Ya-Jun He, Kenny Kuchta*, Xia Lv, Yu Lin, Guo-Rong Ye, Xu-You Liu, Hui-Dong Song, Le-Xin Wang, Yuta Kobayashi and Jian-Chang Shu*

Curcumin, the main active constituent of turmeric (*Curcuma longa* L.), induces apoptosis in hepatic stellate cells by modulating the abundance of apoptosis-related growth factors

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Abstract: In order to elucidate the mechanism of action of curcumin against hepatic fibrosis, cultured rat hepatic stellate cells (HSC) (HSC-T6) were incubated with curcumin for 24 h, after which apoptosis was measured by flow-cytometry. The protein levels of the pro-apoptotic factors Fas and p53b as well as of the anti-apoptotic factor Bcl-2 were monitored by immunocytochemical ABC staining after incubation with curcumin for 24 h. In the case of 20 μM curcumin, not only was the respective apoptosis index increased, but also the abundance of the pro-apoptotic factors Fas and p53 were amplified, whereas that of the anti-apoptotic factor Bcl-2 decreased. All these effects were highly reproducible ($P<0.05$). Consequently, curcumin has an up-regulating effect on pro-apoptotic factors like Fas and p53 as well as a down-regulating effect of the anti-apoptotic factor Bcl-2, thus inducing apoptosis in HSC.

Keywords: apoptosis; curcumin; hepatic stellate cells.

***Corresponding authors: Jian-Chang Shu,** Department of Gastroenterology, Guangzhou Red Cross Hospital, Jinan University, 396 Tong Fu Zhong Lu, Guangzhou 510220, PR China, Phone: +81-(0)90-1334-6183, E-mail: shujc0328@163.com; and School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, New South Wales 2678, Australia; and **Kenny Kuchta,** National Institute of Health Sciences, Division of Pharmacognosy, Phytochemistry and Narcotics, Setagaya-ku, Kamiyoga 1-18-1, 158-8501 Tokyo, Japan, E-mail: kkuchta@rz.uni-leipzig.de
Ya-Jun He, Xia Lv, Guo-Rong Ye, Xu-You Liu and Hui-Dong Song: Department of Gastroenterology, Guangzhou Red Cross Hospital, Jinan University, Guangzhou 510220, PR China
Yu Lin: Medical Corporation Soujikai, 541-0046 Osaka, Chuo, Hirano 2-2-2, Japan
Le-Xin Wang: School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, New South Wales 2678, Australia
Yuta Kobayashi: Faculty of Medicine, Shimane University, 693-8501 Izumo, Enya 89-1, Japan

1 Introduction

Hepatic stellate cells (HSCs) are the major type of mesenchymal cells in liver tissue and play a crucial role in the pathogenesis of hepatic fibrosis. The latter is characterized by their massive proliferation, accompanied by the production and deposition of an excess of extracellular matrix (ECM) in the perisinusoidal space. This proliferation later results in liver cirrhosis and finally in hepatocellular carcinoma unless interrupted or treated [1]. Being capable of both autocrine and paracrine signalling, HSCs can further synthesize pro-inflammatory cytokines and growth factors such as transforming growth factor (TGF), tumor necrosis factor (TNF), and connective tissue growth factor (CTGF).

Nevertheless, only very few effective antifibrogenic therapies have been developed until now in conventional medicine. On the other hand, several medicinal plants of Traditional Chinese Medicine (TCM) have a long history of successful application for curing chronic liver diseases. Among these, the famous turmeric (*Curcuma longa* L.) and its major constituent curcumin (Figure 1) exert anti-inflammatory, anti-tumor, immune modulatory, as well as tissue protection and repair effects [2–9].

Curcumin was recently found to possess significant curative potential against hepatic fibrosis not only in *in vitro* studies but also in an *in vivo* model system developed in our laboratory during preliminary experiments for the present study. This activity is directly connected with the above mentioned liver tissue protective effects and attenuates hepatic fibrosis by inhibiting HSC proliferation while inducing HSC apoptosis [10, 11]. Other pharmacological targets like the prevention of lipid peroxidation play a minor, less well understood role [1, 12, 13].

A very important, but as yet unknown, point are the signalling pathways leading to apoptosis after contact with curcumin. However, these are essential for developing a strategy for curing hepatic fibrosis by inducing

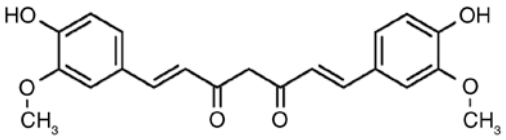

Figure 1: Structure of curcumin.

apoptosis in HS cells. In a previously published *in vitro* study, proliferation inhibition and apoptosis induction in HSCs appeared to be related to the activation of the peroxisome proliferator [14], a key regulator of both apoptosis and the normal cell cycle. In studies on fully malign cancer cells, curcumin has been shown to induce apoptosis by down-regulating the expression of the anti-apoptotic factors Bcl-2 and Bcl-xL [15] as well as up-regulating the caspases and the pro-apoptotic factors Bax and Bak [16]. It is presently unknown if these pharmacological targets are also responsible for the induction of apoptosis in HSCs before genuine malign cancer cells are developed.

The present study aims to clarify the pathway between curcumin induced HSC apoptosis and the modulation of the expression of the apoptosis related growth factors Fas, p53, and Bcl-2.

2 Materials and methods

2.1 Preparation of the curcumin stock solution

Curcumin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 40 μL of dimethyl sulfoxide (DMSO) equivalent to <0.1 % of the final volume. This solution was mixed rapidly with Dulbecco's modified Eagle's medium (DMEM) (Hyclone-Gibco; ThermoFisher, Grand Island, NY, USA) to provide a solution of 10 mM curcumin, which was sterilized by filtration through a 0.22 μm syringe filter. Aliquots were stored in the dark at –20°C. For the negative-controls, a corresponding control solution with equal amounts of DMSO and DMEM but without curcumin was prepared.

2.2 HSC-T6 culture

The rat HSC cell line HSC-T6, which possesses an activated phenotype, was a generous gift from Professor Lie-Ming Xu (Division of Liver Diseases, Shanghai TCM University, China). These cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 10 % new-born calf serum (Hyclone-Gibco). Cells were incubated in a cell culture flask at 37°C under 5 % CO_2/95 % air with saturated humidity. The culture medium was changed every 2 days. When cell density reached 80 %–90 %, cultures were transferred into new medium at a ratio of 1:4 after digestion with 0.02 % EDTA and 0.25 % trypsin.

2.3 Cytotoxicity test

Cells in logarithmic growth phase were transferred to a 96-well plate, applying 100 μL of the culture to each well, resulting in an initial cell density of 1.25×10^5/ml. Twenty-four hours later, they were treated and subsequently cultured with different concentrations of curcumin, namely 0, 20, 30, 40, 50, 60, 70, 80, 90, 100 μM with five repetitions for each concentration. The morphology of the HSC-T6 cells was observed using an inverted microscope.

2.4 Detection of apoptosis by flow cytometry

Cells in logarithmic growth phase were transferred into T-25 culture flasks at an initial cell density of 1.25×10^5/ml and incubated for 24 h before treatment with 0, 10, 20, 30, or 40 μM curcumin for the next 24 h. Both suspended and adherent cells were harvested completely and washed once with cold phosphate-buffered saline (PBS). Subsequently, cells were incubated using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Penzberg, Germany) in accordance with the instructions of the manufacturer. After this treatment, the amount of apoptotic cells was measured using a BD Biosciences FACS Calibur flow cytometer (San Jose, CA, USA).

2.5 Immunocytochemical experiment

HSCs in logarithmic growth phase were transferred into six-well plates at an initial cell density of 1.25×10^5/ml and incubated for 24 h. Then they were incubated with 20 μM curcumin for another 24 h. HSCs were washed three times with PBS after which 4 % (w/v) paraformaldehyde was added to fix the cells. Negative-control groups of HSCs were treated identically during the entire experiment with the above described control solution without curcumin, applying a dose of DMSO equivalent to that used with the highest curcumin doses. Amongst these negative-control groups, an additional blank-control (Figure 2) was treated identically to all other experimental setups but without the addition of the primary antibody described in the following paragraph. Subsequent immunocytochemical tests were performed according to the manual of the respective reagent kit, namely the Bcl-2 Antibody kit (Santa Cruz Biotechnology, Dallas, TX, USA) and the p53 and Fas Antibody kits (both from BosterBio, Pleasanton, CA, USA). After immunological staining was completed, the cells were counterstained with haematoxylin. Positive staining for p53, Bcl-2, or Fas is visible as brown grains in the nucleus, at the membrane, or both at the membrane and in the cytosol, respectively. Both the absolute number of cells and the amount of positively stained cells were counted using an Olympus BX50-32H01 optical microscope (Olympus, Tokyo, Japan) for a total of 400 samples from each of which 10 individual fields of vision were selected randomly. The average percentage of positively stained cells in comparison to the total cell count was defined as the ratio of positive expression.

2.6 Statistical analysis

The SPSS11.0 software package was used for statistical analysis. All results were expressed as means ± SD. Multiple comparisons between

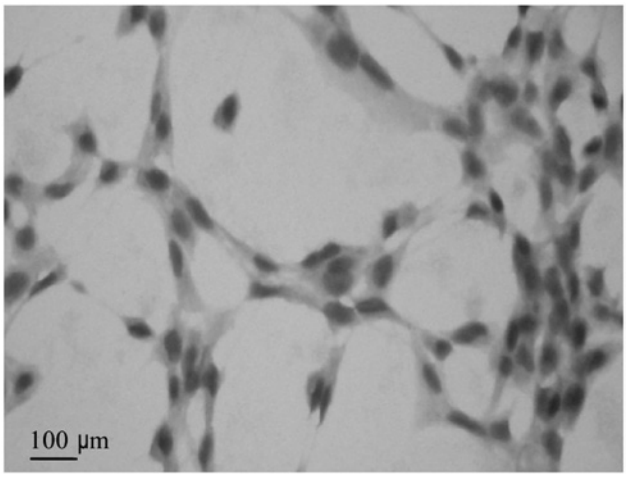

Figure 2: Blank-control. HSCs of a negative-control group that was treated identically to all other experimental setups but without the addition of the primary antibody.

different groups were performed by ANOVA, whereas paired comparisons were performed by the t-test. Categorical data were compared via χ^2-test. $P<0.05$ was considered statistically significant.

3 Results

3.1 Cytotoxicity test of curcumin

When observed through an inverted microscope, each group of cultured cells displayed their normal cellular morphology as fusiform, stellate or irregular cells with intact cellular membranes. The cells looked like typical fibroblasts and neither cell debris nor cells with deformed nuclei were observed. No significant differences between normal and curcumin treated cells could be detected for those groups which received the 20 μM and 80 μM dosages, suggesting that curcumin did not harm the HSCs at concentrations less than or equal to 80 μM.

3.2 Apoptosis of HSCs

After treatment with 10, 20, 30, and 40 μM curcumin for 24 h, the apoptosis indices of the respective HSCs were 6.5 ± 1.9 %, 11.6 ± 2.8 %, 52.0 ± 4.4 %, and 87.1 ± 12.6 %, respectively, demonstrating a clear statistically significant concentration-dependence ($P<0.05$). Compared with the apoptosis index of 3.8 ± 0.6 % measured for the negative-control group, which was lower than that of all curcumin groups, there was an even clearer statistical difference ($P<0.01$).

3.3 Abundance of the proteins Fas, p53 and Bcl-2

Following the treatment of HSCs with 20 μM curcumin, a substantial increase in the abundance of both Fas and p53 was observed, while that of Bcl-2 was reduced (Figure 3). When compared with the negative-control group, these differences were clearly significant ($P<0.05$) for all three proteins (Table 1). In contrast, no significant changes were observed in the 10 μM samples. In the case of the 30 and 40 μM samples, apoptosis occurred so fast that the ABC staining could not be carried out properly.

4 Discussion

In the present study, curcumin was clearly demonstrated to induce apoptosis of HSCs in a concentration-dependent manner. Furthermore, this curcumin-induced apoptosis was shown to be related to increased abundance of the pro-apoptotic proteins Fas and p53 as well as on the reduced level of the anti-apoptotic protein Bcl-2.

Hepatic fibrosis has been demonstrated to be completely reversible if the factor(s) of continuous injury can be eliminated and anti-fibrotic therapy for protecting hepatic tissue is performed. In contrast, untreated chronic hepatic injury inevitably progresses, leading to hepatic cirrhosis and finally hepatocellular carcinoma. Nevertheless, no fully effective method for the treatment of hepatic fibrosis has been developed to date. The further proliferation of HSCs is considered a key element in the pathogenesis of hepatic fibrosis. Consequently, preventing proliferation or inducing apoptosis of HSCs has become a prime target of research for anti-fibrotic therapies.

In this context, medicinal plant extracts used in TCM, which constitute a variety of multi component mixtures, have received increasing attention due to their parallel action on multiple pharmacological targets on multiple levels of disease mechanisms, which is in strong contrast to the single target activity of chemical monomolecular drugs.

Curcumin, the main phenolic constituent of turmeric (*C. longa* L.), plays an important role in TCM due to its anti-inflammatory, anti-oxidative, anti-neoplastic, and anti-fibrotic activity. The present study demonstrates that after treatment with curcumin for 24 h, apoptosis of HSC increases significantly and in close correlation with the applied curcumin concentration.

Apoptosis of HSCs is a complex process which is regulated by several molecular pathways. It has already been demonstrated in experiments with cultured HSCs that

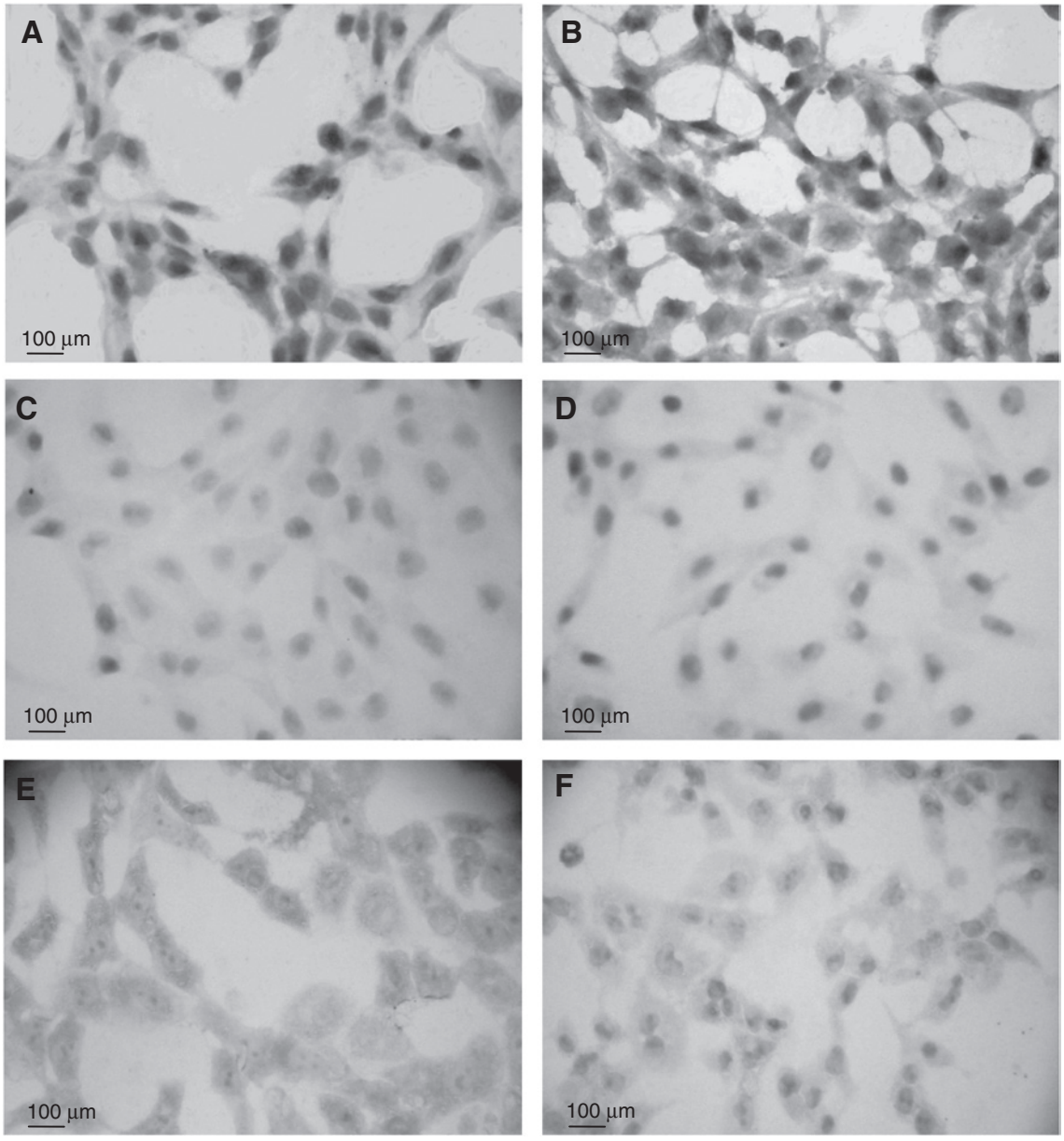

Figure 3: Abundance of Fas, p53 and Bcl-2 in control and curcumin-treated HSCs. Fas (A, B): HSCs of the negative-control group (A), which was treated identically to the other groups, but in the absence of curcumin. HSCs treated with 20 µM curcumin (B). Dark grains, revealing the presence of Fas in both membrane and cytosol after immunocytochemical staining, are significantly more numerous in B as compared to A. p53 (C, D): HSCs of the negative-control group (C), and of the curcumin-treated group (D). Dark grains, revealing the presence of p53 in the nuclei after immunocytochemical staining, are significantly more numerous in D as compared to C. Bcl-2 (E, F): HSCs of the negative-control group (E) and of the curcumin-treated group (F). Dark grains, revealing expression of Bcl-2 at the cell membrane after immunocytochemical staining, are significantly more numerous in E as compared to F.

both CD95 (APO-1/Fas) and CD95L (APO-1-/Fas-ligand) become increasingly abundant during apoptosis. Furthermore, apoptosis could be blocked completely by CD95-binding antibodies, even in HSCs already entering the apoptotic pathway [17]. Conversely, CD95-activating antibodies induced apoptosis in more than 95 % of the HSCs [17]. The expression of the apoptosis-regulating proteins Bcl-2 and p53 was found to increase during apoptosis, which is especially interesting as p53 expression could be induced by CD95-activating antibodies [18].

In the present study, we have demonstrated that the abundance of Fas and p53 increased significantly upon treatment with curcumin, whereas in the case of Bcl-2 an equally significant decrease was observed. These data

Table 1: Abundance of Fas, p53, and Bcl-2 following curcumin treatment (20 μM).

Groups	Positively stained cells in percent of total number of cells counted		
	Fas	p53	Bcl-2
Negative-control group	33.1 ± 3.1 %	20.6 ± 7.2 %	95.4 ± 3.6 %
Curcumin group	87.4 ± 2.8 %[a]	43.1 ± 7.3 %[a]	28.7 ± 5.9 %[a]

[a]Significantly different from the negative-control group ($P<0.05$). For experimental details, see text.

suggest that effects on the expression of apoptosis related growth factors are at least partly responsible for the curcumin – and by extension, the *C. longa* extract – induced apoptosis of HSCs.

In conclusion, the present *in vitro* study revealed the concentration-dependent induction of apoptosis in HSCs by exposure to curcumin, accompanied by enhanced abundance of Fas and thp53, in addition to a reduced abundance of Bcl-2. These data suggest that upregulation of the pro-apoptotic factors Fas and p53 as well as down regulation of the anti-apoptotic factor Bcl-2 may be involved in curcumin-induced apoptosis of HSC cells, similar to the processes previously reported for fully malign cancer cells [15, 16].

References

1. O'Connell MA, Rushworth SA. Curcumin: potential for hepatic fibrosis therapy? Br J Pharmacol 2008;153:403–5.
2. Jayaprakasha GK, Jena BS, Negi PS, Sakariah KK. Evaluation of antioxidant activities and antimutagenicity of turmeric oil: a byproduct from curcumin production. Z Naturforsch 2002;57c:828–35.
3. Egan ME, Pearson M, Weiner SA, Rajendran V, Rubin D, Glöckner-Pagel J, et al. Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. Science 2004;304:600–2.
4. Mall M, Kunzelmann K. Correction of the CF defect by curcumin: hypes and disappointments. Bioessays 2005;27:9–13.
5. Fiala M, Liu PT, Espinosa-Jeffrey A, Rosenthal MJ, Bernard G, Ringman JM, et al. Innate immunity and transcription of MGAT-III and Toll-like receptors in Alzheimer's disease patients are improved by bisdemethoxy curcumin. Proc Natl Acad Sci USA 2007;104:12849–54.
6. Hsu CH, Cheng AL. Clinical studies with curcumin. Adv Exp Med Biol 2007;595:471–80.
7. Ibrahim MA, Elbehairy AM, Ghoneim MA, Amer HA. Protective effect of curcumin and chlorophyllin against DNA mutation induced by cyclophosphamide or benzo[a]pyrene. Z Naturforsch 2007;62c:215–22.
8. Miriyala S, Panchatcharam M, Rengarajulu P. Cardioprotective effects of curcumin. Adv Exp Med Biol 2007;595:359–77.
9. Weisberg SP, Leibel R, Tortoriello DV. Dietary curcumin significantly improves obesity-associated inflammation and diabetes in mouse models of diabesity. Endocrinology 2008;149:3549–58.
10. Shu JC, He YJ, Lv X, Ye GR, Wang LX. Curcumin prevents liver fibrosis by inducing apoptosis and suppressing activation of hepatic stellate cells. J Nat Med 2009;63:415–20.
11. Shu JC, He YJ, Lv X, Zhao JR, Zhao J, Shen Y, et al. Effect of curcumin on the proliferation and apoptosis of hepatic stellate cells. Braz J Med Biol Res 2009;42:1173–8.
12. Park EJ, Jeon CH, Ko G, Kim J, Sohn DH. Protective effect of curcumin in rat liver injury induced by carbon tetrachloride. J Pharm Pharmacol 2000;52:437–40.
13. Bruck R, Ashkenazi M, Weiss S, Goldiner I, Shapiro H, Aeed H, et al. Prevention of liver cirrhosis in rats by curcumin. Liver Int 2007;27:373–83.
14. Xu J, Fu Y, Chen A. Activation of peroxisome proliferator-activated receptor-gamma contributes to the inhibitory effects of curcumin on rat hepatic stellate cell growth. Am J Physiol-Gastrointest- Liver Physiol 2003;285:G20–30.
15. Mackenzie GG, Queisser N, Wolfson L, Fraga CG, Adamo AM, Oteiza PI. Curcumin induces cell-arrest and apoptosis in association with the inhibition of constitutively active NF-kappaB and STAT3 pathways in Hodgkin's lymphoma cells. Int J Cancer 2008;123:56–65.
16. Shankar S, Srivastava RK. Bax and Bak genes are essential for maximum apoptotic response by curcumin, a polyphenolic compound and cancer chemopreventive agent derived from turmeric, Curcuma longa. Carcinogenesis 2007;28:1277–86.
17. Saile B, Knittel T, Matthes N. CD95/CD95L-mediated apoptosis of hepatic stellate cell. A mechanism terminating uncontrolled hepatic stellate cell proliferation during hepatic tissue repair. Am J Pathol 1997;151:1265–72.
18. Michael W, Stephan W, Christine S, Esser P, Fontana A, Dichgans J, et al. Topoisomerase-I inhibitors for human malignant glioma: differential modulation of p53, p21, Bax and Bcl-2 expression and of CD95-mediated apoptosis by camptothecin and β-lapachone. Int J Cancer 1997;73:707–14.