Chinese Journal of Cancer Research 10(3):162–168,1998.

EFFECTS OF LIMONENE, SALVIA MILTIORRHIZA AND TURMERIC DERIVATIVES ON H-RAS ONCOGENE EXPRESSION AND GAP JUNCTION INTERCELLULAR COMMUNICATION IN HUMAN SOLID TUMOR CELL LINES

Chen Xiaoguang[1] 陈晓光 Taday oshi Hasuma[2] 连间忠芳 Yoshihisa Yano[2] 矢野善久
Toshiko Yoshimata[2] 吉都 俣 士子 Hiyoshi Kamoi[2] 鸭井博 Shuzo Otani[2] 大谷周造

[1]*Department of pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, 100050;* [2]*Department of Biochemistry, Osaka City University Medical Schooll, 545 Osaka, Japan*

Objective: To study gap junction intercellular communication (GJIC), H-ras oncogene expression and ras oncogene product (P^{21} ras protein) expression in four human solid tumor cell lines, W1–38, $CACO_2$, A549 and PaCa, and the effects of four compounds, Salvia miltiorrhiza derivative (SMD), d-Limonene, Turmeric derivative I (TD-I) and Turmeric derivative II (TD-II), on them. Methods: The abilities of the four solid tumor cell lines to transfer dye to adjacent cells were examined by the scrape-loading/dye transfer technique, and the H-ras oncogene expression by Northern blotting and P^{21} ras protein expression by Western blotting. Results: The results showed the loss of intercellular coupling in PaCa cells, slight GJIC in A549 and $CACO_2$ cells, and a good GJIC in W1–38 cells. The four compounds could improve the GJIC of PaCa to different extents. The amount of total and membrane associated P^{21} ras in PaCa cells were decreased after treatment with SMD, d-Limonene and TD-I (2.5 μg/ml) for 48 h. Concomitantly, the growth of PaCa cells decreased in soft agar and had enhanced GJIC. The relative potency was found to be:d-Limonene>SMD >TD-I=TD-II. There was no significant effect of the four compounds on H-ras oncogene expression. Conclusion: It was suggested that there was an excellent correlation between loss of Lucifer Yellow dye transfer and ras gene mutation rate in the four solid tumor cell lines (ras gene mutation rate inversely correlated with average cell number coupled, r=0.98) i.e., the high ras gene mutation was closely correlated with loss of GJIC in these malignant human tumor cells; The antitumor effect of the monoterpene d-Limonene and the phenol compound, SMD, might be related to inhibition of P^{21} ras membrane association and enhancement of GJIC, whilst that of the others may be by a different mechanism; The inhibition of P^{21} ras membrane association was directly related to the enhancement of gap junction intercellular communication.

Key words: d-Limonene, Salvia miltiorrhiza derivative (SMD), Turmeric derivatives, H-ras oncogene, Gap, Junction Intercellular Communication (GJIC).

Oncogenic ras proteins are causally implicated certain human malignancies with about 30–40% of human lung adenocarcinomas, 50% human colon carcinomas and 90–100% of pancreas carcinomas producing mutant ras proteins.[1] The mutant proteins are constitutively active, and this constant signal, coupled with other regulatory abnormalities, leads to malignant transformation.[2–4] These observations indicate that ras functions in the pathogenesis of

Accepted March 5, 1998

*The abbreviations used are: GJIC, gap junction intercellular communication; SMD, salvia miltiorrhiza derivative; TD-I and TD-II, turmeric derivative I and II.

human cancers and emphasize the potential broad utility of anticancer agents directed against these tumor cell.

Recently, many studies have been focused on the relationship between ras oncogene product and GJIC.[5,6] There has been evidence that the ras protein serves as mediator of growth factor induction of inositol phospholipid turnover.[1] The viral v-Ki-ras oncogene has been shown to inhibit GJIC between NRK cells, and the activated H-ras-1 oncogene inhibited GJIC between epithelial clone rat liver cells.

Salvia miltiorrhiza derivative (SMD) is a potent compound. Our previous works have demonstrated that it has a stronger antioxidation and antitumor activities. d-Limonene, a geranyl pyrophosphate-derived monoterpene found in orange peel and other plant essential oils,[7] is one of the few compounds known to both inhibit protein isoprenylation[8] and has *in vivo* antitumor activity. d-Limonene has chemo-preventive activity against chemically induced rodent mammary, lung, forestomach and liver[9,10] tumors, and against v-Ha-ras induced rat mammary tumors[11] at doses that cause no toxicity to the host. In addition, d-Limonene is efficacious as a chemotherapeutic agent, causing >80% of chemically induced rat mammary carcinomas to regress completely. The antitumor activity of d-Limonene may be due, in part, to its ability of inhibiting the isoprenylation of cell growth-associated small proteins such as ras.[8] Turmeric, a compound isolated from ginger, curry, and mustard, has been shown to possess antioxidative and anti-inflammatory properties, and possesses antitumor-promoting effect in a mouse skin tumorigenesis model.[12] Turmeric derivatives (TD-I and TD-II) are a series analogues of turmeric. Our previous research[13] has shown that turmeric derivative I and II possess antitumor activities on S180 solid type and H22 hepatoma in mice, enhance GJIC and counteract the inhibition of TPA-induced GJIC in V79, Balb/c-3T3, WB and 2BS normal cells. In view of the antioxidative, antitumor-promoting and antitumor activities of Salvia miltiorrhiza derivative, d-Limonene and the turmeric derivatives, we considered that these compounds may possess significant antitumor potentials in human solid tumor cell lines. In this study, we assessed the antitumor activities of these compounds in the four human solid cell lines, viz, W1–38, $CACO_2$, A549 and PaCa cells. We examined H-ras oncogene expression and GJIC in these cell lines and the relationship between them when these cells were treated with the experimental agents to determine the mechanism of the effect produced.

MATERIALS AND METHODS

Chemicals

Salviol derivative was synthesized by Professor Li. Lianniang (Institute of Materia Medica, Chinese Academy of Medical Sciences); d-Limonene was purchased from the Aldrich Chemical Co (Milwaukee, WI) at the highest chemical purity available; Turmeric derivatives were synthesized by Professor Liu Dakuan (Institute of Materia Medica, Chinese Academy of Medical Sciences); Lucifer Yellow CH was obtained from Sigma Chemical Co; Rhodamine dextran was purchased from Molecular Probe; H-ras cDNA (1.02 kb) was prepared in a bacterial expression system with H-ras provided by Health Science Research Resources Bank; Pan anti-P^{21} ras mouse monoclonal antibody was obtained from Calbiochem (Cambridge, Massachusettes); Biotin-conjugated anti-mouse IgG antibody was purchased from Vector Laboratories Inc. (Burlingame, CA); [α-^{32}P] dCTP (3000 ci/mol) was purchased from Amersham; Tissue culture media and fetal calf serum were products of Gibco Laboratories (Grand Island, NY), and all of other reagents were special grade commercial products.

Cell Culture

A549, PaCa, W1–38 and $CACO_2$ tumor cells were obtained from ATCC. PaCa human pancreas cancer cells, A549 human lung adenocarcinoma cells and W1–38 human lung squamous cells were grown on 50 ml plastic bottle in modified Eagle's medium with 1% non-essential amino acid and 10% fetal calf serum. $CACO_2$ human colon carcinoma cells were cultured in MEM medium with 20% fetal calf serum. All cells were cultured at 37℃ in humidified air with 5% CO_2.

Clonal Assay

One hundred cells were plated per 35 mm plastic dish and treated 9 days with the test compounds in DMSO or 0.1% DMSO (control group). After 9 days of treatment, the cells were fixed in 10% buffered formalin and stained with 0.25% crystal violet. Colonies of more than 50 cells were counted.

Anchorage-Independent Growth Assay

One hundred cells were plated in culture medium containing 0.3% agar onto a base of 0.6% agar in culture medium. The cultures were gently overlaid with the test compounds. After 10 days of treatment, colonies of more than 50 cells were counted.

Scrape-loading/dye Transfer Assay

Four types of monolayer of tumor cells in 35 mm dishes were treated with the test compounds or 0.1% DMSO for 48 h, and then the culture medium was removed and the cells were rinsed with phosphate-buffered saline (PBS), Lucifer Yellow CH and Rhodamine dextran (both of 0.05% in PBS, 2 ml/dish) were added to each dish, and the cell were scraped with a fine surgical blade. The scraping causes a transient rupture of the cell membranes and permits entrance of the dye. In GJIC-competent cells, dye spreads from these dye-loading cells to neighboring cells; in GJIC-incompetent cells, dye remains in the dye-loaded cells. Three minutes after scraping, the cells were washed with PBS, refed with fresh PBS, and photographed (phase-contrast micrograph and epifluoresence image).

Isolation of Total RNA and Northern Blot analysis

Cells were harvested and lysed in 4M guanidine thiocyanate lysis buffer, and total RNA was extracted by the method of Chomczynski and Sacchi with some modification as described earlier.[14] The concentration of total RNA was determined by measuring the absorbance at 260 nm with a spectrophotometer. Total RNA (20 μg/lane) was electrophoresed through a 1% agarose gel containing 6% formaldehyde and transferred onto a Hybone-N membrane. The membrane was UV cross-linked (1000 millijoules/cm^2), pre-hybridized at 42°C for 2 h, and hybridized to the ^{32}P-Labelled H-ras cDNA probe overnight at 42°C. The H-ras cDNA, a fragment of about 1.02 kb, was labelled with [^{32}P] dCTP using a random DNA labelling Kit (Amersham LIFE SCIENCE, England). After hybridization, the Hybond-N membrane was washed three times for 20 min each time in 2×SSPE and 0.1% SDS buffer at 42°C, once for 30 min in 1×SSPE and 0.1% SDS at 42°C and once for 15 min in 0.1×SSPE and 0.1% SDS at room temperature. The membrane was autoradiographed using Fuji film with intensifying screen at -70°C for 96 h. After stripping, the same membrane was rehybridized to ^{32}P-labelled GAPDH cDNA to verify equal loading of RNA onto the gel. The autoradiograph was scanned with a densitometer, and the results were integrated and normalized to the value of the GAPDH.

Western Blotting of P^{21} Ras

For total cellular P^{21} ras analysis by Western blotting, cells were harvested by scraping, pelleted by centrifugation (1500×g), and solubilized in lysis buffer (1% Triton X-100; 0.1% sodium dodecyl sulfate (SDS); 10 mM sodium phosphate buffer, pH 7.2; 50 mM Tris base, pH 7.6; 2 mM phenylmethylsulfonyl fluoride (PMSF) and 250 kU/ml aprotinin) by vortexing and incubating on ice for 10 min. The lysates were clarified by centrifugation at 100,000×g for 30 min. For Western blotting analysis of cytosolic and membranous P^{21} ras, the pelleted cells were solubilized in ice-cold PBS-PMSF (10 mM phosphate buffer, pH 7.2 and 2 mM PMSF) by homogenization and centrifuged at 100,000×g for 30 min. The supernatants (cytosolic fractions) were saved, and the pellets (membranous fractions) were solubilized in lysis buffer as described above. The protein levels in the cell fractions were determined with the Bio-Rad Protein Assay using bovine serum albumin as a standard. Twenty micrograms of protein per lane was electrophoresed on 15% polyacrylamide-SDS minigels in SDS sample buffer. The proteins were then transferred to PVDF membrane. After electrophoretic transfer, membrane was blocked for 2 h in blocking buffer (3% BSA and 0.25% normal horse serum). The blots were then analyzed using anti- P^{21} ras (primary antibody) and biotin-conjugated anti-mouse IgG (secondary antibody) and developed in AEC solution (0.8% amino-ethylcarbazole and 0.1M acetate, pH 5.2). The pan-ras antibody recognizes both normal and oncogenic forms of P^{21} ras. P^{21} ras bands were scanned with a densitometer.

RESULTS

Inhibition of the Four Test Compounds on Human Solid Tumor Cells

The concentrations of SMD, d-Limonene, TD-I and TD-II that inhibit the growth of W1–38, $CACO_2$, A549 and PaCa cells were determined by MTT and

clonal assay. SMD, d-Limonene, TD-I and TD-II could significantly inhibit W1–38, $CACO_2$, A549 and PaCa cell proliferation at concentrations greater than 10 μg/ml after 4 days of exposure, and inhibited clonal growth at concentrations greater than 5 μg/ml after 9 days of exposure. Colony formations of the four tumor cells were also inhibited by the four compounds dose-dependently. At higher doses of the four test compounds, all four types of tumor cell rounded up and got detached from the dishes.

Enhancement of Test Compounds on Gap Junction Intercellular Communication (GJIC) of Human Tumor Cells

The concentration of the four test compounds (2.5 μg/ml) used did not significantly inhibit clonal growth of four types of tumor cell. Figure 1 however, shows the effects of these compounds on GJIC in the four tumor cell lines. Among these human tumor cells, W1–38 cells possessed a better GJIC, slightly good GJIC in A549 and $CACO_2$ cells and loss of GJIC in PaCa cells. SMD and d-Limonene improved GJIC of A549 and $CACO_2$, and increased A549 and $CACO_2$ cell dye coupling (Figure 1). No effects were seen in W1–38 cells after 48 h exposure to the four test compounds, but significant increase was noted in PaCa cells (Figure 1). The potency is d-Limonene >SMD>TD-I=TD-II.

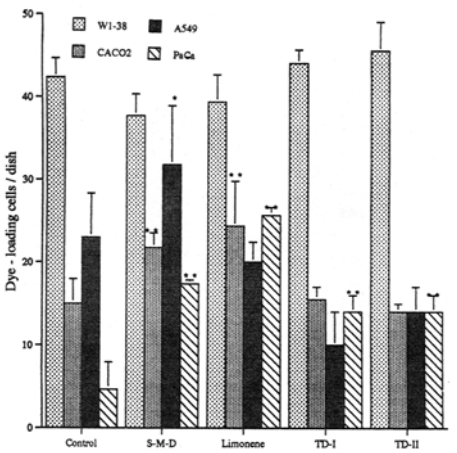

Fig. 1. Enhancement of GJIC in W1–38, $CACO_2$, A549 and PaCa tumor cell lines by test compounds after 48 h of treatment. Results are $\bar{x}\pm s$ of triplicate determinations. Significantly different from control; $^{*}P<0.05$, $^{**}P<0.01$.

Effect of Compounds on the H-ras Oncogene Expression

Figure 2 showed that SMD, TD-I and TD-II (2.5 μg/ml for 48 h) significantly and d-Limonene (2.5 μg/ml for 48 h) slightly inhibited H-ras oncogene expression in A549 cells, but no obvious effects were found in other tumor cells.

Effect of Test Compounds on P^{21} Ras Protein Expression

By Western-blot analysis, and increase in cytosolic P^{21} ras was evident in W1–38 cells treated for 48 h with 2.5μg/ml SMD, d-Limonene, TD I and TD II (Figure 3, A. B.). Only TD-I and TD-II decreased total P^{21} ras, but there was no significant change in membrane P^{21} ras (Figure 3, A. B.). There was an increase in cytosolic P^{21} ras by SMD, TD-I and TD-II in $CACO_2$, but no evident effects were seen in total and membrane P^{21} ras (Figure 3, C. D.). Only d-Limonene reduced memebrane P^{21} ras expression in A549 cells after 48 h of exposure of test compounds (Figure 3, E. F.). The significant cytosolic accumulation of P^{21} ras and the decrease in total and membranous P^{21} ras were also noted in PaCa cells with 2.5μg/ml SMD and d-Limonene of treatment for 48 h (Figure 3, G. H.). The changes in PaCa cell P^{21} ras membrane association and GJIC induced by d-Limonene and SMD were accompanied by changes in morphology (data not shown) and reduced ability to grow in soft agar. The inhibition of anchorage-independent growth by the test compounds was at lower concentration than required to inhibit growth on plastic dishes.

This was manifested as enhanced GJIC, and reduced ability to grow in soft agar. These effects occurred with d-Limonene and SMD at doses that did not inhibit the growth of PaCa cells on plastic dishes. These data indicated that Limonene and SMD reduced P^{21} ras membrane association and enhanced GJIC at nontoxic, and non-growth-inhibitory concentrations. Our results and those of Ruch[15] and De Feijter et al,[16] support the hypothesis that pharmacological inhibition of P^{21} ras membrane association is correlated with enhancement of GJIC at non-growth-inhibitory drug concentrations.

Our data suggest that the effects of d-Limonene and SMD on PaCa cells were due to the inhibition of P^{21} ras membrane association and the enhancement of gap

junction intercellular communication. D-Limonene, however, also inhibited the isoprenylation of 21–26 kda small g proteins. Our data do not exclude the possibility that drugs inhibited posttranslation modifications or inhibited farnesyl protein transferase.

In the last few years, many lines of evidence have been accumulated showing the important role of oncogene in the process of malignant progression. On the other hand, a lack of ability to communicate has been suspected to be involved in the anarchical behavior of cancer cells. It was thus all the more interesting to investigate the effects of the ras oncogene (which has been found activated in 30% of the investigated cancer cells) on gap junction intercellular communication. In our experiment, we found that cancer cell lines with higher ras gene mutation rate communicate less than those with lower ras gene mutant rate, i.e., ras gene mutation rate is inversely correlated with gap junction intercellular communication. It suggests an important effect of the ras oncogene on gap junction intercellular communication.

DISCUSSION

The test compounds used in our study possessed stronger inhibition of proliferation on the four human solid tumor cell lines W1–38, $CACO_2$, A549 and PaCa cells *in vitro*. d-Limonene and SMD reduced P^{21} ras membrane association and concomitantly accumulated cytosolic P^{21} ras in PaCa cells.

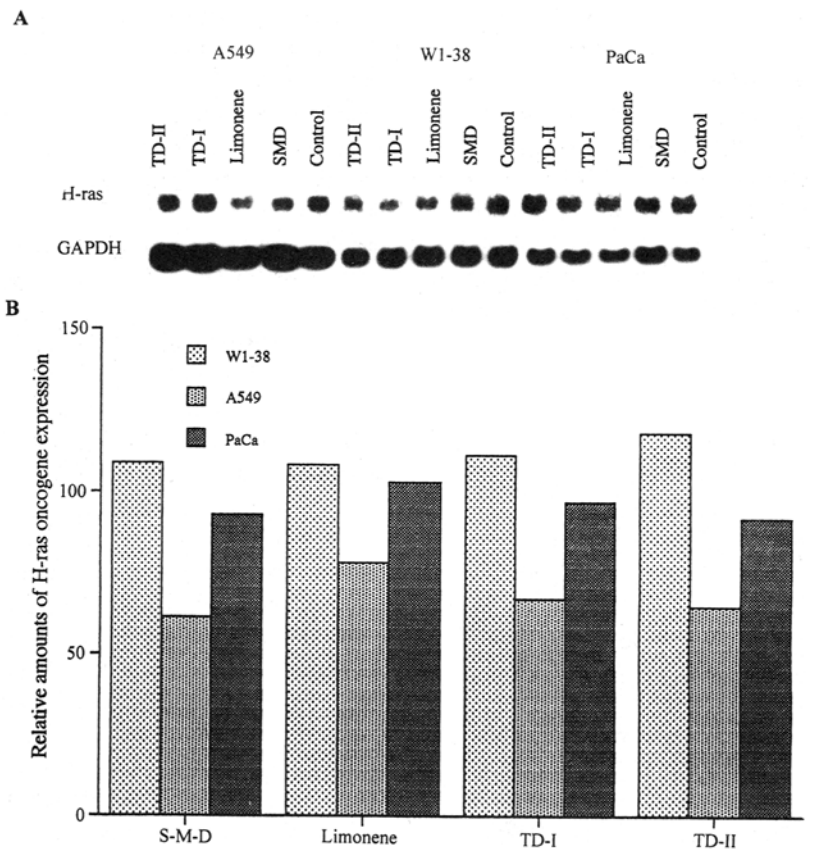

Fig. 2. Effect of test compounds on H-ras oncogene expression from W1–38, A549 and PaCa cells.

A: The total RNA was isolated. Fractionation of RNA by agarose gel electrophoresis, Northern blotting and hybridization to the ^{32}P-labeled H-ras cDNA probe are described in "materials and methods". Loading of RNA was monitored by hybridization to labeled GAPDH.

B: Relative amounts of H-ras oncogene expression, as determined by laser densitometer.

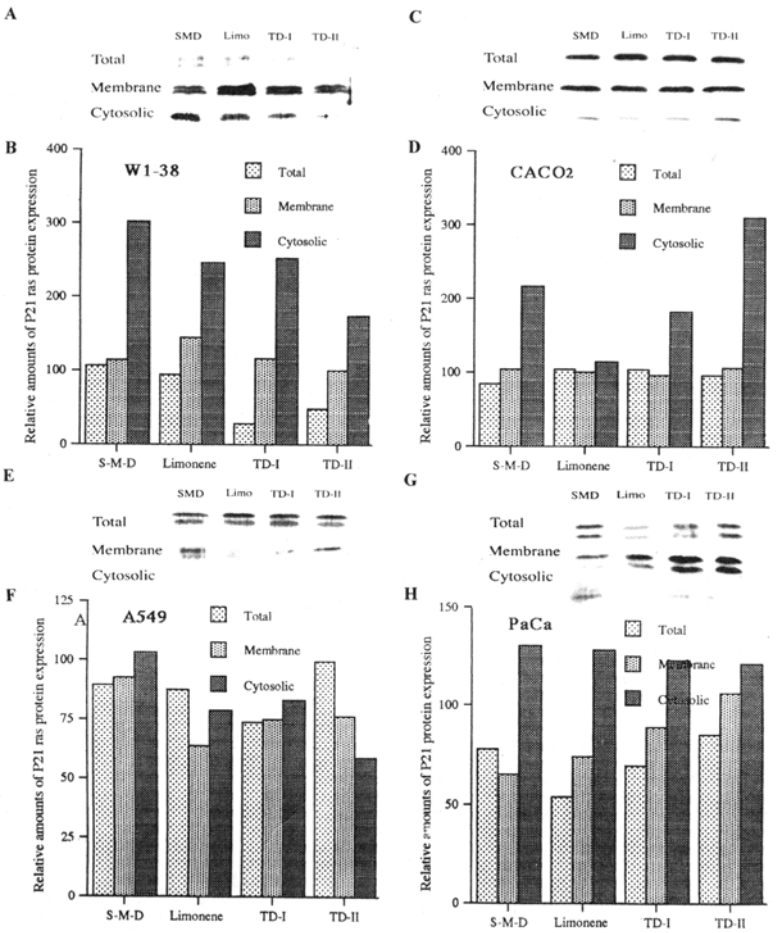

Fig. 3. A. C. E. G: Effect of test compounds on P^{21} ras protein expression in W1–38, $CACO_2$, A549 and PaCa. The total cellular, membrane and cytosolic proteins were extracted and separated by SDS-PAGE electrophoresis. P^{21} ras proteins were detected by western blotting with pan anti-P^{21} ras antibody as described in materials and methods. Molecular size of protein was measured using protein molecular weight marker (Daiichi Pure Chemicals, Japan) as the standard; B. D. F. H: Densitometric analysis of relative amounts of P^{21} ras protein expression.

How could the ras gene product interfere with gap junction intercellular communication? According to the reports of Musil et al.[17] and Ruch et al.,[15] cAMP quickly increases junctional conductance and stimulates phosphorylation of the principal gap junction 26-kDa protein; gap junction intercellular communication might thus be dependent on cAMP-dependent phosphorylation of the principal gap junction polypeptide.[18] Moreover, the activated ras oncogene is known to interfere with two metabolic pathways that are known to control intercellular protein phosphorylations: it down-modulates enzymatic activities of the adenylate cyclase.[19,20] Ras is indeed known to enhance diacylglycerol (a stimulator of protein kinase C) levels. Furthermore, diacylglycerol inhibits gap junction communication between epithelial cells. These data suggest the possibility that ras product can diminish cAMP-dependent phosphorylation of major gap junction polypeptide. In addition, by stimulating protein kinase C,[21] it could induce phosphorylation of the gap junction proteins (connexins) in a manner distinct from that of cAMP-dependent phosphorylation thereby deregulating the function of this protein,[22] and resulting in reduced gap junction permeability. The possibility is that up and down regulation of gap junction intercellular communication could be modulated by alternative phosphorylations.

In summary, our results showed that high ras gene

mutation cells may exhibit the loss of gap junction intercellular communication; the inhibition of ras product is closely correlated with the enhancement of GJIC. We postulate that the effects of ras gene mutation or ras product expression may be due to interferences with the phosphorylation of the gap junction protein connexin.

Acknowledgments

The support of the Japan Society for the Promotion of Science is gratefully acknowledged.

REFERENCES

1. Barbacid M. Ras genes. Annu Rev Biochem 1991; 56:827.
2. James GL, Galdstein JL. Benzodiazapine peptidomimetics: potent inhibitors of ras farnesylation in animal cells. Science 1993; 260:1937.
3. Schafer WR, Rine J. Protein prenylation: Genes, enzymes, targets, and functions. Annu Rev Genet 1992; 30:209.
4. Itoh T, Kaibuchi K, Masuda T, et al. The posttranslational processing of ras P^{21} is critical for its stimulation of mitogen-activated protein kinase. J Biol Chem 1993; 268:3025.
5. Dotto GD, El-Fouly MH, Nelson C, et al. Similar and synergistic inhibition of gap junctional communication by ras transformation and tumor promoter treatment of mouse primary keratinocytes. Oncogene 1989; 4: 636.
6. Brissette JL, Kumar NM, Gilula NB, et al. The tumor promoter 12-0-tetradecanoylphorbol-130acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. Mol Cell Biol 1991; 11:5364.
7. Croteau R. Biosynthesis and catabolism of monoterpenes. Chem Rev 1987; 87:929.
8. Crowell PL, Chang RR, Ren Z, et al. Selective inhibition of isoprenylation of 21–26 kDa proteins by the anticarcinogen d-Limonene and its metabolites. J Biol Chem 1991; 266:17679.
9. Wattenbery LW, Sparnins VL, Barany G. Inhibition of N-nitrosodiethylamine carcinogenesis in mice by naturally occurring organosulfur compounds and monoterpenes. Cancer Res 1989; 49:2689.
10. Wattenberg LW, Coccia JB. Inhibition of 4-(methylnitrosoamino)-1-(3-pyridy)-1-butanone carcinogenesis in mice by d-Limonene and citrus fruit oils. Carcinogenesis 1991; 12:115.
11. Moore CJ, Kennan WS, Wang B, et al. Inhibition of ras-induced rat mammary carcinogenesis by Limonene. Proc Am Assoc Cancer Res 1991; 32:131.
12. Katiyar SK, Agarwal R, Mukhtar H. Inhibition of tumor promotion in SENCAR mouse skin by ethanol extract of zinginber officinale rhizome. Cancer Res 1996; 56:1023.
13. Li Y, Fu ZD, Chen XG. Effects of curcumin derivatives on the GJIC of normal and tumor cells. Acta Acad Med Sinicae 1996; 18(2):111.
14. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guandidinium thiocyanate-phenochlorform extraction. Anal Biochem 1987; 162:156.
15. Ruch RJ, Madhukar BV. Reversal of ras-induced inhibition of gap-junctional intercellular communication, transformation, and tumorigenesis by lovastatin. Molecular Carcinogenesis 1993; 7:50.
16. De Feijter AW, Ray JS, Weghorst CH. Infection of rat liver epithelial cells with v-Ha-ras: correlation between oncogene expression, gap junctional communication, and tumorigenecity. Mol Carinog 1990; 3:54.
17. Musil LS, Beyer EC, Goodenough DA. Expression of the gap junction connexin 43 in embryomic chick lens: molecular cloning, ultrastructural localization and posttranslational phosphorylation. J Membr Biol 1990; 116:163.
18. Crow DS, Beyer EC, Paul DL, et al. Phosphorylation of connexin 43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian fibroblasts. Mol Cell Biol 1990; 10:1754.
19. Filson AJ, Azarnia JR, Eyer EC, et al. Tyrosine phosphorylation of a gap junction protein correlated with inhibition of cell-to-cell communication. Cell Growth Differ 1990; 1:661.
20. Musil LS, Cunningham BA, Edelman GM, et al. Differential phosphorylation of the gap junction protein connexin 43 in junctional communication-competent and deficient cell lines. J Cell Biol 1990; 111:2077.
21. Lioyd AC, Paterson HF, Morris JDH, et al. P^{21} H-ras-induced morphological transformation and increased c-*myc* expression are independent of functional protein kinase C. EMBO J 1989; 8:1099.
22. Guo HM, Acevedo P, Parsa FD, et al. Gap-junctional protein connexin 43 is expressed in dermis and epidermis of human skin: Differential modulation by retinoids. J Investigative Dermatology 1992; 99: 460.