Potential role of β-elemene on histone H1 in the H22 ascites hepatoma cell line

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Abstract. β-elemene is extracted from the Chinese medicinal herb Curcuma Wenyujin. It has a broad-spectrum antitumor effect on many types of cancer. However, the exact mechanism of action of β-elemene in hepatocellular carcinoma (HCC) remains unknown. Histone H1 is thought to act as a repressor of transcription by promoting the compaction of chromatin into higher order structures. We found that histone H1 plays an important role in the antitumor function of β-elemene. In this study, histone H1 expression in the H22 murine hepatocellular carcinoma cell line was confirmed, with P388D1 cells serving as a positive control. Furthermore, H22 cells were cultured with β-elemene for different time-points *in vitro* and treated with different dose-dependent β-elemene in an experimental H22 HCC xenograft transplantation model to confirm whether β-elemene inhibited the growth of H22 tumor cells. In addition, measurements of histone H1 expression both *in vitro* and *in vivo* enabled us to demonstrate that β-elemene affects the expression of histone H1 only at the protein level, but is not involved in regulation at the gene level. Our results clearly show that the effect of β-elemene on inhibiting the growth of H22 tumor cells is time- and dose-dependent. In conclusion, β-elemene inhibits the growth of H22 cells by enhancing the expression of histone H1 only at the protein level. This finding may provide insight into a new mechanism of the antitumor action of β-elemene.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide and is responsible for approximately one million deaths each year (1). HCC is especially frequent in Asia due to the high prevalence of chronic HBV and HCV infections. During most of the long pre-neoplastic stage leading to HCC, alterations in gene expression are almost entirely quantitative, occurring by epigenetic mechanisms in the absence of detected changes in the structures of chromosomes (2). Therefore, identifying a way to maintain normal chromatin structure and effectively treat this disease is crucial.

The basic structural unit of chromatin, known as the nucleosome, comprises DNA and histone proteins (3). There are five types of histone proteins: H1, H2A, H2B, H3 and H4 (4-6). Histone H1 is known as the linker histone, whereas the remaining four histone proteins are collectively known as the core histones. Human and mouse genomes contain up to 11 histone H1 variants, all consisting of a short N-terminal tail, a globular core domain and a C-terminal tail, making up approximately 50% of the whole protein (7,8). These tails are post-translationally modified, mostly by phosphorylation, but also by acetylation and methylation (9,10). The linker histone H1 binds to the DNA between the nucleosomal core particles, and has a key role in establishing and maintaining higher order chromatin structures and in regulating gene expression (11). Other studies have described the principle role of histone H1 in autoimmune disease (12), breast cancer (13) and pancreatic cancer (14). The main biological and physiological function of histone H1 possibly involves marked cytotoxicity to abnormal cells. Recent studies have shown that abundance of histone H1 is closely associated with chromatin configuration and may be altered in cancer cells (15).

Elemene, isolated from the Chinese medicinal herb Curcuma Wenyujin, is a mixture of β-, δ- and γ-elemene. Accounting for 60-72% of the three isoforms, β-elemene is the main component of elemene. β-elemene has been shown to inhibit tumor cell growth *in vitro* and *in vivo*, and has been employed in clinical trials in cancer patients with observed efficacy (16). Previous studies showed that β-elemene exerts effects on the SMMC-7721 cell line of human HCC by inhibiting cell proliferation to induce apoptosis (17). Other authors reported that the apoptosis triggered by β-elemene was via the mitochondrial-mediated pathway, as it was accompanied by a reduction of Bcl-2, Bcl-X(L) and XIAP (18,19). However, the exact mechanism of action of β-elemene in HCC remains to be determined.

In this study, we found that histone H1 is expressed in H22 cells both *in vivo* and *in vitro*. The effect of β-elemene on

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histone H1 in H22 cells was assessed by PCR, western blotting/protein electrophoresis, immunofluorescence and immunohistochemistry in *in vitro* and *in vivo* experiments. Based on the fact that elemene and histone H1 are closely associated with antitumor properties, we speculated that β-elemene may also affect the expression of histone H1. A new mechanism underlying the antitumor effects of β-elemene was also confirmed.

Materials and methods

Materials. β-elemene (5 mg/ml) was purchased from Dalian Holley Kingkong Pharmaceutical Co., Ltd. (Dalian, China). 5-Fluorouracil (5-FU) injection (0.25 g/ml) was purchased from the Second Affiliated Hospital of Dalian Medical University, China. RPMI-1640 medium and fetal bovine serum (FBS) were provided by Gibco Corp, Grand Island, NY, USA. Murine ascites hepatocarcinoma cell strain H22 cells were preserved by our laboratory. BALB/c mice were obtained from the Dalian Medical University Laboratory Animal Center.

Cell culture. H22 cells were injected into BALB/c mice in duplicate in order to collect a large amount of cells in <2 weeks, and incubated. H22 cells were maintained in RPMI-1640 medium buffer, with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown to 85-95% confluence prior to use.

RNA isolation and reverse transcription-PCR. Reverse transcription-PCR was used to measure the mRNA expression of histone H1. Total RNA was isolated from H22 cells or mouse-borne tumor tissues after treatment with β-elemene using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The samples were prepared and mRNA was added to each PCR tube containing the reaction mixture. The sequence-specific primers used were: forward: 5'-ATGACTCCTTTTTCCTTAAC-3' and reverse: 5'-CAAGCAGTCTAAGAAAGTCT-3'.

A PCR system with the following settings was employed: denaturation at 94˚C, 5 min for 1 cycle, followed by 35 cycles of 95˚C for 30 sec, annealing 55˚C for 1 min, 72˚C for 1 min, and an extension at 72˚C for 10 min.

Western blotting. Cells or tissues were lysed in sample buffer supplemented with mercaptoethanol after treatment as previously reported (18). Proteins (20 μl) from each sample were separated by SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes. Western blots were performed according to standard methods, which involved normalization to β-actin. In this study, the primary antibodies were directed against histone H1 (1:200; Bioworld Technology, Inc., Minneapolis, MN, USA) and mouse anti-actin (1:500). The membranes were then incubated with the appropriate secondary antibody (1:2,500; anti-rabbit or anti-mouse IgG). Reactive bands were visualized using an enhanced chemiluminescence system (Amersham).

Immunofluorescence analysis. Aliquots of $4x10^7$ cells/ml were seeded into a 5-ml elemene medium with 100 μg/ml elemene in tubes in a 5% CO_2 atmosphere at 37˚C for 0, 4, 8, 12 and 24 h. Cells were then washed with PBS (3x5 min) and blocked with 10% FBS for 30 min at room temperature. Cells were incubated with the polyclonal anti-histone H1 antibody in PBS (1:100) for 45 min and washed with ice-cold PBS. The cells were incubated with goat anti-rabbit IgG-FITC (1:100) for 45 min at 4˚C. Observation was carried out and images were captured with an Olympus multifunction microscope (Olympus BX51, Japan).

Immunohistochemistry. Tissue sections were fixed in 10% formalin. Paraffin-embedded 4-μm sections of the bearing tumor were used for immunohistochemical studies. The slides were incubated for 10 min in 3% H_2O_2, rinsed with PBS and incubated for 15 min in blocking solution (5% fetal calf serum). Hybridization with histone H1 antibody (1:100 dilution) was carried out for 1 h at 37˚C. After rinsing with PBS, secondary antibody (goat anti-rabbit IgG) was incubated for 20 min. The slides were then incubated with diaminobenzidine (DAB) and counterstained with hematoxylin. Microscopic examination was performed using an Olympus multifunction microscope (Olympus BX51).

Tumor xenografts in mice. Six- to seven-week-old BALB/c female mice maintained under sterile conditions were used. Experiments were approved and conducted in accordance with the ethics guidelines set by the University's Committee on Using Live Animals. The mice were kept for 5 days before experimentation. The H22 cells obtained from mice ascites (approximately $4x10^7$) were suspended in sterile saline. Each mouse was injected via the armpit vein with 0.1-ml cell suspension and the treatments were initiated after 24 h. Mice were individually identified and randomly assigned to the control and treatment groups (n=5 and n=7, respectively) according to the following regimens: 1, elemene-higher group (100 mg/kg); 2, elemene-lower group (50 mg/kg); 3, 5-FU group (20 mg/kg); 4, physiological saline, 0.2 ml each mouse. Tumors became palpable 4 days after xenografting. The animals received treatment regimens every day from day 1 for 10 days. The mice were sacrificed by cervical dislocation on the day following the final injection. Tumors were dissected, weighed individually and the percentage of the inhibition rate (IR) was calculated: IR = (1 - tumor weight drug/tumor weight control). The expression levels of histone H1 in tumors from different groups were detected by RT-PCR, western blotting and immunohistochemistry.

Statistical analysis. Data were shown as the means ± SD. SPSS 11.5 statistical software and Student's t-test were used for statistical analysis. $P<0.05$ denoted a statistically significant difference. The experiments in this study were repeated three times.

Results

Histone H1 expression in the H22 murine hepatocellular carcinoma cell line. To discover the biological function of histone H1 in H22 murine HCC cells, we initially investigated the expression of histone H1 in H22 cells by western blotting (Fig. 1A), RT-PCR (Fig. 1B) and immunofluorescence (Fig. 1C). P388D1 cells were used as a control. The specificity protein stripe was evident at 27 kDa and amplified at ~205 bp bands

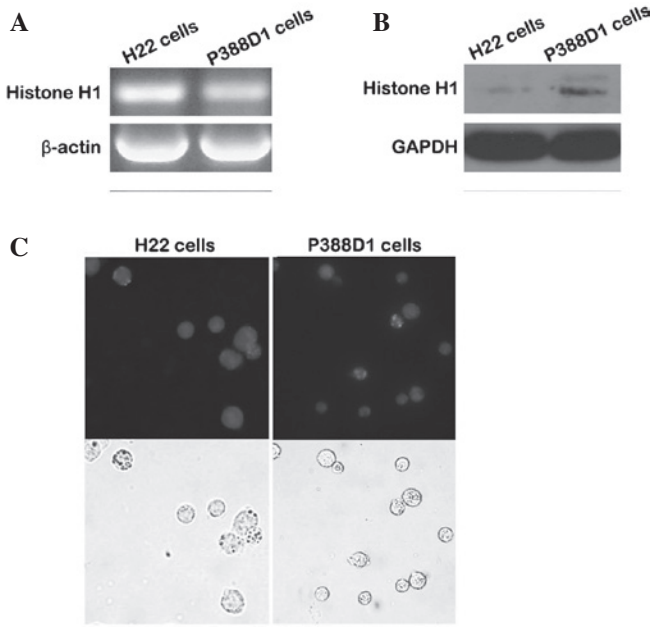

Figure 1. Histone H1 expression in the H22 murine hepatocellular carcinoma cell line. H22 cells were analysed by RT-PCR, western blotting and an immunofluorescence assay. (A) Electrophoresis in 1.2% (w/v) agarose gel reveals a single 205-bp product following amplification by PCR using histone H1-specific primers. (B) Western blot analysis of histone H1 in H22 cells. GAPDH loading control is shown in the bottom panel. (C) The expression of histone H1 on H22 cells was determined by an immunofluorescence assay (magnification, x400).

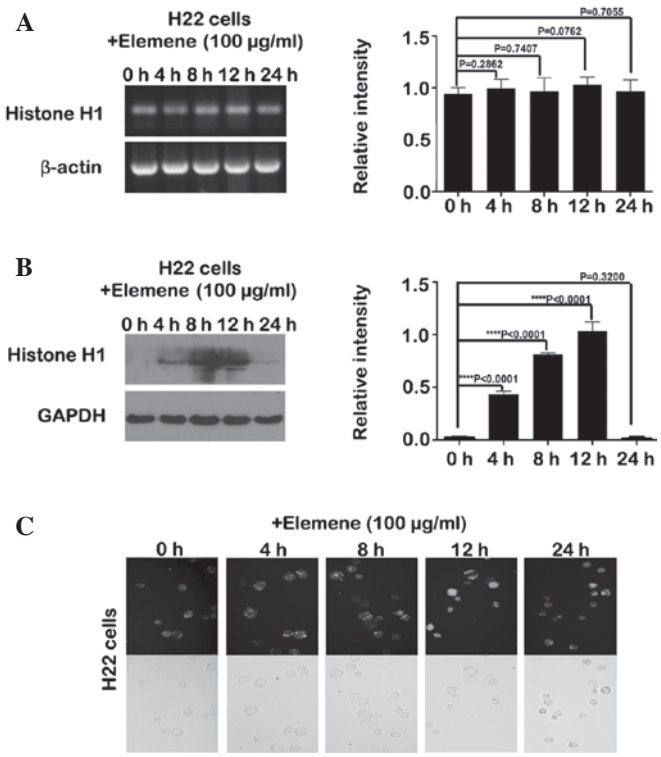

Figure 2. β-elemene induces an increased expression of histone H1 *in vitro*. (A) H22 cells were pre-treated with β-elemene (100 μg/ml) and RT-PCR-amplified products were separated on agarose gel. We processed products for RT-PCR analysis at the indicated time-points (0, 4, 8, 12 and 24 h). Densitometric analysis of histone H1 bands was normalized to β-actin. Results are shown as the means ± SD (n=4). (B) H22 cells were pre-treated with β-elemene (100 μg/ml) and processed for western blot analysis at the indicated time-points (0, 4, 8, 12 and 24 h). Densitometric analysis of histone H1 bands was normalized to GAPDH. Results are shown as the means ± SD (n=4). (C) Following treatment of β-elemene (100 μg/ml), H22 cells were stained for histone H1 protein by FITC at the indicated time-points (0, 4, 8, 12 and 24 h). H22 cells were confirmed by fluorescence microscopy.

(Fig. 1). Histone H1 showed lower protein expression levels in H22 compared to P388D1 cells, whereas its gene expression levels were relatively higher. Immunofluorescence analysis revealed an abundant concentration of histone H1 not only in the karyotheca, but also on the membrane of H22 and P388D1 cells. Thus, histone H1 is expressed in H22 murine HCC cells.

β-elemene induces increased expression of histone H1 in H22 cells in vitro. To determine whether β-elemene regulates the expression of histone H1 in H22 cells, which may play an important role in inhibiting tumor cell growth or in leading to tumor cell apoptosis, H22 cells were randomized into six groups (~5x10^6 cells/ml/group). Each group was cultured in culture medium containing β-elemene with 100 μg/ml for set periods of time (0, 4, 8, 12 and 24 h), while the control groups were cultured in culture medium without β-elemene. The variation of expression of histone H1 after exposure to β-elemene *in vitro* was analyzed using RT-PCR (Fig. 2A), western blotting (Fig. 2B) and immunofluorescence (Fig. 2C). The RT-PCR results showed that the gene expression levels of histone H1 remained constant after treatment with β-elemene at different time-points, but the protein levels of histone H1 significantly increased after treatment with β-elemene. H22 cells were cultivated and it was found that there was a marked increase in the histone H1 expression at the 12 h point. Concomitantly, green fluorescence and histone H1 protein levels were significantly increased with β-elemene treatment up to 12 h (Fig. 2C). This indicates that β-elemene has obvious effects on histone H1 expression in a time-dependent manner in H22 cells.

Suppression effect of β-elemene on the growth of H22 cell-transplanted tumors in vivo. To confirm whether β-elemene increased the expression of histone H1 in the H22 murine HCC cell line, suggesting a new mechanism of the antitumor function of β-elemene, we analyzed whether β-elemene inhibited H22 tumor cell growth *in vivo*. For this purpose, an H22 HCC cell line xenograft transplantation model was constructed. We set up normal saline (NS) and 5-FU groups as control groups for β-elemene-treated groups. In tumor-bearing BALB/c mice, tumors of mice within the NS group grew rapidly and became significantly bigger compared to the elemene and 5-FU groups, while mice in the NS group died within 15 days. Consistent with the significant regression of tumor growth in the mice treated with 100 and 50 mg/kg elemene and 5-FU, respectively, these drug groups showed higher survival rates than the control group (Fig. 3A). The β-elemene-induced reductions of tumor weight were progressive during the period of the experiment. The mice tumor weight after treatment with β-elemene or 5-FU was decreased, but there were no significant differences between the different drug-treated groups (Fig. 3B). The IRs of the β-elemene-treated groups ($IR_{20\ mg/kg\ 5\text{-}FU}$ = 50.9%; $IR_{100\ mg/kg\text{-}elemene}$ = 52.71%; $IR_{50\ mg/kg\text{-}elemene}$ = 44.77%) were higher than those of the physiological saline control group ($p<0.05$)

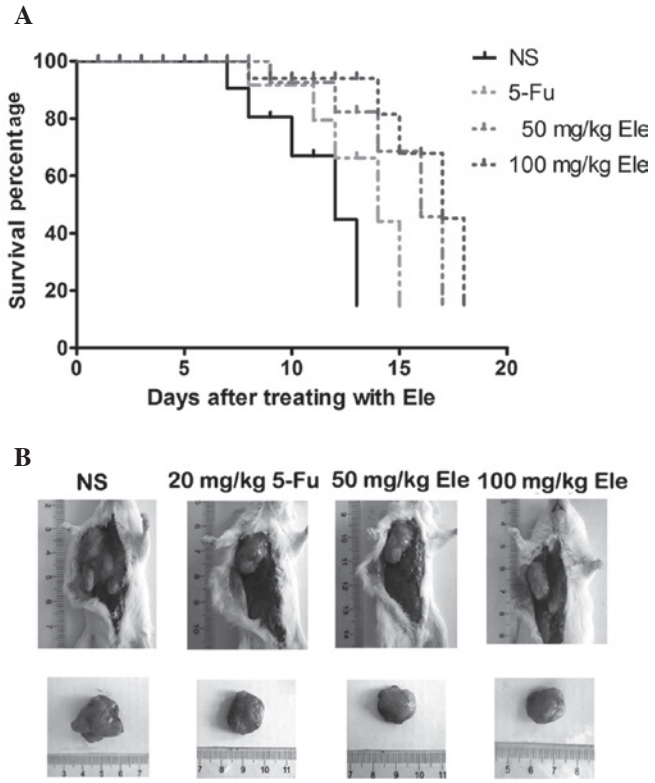

Figure 3. Suppression effect of β-elemene on the growth of H22 cell-transplanted tumors *in vivo*. (A) Comparison of the survival percentage of β-elemene (50 and 100 mg/kg) in the 5-FU treatment group and the NS control group. Values are the means ± SD (n=5; *p<0.05). (B) Tumors in the drug-treated group (50 and 100 mg/kg and 5-FU) were significantly smaller than those in the NS control group.

Table I. Inhibition rate (IR) of all the drug-administered groups.

Group	Tumor weight (g, $\bar{\chi}$ ± s)	IR (%)
Nacl	2.77±0.38	-
5-FU	1.36±0.43	50.90[a]
β-elemene (100 mg/kg)	1.31±0.28	52.71[a]
β-elemene (50 mg/kg)	1.53±0.65	44.77[a]

n=5; [a]p<0.05. All the drug-administered groups showed significant inhibition of tumor growth compared to the control group ([a]p<0.05). There was no significant difference between the IR of the β-elemene 100 mg/kg group and the β-elemene 50 mg/kg group (p>0.05). The group receiving 100 mg/kg of β-elemene showed the highest IR.

(Table I). However, the IR of the 100 mg/kg β-elemene-treated group was not significantly different from that of the 20 mg/kg 5-FU-positive control group (p>0.05).

β-elemene induces increased expression of histone H1 in H22 cells in vivo. Histone H1 was found to be increased *in vitro* (Fig. 2), thus we investigated whether this would also occur *in vivo*. Histone H1 expression in transplanted tumors of H22 cells was measured by RT-PCR, western blotting and immunohistochemistry. The results of RT-PCR (Fig. 4A) revealed

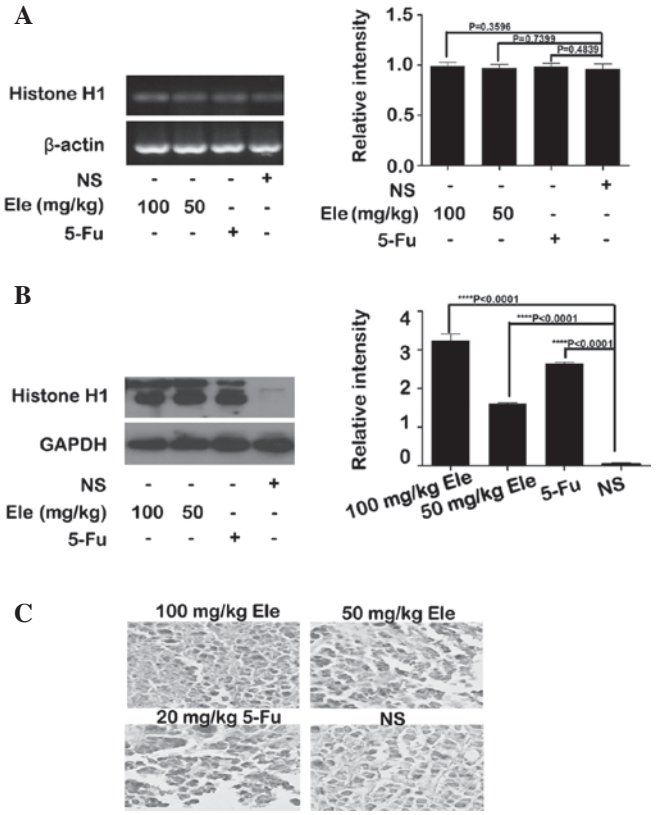

Figure 4. β-elemene induced a decreased expression of histone H1 *in vivo*. H22 cells were injected subcutaneously into the abdominal cavity of the mice. Following treatment with β-elemene (50 and 100 mg/kg), 5-FU and NS, the carcinoma was separated carefully from the abdominal cavity for analysis of histone H1. (A) RT-PCR analysis of histone H1 in the β-elemene (50 and 100 mg/kg), 5-FU and NS groups (top panel); the β-actin loading control is shown in the bottom panel. Densitometric analysis of histone H1 bands was normalized to β-actin. Results are shown as the means ± SD (n=4). (B) Western blot analysis of histone H1 in the β-elemene (50 and 100 mg/kg), the 5-FU and NS groups (top panel); the β-actin loading control is shown in the bottom panel. Densitometric analysis of histone H1 bands was normalized to GAPDH. Results are shown as the means ± SD (n=4). (C) The expression of histone H1 on H22 cells in the β-elemene (50 and 100 mg/kg), the 5-FU and NS groups was determined by immunohistochemistry (magnification, x400).

that histone H1 expression in transplanted tumors of H22 cells had similar levels when comparing the 5-FU control to the β-elemene-treated groups. Moreover, compared to the NS group, we found that the NS control group had lower expression levels. The western blot data (Fig. 4B) shows that increased levels of histone H1 were detected after treatment with 100 and 50 μg/ml β-elemene and in the 5-FU control groups, and this effect was confirmed by immunohistochemistry (Fig. 4C). We also proved that different concentrations of β-elemene have different effects. The 100 μg/ml-β-elemene group was more effective than the 50 μg/ml and the 5-FU groups in inducing increased levels of histone H1. Based on these data, we proved that β-elemene also increased the expression of histone H1 *in vivo* in a dose-dependent manner.

Discussion

In this study, we have demonstrated for the first time that enhancing the expression of histone H1 may be one of the

mechanisms of the antitumor effects of β-elemene. In the PCR test, there was no obvious difference of the histone H1 expression level following the different concentrations of β-elemene injection in the *in vivo* and *in vitro* experiments. In the western blot/agarose gel electrophoresis test, however, a positive correlation was revealed between the β-elemene concentration and the control groups. These findings may indicate that β-elemene affects histone H1 expression only at the protein, and not at the gene, level. Specifically, β-elemene influences histone H1 only at the protein level, without changing the base composition or proportion or intrinsic structure of DNA.

Histone H1 is involved in the nucleosome positioning and formation of the higher-order chromatin structure. Histone H1-containing chromatin shows a strong inhibition of nucleosome sliding and is more resistant to nuclease digestion (20). Consequently, histone H1 is regarded as a structural component associated with chromatin compaction and inaccessibility to transcription factors or RNA polymerase. Although originally thought to be a general repressor of transcription, histone H1 has since been shown to have more specific effects on RNA pol II and pol III transcription. A number of studies have demonstrated an inhibitory effect of histone H1 on RNA pol II or pol III transcription (21-23). Pol III directs transcription of small non-coding RNAs that are involved in translation, splicing and other cell processes. Transcription by pol III is closely regulated in normal cells, but this regulation is lost during tumorigenesis. Thus, pol III transcription is negatively regulated in normal cells by tumor suppressor gene products, such as Rb, p53 or PTEN or other factors (MAF1), and activated via signal transduction cascades (24). Previous studies have indicated that an enhanced pol III transcription is required for cell growth and transformation by the MYC oncogene (25). In our study, after treatment with β-elemene, notable differences in histone H1 concentration were observed in western blotting. Compared to the control group, the experimental group had a significantly high level of histone H1. Enhanced histone H1 may cause the chromatin structure to remain condensed and stabilize the higher-order chromatin structure; thus, histone H1 may be regarded as a general repressor of chromatin activity. However, enhanced histone H1 may have positive effects on the functions of tumor suppressor gene products or signal transduction cascades, which suppress the transcription of pol III and block the actions of some oncogenes.

Footprinting analysis and *in vitro* binding assays (26,27) showed that the chromatin binding sites of histone H1 are similar to those of the high mobility group (HMG) proteins, and suggested that histone H1 and HMG proteins compete for the same binding sites.

As with histone H1, HMG is also a chromatin-binding protein. It regulates the gene expression by modulating the compactness of the chromatin fiber and affecting the ability of regulatory factors to access their nucleosomal targets. Histone H1 stabilizes the higher-order chromatin structure and decreases nucleosomal access, while the HMG proteins decrease the compactness of the chromatin fiber and enhance the accessibility of chromatin targets to regulatory factors. The HMG superfamily comprises three families: HMGB, HMGA and HMGN. There is a network of dynamic and competitive interactions involving HMG proteins, and histone H1 constantly modulates nucleosome accessibility and the local structure of the chromatin fiber (28). As an important membrane of the HMG family, HMGB1 is important in cancer development and metastasis, with RAGE-HMGB1 signaling promoting the spread of most tumor types. The serum HMGB1 levels in patients with hepatocellular carcinoma (84.2±50.4 ng/ml) were significantly higher than those with chronic hepatitis (39.8±10.5 ng/ml), liver cirrhosis (40.2±11.6 ng/ml) or healthy controls (7.0±5.9 ng/ml, p=0.0001, respectively) (29). The downregulation of HMGB1 expression resulted in apoptosis in LNCaP prostate cancer cells (30). Following treatment with β-elemene, enhanced histone H1 may have negative effects on the expression of HMGB1. Additionally, the downregulation of HMGB1 expression inhibited the development of H22 cells. Thus, H22 cell growth is negatively influenced.

There are also other ways to explain the antitumor effect of histone H1. Class *et al* (31) suggested that histone H1 cytotoxicity is based on the presence of histone H1-binding proteins on the cell surface, which trigger cellular responses, such as apoptosis. Widlak *et al* (32) showed that the C-terminal domain of histone H1 activates the apoptotic nuclease DNA fragmentation factor DFF40/CAD via protein-protein interactions. Moreover, the entire histone H1 molecule may be required to obtain the observed cellular response, since different peptides derived from histone H1 have no inhibitory effect (31).

Taken together, these data suggest a new mechanism of β-elemene. β-elemene may cause the chromatin structure to remain condensed and suppress the transcription of pol III by upregulating the levels of histone H1. However, it may inhibit the expression of HMG proteins, which have positive effects on cancer development. Both of these functions are responsible for the antiproliferative effect of β-elemene on H22 cells.

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