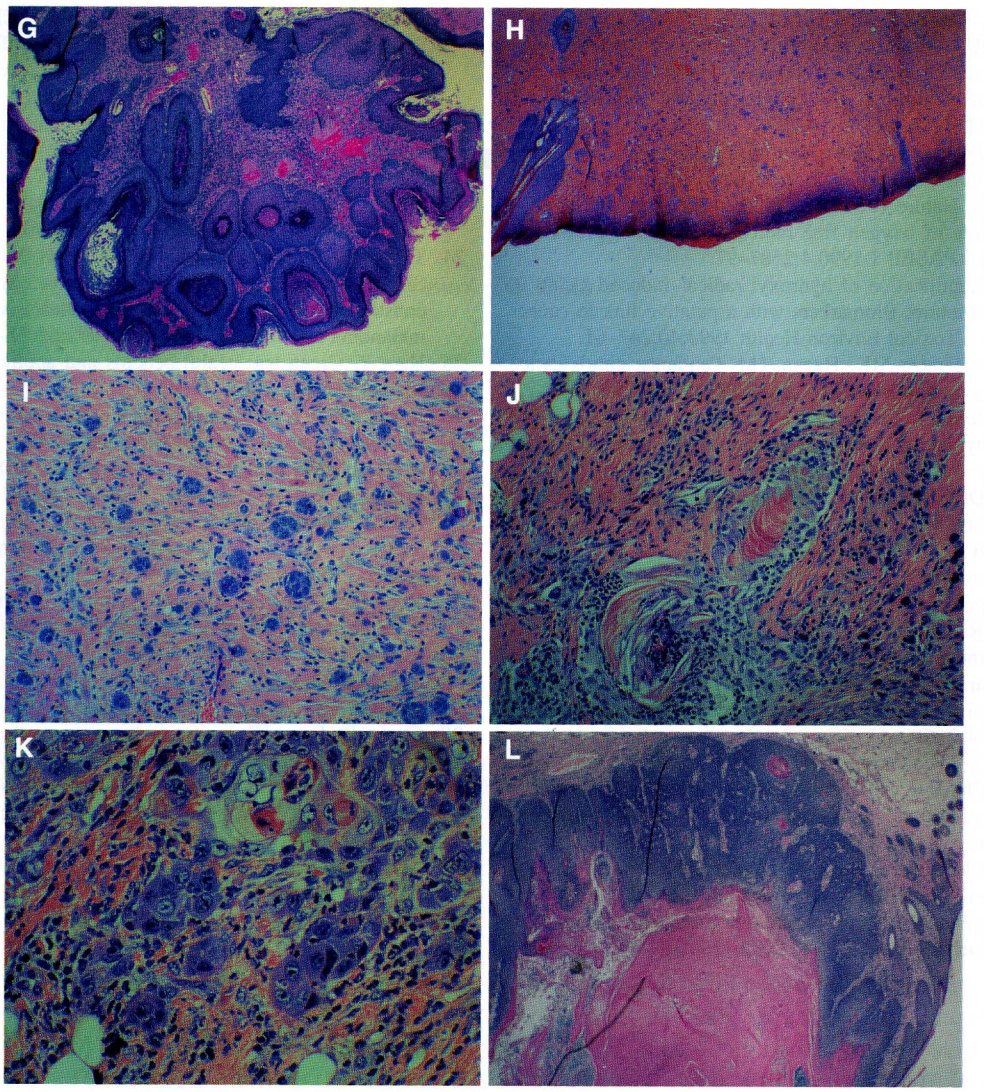


**Fig. 4** continued

and kidney) nor on the associated functional serum enzymes [31].

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## References

1. National Cancer Institute. Skin Cancer. (2007) http://www.cancer.gov/cancertopics/types/skin. Accessed 30 July 2007.
2. Boukamp P. Non-melanoma skin cancer: what drives tumor development and progression? Carcinogenesis 2005;26(10): 1657–67.
3. Slaga TJ, O'Connell J, Rotstein J, Patskan G, Morris R, Aldaz M, Conti C. Critical genetic determinants and molecular events in multistage skin carcinogenesis. Symp Fundam Cancer Res 1987;39:31–4.
4. Slaga TJ, Fischer SM, Weeks CE, Klein-Szanto AJP, Reiners J. Studies on the mechanisms involved in multistage carcinogenesis in mouse skin. J Cell Biochem 2004;18(1):99–119.
5. DiGiovanni J. Multistage carcinogenesis in mouse skin. Pharmacol Ther 1992;47:63–128.
6. Klein-Szanto AJP, Slaga TJ. Effects of peroxides on rodent skin: epidermal hyperplasia and tumor promotion. J Invest Dermatol 1982;79:30–4.
7. Rath M, Pauling L. Plasmin-induced proteolysis and the role of apoprotein(a), lysine and synthetic analogs. Orthomol Med 1992;7:17–23.
8. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. In vitro and in vivo antitumor effect of ascorbic acid, lysine, proline, and green tea extract on human melanoma cell line A2058. In Vivo 2006;20(1):25–32.
9. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. Inhibition of pulmonary metastasis of melanoma B16FO cells in C57BL/6 mice by a nutrient mixture consisting of ascorbic acid, lysine, proline, arginine, and green tea extract. Exp Lung Res 2006;32(10):517–30.

10. Hanausek M, Ganesh P, Walaszek Z, Amtzen CJ, Slaga TJ, Gautterman JU. Avicins, a family of triterpenoid saponins from Acacia victoriae (Bentham), suppress H-ras mutations and aneuploidy in a murine skin carcinogenesis model. PNAS 2001;98(20):11551–6.
11. Quintanilla M, Brown K, Ramsden M, Balmain A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature 1986;322:78–80.
12. Floyd RA. Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J 1990;4:2587–97.
13. Malins DC. Identification of hydroxyl radical–induced lesion in DNA bases structure: Biomarkers with a putative link to cancer development. J Toxicol Environ Health 1993;40:247–61.
14. Ames BN, Shipenaga MK, Hagan TM. Oxidants, antioxidants, and the degenerative diseases of aging. PNAS 1993;90:7915–22.
15. Slaga TJ. Inhibition of skin tumor initiation, promotion, and progression by antioxidants and related compounds. Crit Rev Food Sci Nutr 1995;35(1–2):51–7.
16. Hu C, Kitts DD. Evaluation of antioxidant activity of epigallocatechin gallate in biphasic model systems in vitro. Mol Cell Biochem 2001;218(1–2):147–55.
17. Chu FF, Esworthy RS, Doroshow JH. Role of Se-dependent glutathione peroxidases in gastrointestinal inflammation and cancer. Free Radic Biol Med 2004;36(12):1481–95.
18. Ivanov V, Ivanova S, Roomi MW, Kalinovsky T, Niedzwiecki A, Rath M. Naturally-produced extracellular matrix inhibits growth rate and invasiveness of human osteosarcoma cancer cells. Med Oncol 2007;24(2):209–17.
19. D'Armiento J, DiColandrea T, Dalal SS, Okada Y, Huang MT, Conney AH, Chada K. Collagenase expression in transgenic mouse skin causes hyperkeratosis and acanthosis and increases susceptibility to tumorigenesis. Mol Cell Biol 1995;15(10):5732–9.
20. Sun Z, Chen YH, Wang P, Zhang J., Gurewich V, Zhang P, Liu JN. The blockage of high-affinity lysine binding sites of plasminogen by EACA significantly inhibits prourokinase-induced plasminogen activation. Biochem Biophys Acta 2002;1596:182–92.
21. Valcic S, Timmermann BN, Alberts DS, Wachter GA, Krutzsch M, Wymer J, Guillen JM. Inhibitory effect of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. Anticancer Drugs 1996;7:461–8.
22. Mukhtar H, Ahmed N. Tea polyphenols: prevention of cancer and optimizing health. AM J Clin Nutr 2000;71:1698s–702s.
23. Yang GY, Liao J, Kim K, Yurtow EJ, Yang CS. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. Carcinogenesis 1998;19:611–6.
24. Taniguchi S, Fujiki H, Kobayashi H, Go H, Miyado K, Sadano H, Shimikawa R. Effect of (-) epigallocatechin gallate, the main constituent of green tea, on lung metastasis with mouse B16 melanoma cell lines. Cancer Lett 1992;65:51–4.
25. Hare Y. Green tea: health benefits and applications. Basel, NY: Marcel Dekker; 2001.
26. Kawakami S, Kageyama Y, Fujii KK, Oshima H. Inhibitory effects of N-acetyl cysteine on invasion and MMP 9 production of T24 human bladder cancer cells. Anticancer Res 2001;21:213–9.
27. Morini M, Cai T, Aluigi MG, Noonan DM, Masiello L, De Floro S, D'Agostinin F, Albini A, Fassima G. The role of the thiol N-acetyl cysteine in the prevention of tumor invasion and angiogenesis. Int J Biol Markers 1999;14:268–71.
28. Yoon SO, Kim MM, Chung AS. Inhibitory effects of selenite on invasion of HT 1080 tumor cells. J Biol Chem 2001;276:20085–92.
29. Maramag C, Menon M, Balaji KC, Reddy PG, Laxmanan S. Effect of vitamin C on prostate cancer cells in vitro: effect on cell number, viability and DNA synthesis. Prostate 1997;32:188–95.
30. Cooke JP, Dzau VJ. Nitric oxide synthase: Role in the genesis of vascular disease. Annu Rev Med 1997;48:489–509.
31. Roomi MW, Ivanov V, Netke SP, Niedzwiecki A, Rath M. Serum markers of the liver, heart, and kidney and lipid profile and histopathology in ODS rats treated with nutrient synergy. J AM Coll Nutr 2003;22:477 [Abstract 86].