Carnosol Induces Cell Apoptosis and Inhibits Invasion and Proliferation in Skin Epidermoid Cancer in Vitro Through MMP11-EGFR Pathway

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Research

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Abstract

Background and purpose: The anti-tumor effect of carnosol has been revealed in cancers. This research intends to explore the role and underlying mechanisms of carnosol in the skin cancer in vitro.

Methods: Online database GEPIA was used to preliminarily analyze MMP11 expression in skin cancer and further performed overall survival evaluation based on TCGA data. 0, 5μM, 10 μM, 20 μM carnosol was added into the skin cancer A431 cell culture. Later, RT-PCR method detected the mRNA expression of MMP-11 and Elisa kits measured the concentrations of MMP11, phosphor-EGFR and total EGFR proteins as well as the biomarkers related to proliferation (Ki67) and EMT (E-cadherin and Vimentin) as well as Casepase-3. The 20μM carnosol group was selected to further study the regulatory effect of MMP11 in A431 cells.Colony formation examined the cell proliferation. Flow cytometry method checked cell apoptosis while Transwell method explored the cell invasion and migration.

Results: MMP11 is upregulated in skin cancer and lower level of MMP11 or EGFR expression is correlated with higher overall survival rates. Carnosol addition inhibited the mRNA expression of MMP11 and lowered the protein concentrations of MMP11 and phosphor-EGFR. In addition, Ki67 and Vimentin concentration was inhibited by carnosol while E-cadherin was instead promoted. Caspase-3 activity was enhanced by carnosol. Upregulation of MMP11 recovered EGFR activation, cell proliferation and invasion while inhibited apoptosis, partly counteracted the function of carnosol in cells.

Conclusion: Carnosol might induce cell apoptosis and inhibits invasion and proliferation in skin epidermoid cancer in vitro through MMP11-EGFR pathway.

Introduction

Skin cancer is normally divided into two types including squamous cell carcinoma, which originates from spinous layer and leads to 80–85% of the overall non-melanoma skin cancers and, basal cell carcinoma that originates from basal layer of epidermis and leads to 15–20% of the overall non-melanoma skin cancer [1, 2]. The most common cause for skin cancer genesis is excessive exposure to UV rays [3]. Although laser therapy, cryotherapy, radiotherapy, and immunotherapy are available for patients, all the therapies have adverse effects and also the cost of treatment is very high. Therefore, to explore the efficient and cost-effective treatments of skin cancer is of urgent need.

Previous research reported natural essence derived from the plants ,resveratrol, brassinin, withanolides and sulforaphane as potentially effective drug for cancer treatment as well as chemoprevention [4]. Carnosol, an active diterpene found in sage and rosemary, has been discovered as anti-oxidants and anti-inflammatory medication in osteoarthritic chondrocytes in vitro and vivo [5, 6]. Recently, carnosol exerted its anti-tumor role in UVB light-induced skin cancer via ROS/NF-κB pathway [7]. Carnosol was also reported to deter the carcinogenesis by downregulation of aryl hydrocarbon receptor (AhR)-dependent gene [8, 9].

Matrix metalloproteinase (MMP) family play important roles in various cancers and diseases by degrading the ECM and mediating cell invasion, proliferation and angiogenesis, etc[10].MMP2 and MMP9 were discovered to be associated with the occurrence of skin inflammation[11]. It was reported that MMP11, also named Stromelysin-3, is an initiator in skin cancer [12]. It was discovered that MMP11 is upregulated in neoplastic skin than normal skin in patients with non-melanoma cancer[13]. Similarly, RNA sequencing also indicated that in basal cell cancer tissues, MMP11 was notably correlated with this type of skin cancer[14]. However, there are no further studies that explore the specific functions and regulatory system of MMP11 in skin cancer. EGFR, Epidermal growth factor receptor, has been widely unveiled as an oncogenic gene in various cancers and a potential target for cancer therapies and in skin cancer A431 cells, EGFR inhibitors gefitinib, Cetuximab, have been shown in clinical trials to improve the efficacy when used together with radiotherapy with skin responses[15]. EGFR inhibitors have been clinically as an option for therapy among cancer patients although the side effect is not nonnegligible including skin toxicity [15, 16]. With more options for EGFR inhibitors, skin reactions are less serious[17], which motivated researchers to find more suitable inhibitors for EGFR, which might help to ameliorate side effects. Previously, mounting evidence showed that MMP interact with EGFR in cancers including skin cancers[18] [19, 20], prostate cancer[21], esophageal cancer[22]. Therefore, we hypothesized that carnosol might regulate the cell phenotype through regulation of MMP11-EGFR. In this study, we investigated the in-vitro effect of carnosol in skin cancer and explored the underlying mediatory mechanism.

Methods

Bioinformatic analysis

GEPIA online database was utilized to preliminarily analyze the differential expression of MMP11 in skin cancer tissues compared to normal ones. Overall survival analysis was performed on GEPIA based on the shared TCGA data in groups (high MMP11 and low MMP11). In addition, the overall survival analysis was also done similarly on GEPIA with groups (high EGFR and low EGFR). Furthermore, the associations between MMP11 and EGFR expression in skin cancer patients are analyzed using Pearson's method.

Cell culture and transfection

Human epidermoid skin cancer A431 cells were purchased from ATCC, USA. The cells were cultured in DMEM with streptomycin (100 µg/ml), 10% FBS, and penicillin (100 units/ml), at 37^0C with 5% CO_2 (Beyotime, Shanghai, China). The absolute ethanol was used to prepare the stock solution of carnosol followed by the dilution of this stock solution with the growth medium to different concentrations for further use. More specifically, 96-well plate was used to seed (5×10^4 cells/well) and the culture medium was added with carnosol (5, 10, 20 µM) for 24h with normal A431 cells as a control group. Meanwhile, cells were transfected with sh-MMP11 or oe-MMP11 plasmids and then were treated with 20 µM for 24h with the cells skipped the transfection and directly underwent 20µM carnosol treatment for 24h as a

control group. Thereafter, EGFR inhibitor, Cetuximab (250µg/ml, Merck, USA), was added into the cell 20µM carnosol group and incubated for 48h.

Elisa assay

The cell supernatant was selected for Elisa methods to measure the protein changes in different groups of A431 cells. MMP11 ELISA Kit (Matrix Metallopeptidase 11 (Stromelysin 3), Human EGFR ELISA Kit (pY1086) (ab126440), Human EGFR (pY1045)/ total EGFR ELISA Kit (ab126437) and Human Ki67 Elisa kit(ab253221) are commercially designed by Abcam, USA. Human E-Cadherin ELISA Kit (ab233611), Human Vimentin ELISA Kit (ab173190) were used for EMT evaluation. Human Caspase 3, Casp-3 ELISA Kit (Cusabio, Shanghai, China) to detect Caspase-3 activity. The protocols were strictly followed during the detection process. All the groups were detected for three times repetitively.

RT-qPCR

TRIzol reagent (Sigma Aldrich, MO, USA) was employed for the extraction of total RNA from A431 cells and PrimeScript RT Master Mix Kit (Takara Bio, Goteborg, Germany) was used to reversely transcribed RNA into cDNA. The primers were amplified in a Step One Plus real time PCR system by using SYBR Premix Ex Taq II kit (Thermo Fisher, USA) and the primers are listed as below. MMP11, forward, 5'-GAGAAGACGGACCTCACCTACA-3', reverse, 5'- CTCAGTAAAGGTGAGTGGCGTC-3'. β-actin, forward, 5'-CACCATTGGCAATGAGCGGTTC-3', reverse, 5'- AGGTCTTTGCGGATGTCCACGT 3'Following conditions were used for the PCR cycling: 35 s at 95^0C, 50 s at 60^0C, 35 s at 70^0C (30 cycles) followed by the extension at 72^0C for 5 min. 1% of agarose gel was used for the analysis of reaction products and visualized by ethidium bromide. β-actin was taken as an internal control. $2^{-\triangle\triangle Ct}$ method was used to calculate the relative expression of MMP11 in different groups. All groups were evaluated at least for three times.

Colony formation assay

6-well plate was used to seed A431 cells (1×10^5 cells/well) from normal group, 20µM carnosol group, oeMMP11 group followed by 20µM carnosol treatment and EGFR as well as the one treated by both 20µM carnosol and EGFR inhibitor. After 14 days of culture, the plates were dried in air and photographed. In the control and treated group, the colonies were counted by Image J Software. All the procedure was repeated for three times.

Flow cytometry (FCM) assay

A flow cytometry by Annexin-FITC/PI (BD Biosciences, CA, USA) kit was used to analyze the apoptosis induction. The cells were washed with PBS twice and deferred in binding buffer (500 µl) followed by the staining with V-FITC (5 µl), PI (5 µl) and incubated for 5 min in the absence of light. FACScan flow cytometry (LabX, VA, USA) was used to analyze stained cells using statistics of the quadrants for necrotic

cell population, sorting out live, late apoptosis and early apoptosis. All the procedure was repetitive three times.

Transwell invasion experiment

Transwell chambers were prepared for this assay (Beyotime, Shanghai , China). Matrigel (Beyotime, Shanghai, China) was melt at 4℃ overnight diluted to 1mg/ml with serum-free medium on the ice. Then 100μl Matrigel was incubated for 4 h at 37℃Transwell chambers were used in this assay. Serum-free medium washed the digested cells for three times and the resultant cell suspension was added into each pore (100μl/pore). The lower chamber was added with 500μl medium which contained 20% FBS (Beyotime, Shanghai, China). After incubated for 24h, the Transwell chambers were washed using PBS for two times and fixed with 5% glutaral at 4℃. Crystal violet(0.1%) was added into the cell culture. The images were taken under the lab microscope.

Statistical analysis

Data was expressed as the mean ± SD. SPSS 19.0 (IBM, NY, USA) was employed for the data examination. One-way ANOVA followed by post-hoc analysis using Bonferroni's multiple comparisons test for the assessment among two or more groups. The general alpha is 0.05. Graphpad Prism (Graphpad Prism, CA, USA) examined the statistical analysis. All the procedure was repetitive three times.

Results

MMP11 presented higher expression in skin cancer and was correlated with lower survival rate in skin cancer patients

GEPIA online database was used to analyze differential expression of MMP11 in skin cancer tissues and normal tissues based on the TCGA data. It was shown that MMP11 expression was higher in the tumor group compared with the normal group (Fig. 1A). In addition, the overall survival rate is higher in the patient group with low MMP11 expression ($P < 0.05$, Fig. 1B). Furthermore, we also analyzed the association between EGFR expression and the overall survival rates among the skin cancer patients. It was unveiled low expression of EGFR in patients was in positive correlation with high overall survival rate ($P < 0.05$, Fig. 1C). Interestingly, on GEPIA, Pearson's correlation disclosed that MMP11 was correlated with EGFR expression in skin cancer patients ($P < 0.05$, $R = 0.11$, Fig. 1D).

Carnosol inhibited MMP11 expression and inactivated the EGFR signaling pathway in skin cancer cells

RT-PCR detected the mRNA expression changes of MMP-11 in the cell groups after treated with carnosol (0, 5,10,20μM) in A431 cells, which revealed that MMP11 was inhibited by carnosol in response to increasing concentrations (Fig. 2A). Elisa method was also used to observe the protein concentration in cells after treatment with different concentrations of carnosol, whose findings were in accordance with the MMP11 mRNA expression change (Fig. 2B). On the other hand, Elisa assays were conducted to

r1045)and total-EGFR proteins in cells. It was

also found that the phosphor-EGFR concentrations were significantly reduced as the increase of the carnosol addition (Fig. 2C).

Carnosol promoted the caspase-3 activity and EMT process and inhibited Ki67 in skin cancer cells

Elisa results showed that Ki67 protein concentration decreased with the increase of carnosol concentration (Fig. 3A). Similar, E-cadherin protein concentration was enhanced while Vimentin was inhibited as carnosol concentration increased (Fig. 3B), suggesting the inhibition of the EMT process. Furthermore, Caspase-3 activity was detected using the Caspase-3 activity kit and it was displayed that the activity of Caspase-3 was promoted with the rise of carnosol concentration added in cells (Fig. 3C).

MMP11 downregulation inactivated EGFR signaling leading to the Ki67 inhibition, EMT prevention and higher Caspase-3 activity

The A431 cells were transfected to upregulate or reduce the expression of MMP11 or cultured with EGFR inhibitor Cetuximab and then the cells were treated with 20uM carnosol, divided into 4 sub-groups, 20uM (as a control), 20uM + oe-MMP11, 20uM + sh-MMP11 and 20uM + Cetu. RT-PCR detected the mRNA expression of MMP11 in each group. Elisa methods were used to detect the concentration changes of proteins including MMP-11, phospho-EGFR(TyR1068), phosphor-EGFR(TyR1045), total EGFR, Ki67, E-cadherin and Vimentin. Our findings demonstrated that MMP11 mRNA expression and protein concentration was effectively inhibited in the cells transfected with sh-MMP11 and upregulated in the cells transfected with oe-MMP11(Fig. 4A-B). Also, the addition of EGFR inhibitor Cetuximab also contributed to the significant decrease of MMP11 in cells (Fig. 4A-B). Furthermore, the impact of MMP11 on the EGFR signaling was disclosed and it was displayed that the increase of MMP11 in cells led to the increase of phospho-EGFR (TyR1068 & TyR1045), signifying the activation of EGFR signaling (Fig. 4C). On the contrary, the inhibition of MMP11 was correlated with the inactivation of EGFR signaling (Fig. 4C). The curbing effect of EGFR inhibitor on the EGFR signaling was also verified by Elisa method (Fig. 4C). Furthermore, the down-regulation of MMP-11 or the introduction of EGFR inhibitor, resulted in the inhibition of Ki67 (Fig. 4D) and Vimentin and promoted the E-cadherin in protein concentrations (Fig. 4E). Caspase-3 activity in cells were elevated by the inhibition of MMP11 or EGFR inhibitor (Fig. 4F).

Carnosol induced cell apoptosis and inhibited proliferation and invasion and upregulation of MMP11 counteracted the effect of carnosol

The addition of 20uM carnosol inhibited the cell colonies while the upregulation of MMP11 in cells led to an opposite effect in colonies using Colony formation method (Fig. 5A). Transwell method evaluated the invasion ability of the cells and discovered that carnosol inhibited the cell invasion and upregulation of MMP11 in cells suppressed the effect of carnosol notably (Fig. 5B). Furthermore, apoptosis rates of cells were confirmed to be enhanced by carnosol and reversed by the involvement of MMP11 upregulation in cells (Fig. 5C).Taken together, These findings suggested that carnosol induced cell apoptosis and promoted cell proliferation and invasion through MMP11 regulation.

Discussion

In this article, we first pointed out the downregulation of MMP11 is associated with the higher survival rate of skin cancer patients. Then we performed a series of cellular experiments to disclose the potential role of carnosol in skin cancer cells, A431, which showed that carnosol inhibited MMP11, EGFR activation, Ki67 and added to the Caspase-3 activity. MMP11 inhibition could lead to the inactivation of EGFR signaling, downregulation of Ki67 protein, and inhibition of EMT process. Also, it is presented in this study that EGFR inhibitor Cetuximab could suppress MMP11, Ki67 and EMP process in A431 cells. Furthermore, we also unveiled that carnosol could reduce the cell proliferation and invasion but promote the apoptosis while MMP11 upregulation could reverse the effect of carnosol. Taken together, it is suggested that carnosol might inhibit the cell proliferation and invasion and induce the apoptosis by downregulating MMP11 via EGFR signaling pathway.

Previously, carnosol has been reported as a potential chemo-preventive drug against UVB radiation, which could induce skin cancer [7]. Carnosol, as a natural polyphenol, inhibited the cell invasion, migration and proliferation in breast cancer[23]. It was reported as well that carnosol induced ROS, resulting in the inhibition of osteosarcoma in cellular level[24]. In addition, carnosol could notably inhibit MMP3, IL-6 and NO production in human osteoarthritic chondrocytes[6]. In melanoma cells, carnosol was reported to suppress MMP9 via NF-Kβand c-Jun[25]. In macrophages and vascular smooth muscle cells, the involvement of carnosol inhibited the MMP9 activity[26]. However, there has been no record on carnosol and MMP11 in skin cancer. Therefore, we analyzed the differential expression of MMP9 in skin cancer tissues compared to normal ones based on the previous data in GEPIA and found that MMP11 expression was abnormally enhanced in skin cancer, which is in accordance with previous study[13]. Furthermore, the overall survival rate among skin cancer patients with higher MMP11 was significantly lower than that in the ones with lower MMP11. MMPs are also reported to promote the cell growth and invasion in skin cancer cells[12]. Previously, mounting proofs pointed out that MMPs are involved with the EMT process and also cell invasion related to EMT in hepatocellular carcinoma[27]. EMT, short for epithelial-to mesenchymal transition, features the increase of mesenchymal proteins (Vimentin) and decrease of epithelial markers (for instance, E-cadherin)[28]. In cancers, EMT process is closely associated with tumor malignancy[28, 29]. In this study, we further confirmed that carnosol could reduce MMP11 in A431 cells and deter the cell proliferation and invasion, Ki67 and EMT and induce apoptosis while upregulation of MMP11 could partly reverse the effect of carnosol. Hence, we concluded that carnosol inhibited the cell proliferation, invasion and EMT and induced apoptosis in A431 cells probably through the modulation of MMP11.

EGFR is a critical therapeutic target in cancer. In clinical stage, the anti-EGFR therapy trials in colorectal cancer[30], biliary cancer[31], head and neck cancer[32], non-melanoma cancer[33], lung cancer[34], etc. have been performed. In this research, we examined the interplay between MMP11 and EGFR, which supported that the inhibition of MMP11 could inactivate the EGFR signaling by reducing the phosphorylated EGFR EGFR pathway, resultantly leading to the

phenotype change of A431 cells. Meanwhile, this also implied that carnosol or MMP11 might be a potent modulator of EGFR in skin cancer cells, yet it is early to conclude that carnosol could be a natural drug in skin cancer treatment before further animal and clinical studies are involved.

Conclusion

Carnosol might induce cell apoptosis and inhibits invasion and proliferation in skin epidermoid cancer in vitro through MMP11-EGFR pathway.

Abbreviations List

matrix metalloproteinase 11,MMP11; epidermal growth-factor receptor EGFR; EMT, epithelial-mesenchymal transition; extracellular matrix, ECM; epidermal growth factor receptor, EGFR;

Declarations

Acknowledgements

Author contributions

A - Study Design - Shiqiu Jiang Hairong Liu Jie Zhang

B - Data Collection - Shiqiu Jiang Hairong Liu

C - Statistical Analysis - Jie Zhang

D - Data Interpretation -Fang Zhang

E - Manuscript Preparation - Shiqiu Jiang

F - Literature Search - Jiawei Fan

G - Funds Collection - Hairong Liu

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Data Statement

All data, models, and code generated or used during the study appear in the submitted article.

Conflicts of interest

None

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Figures

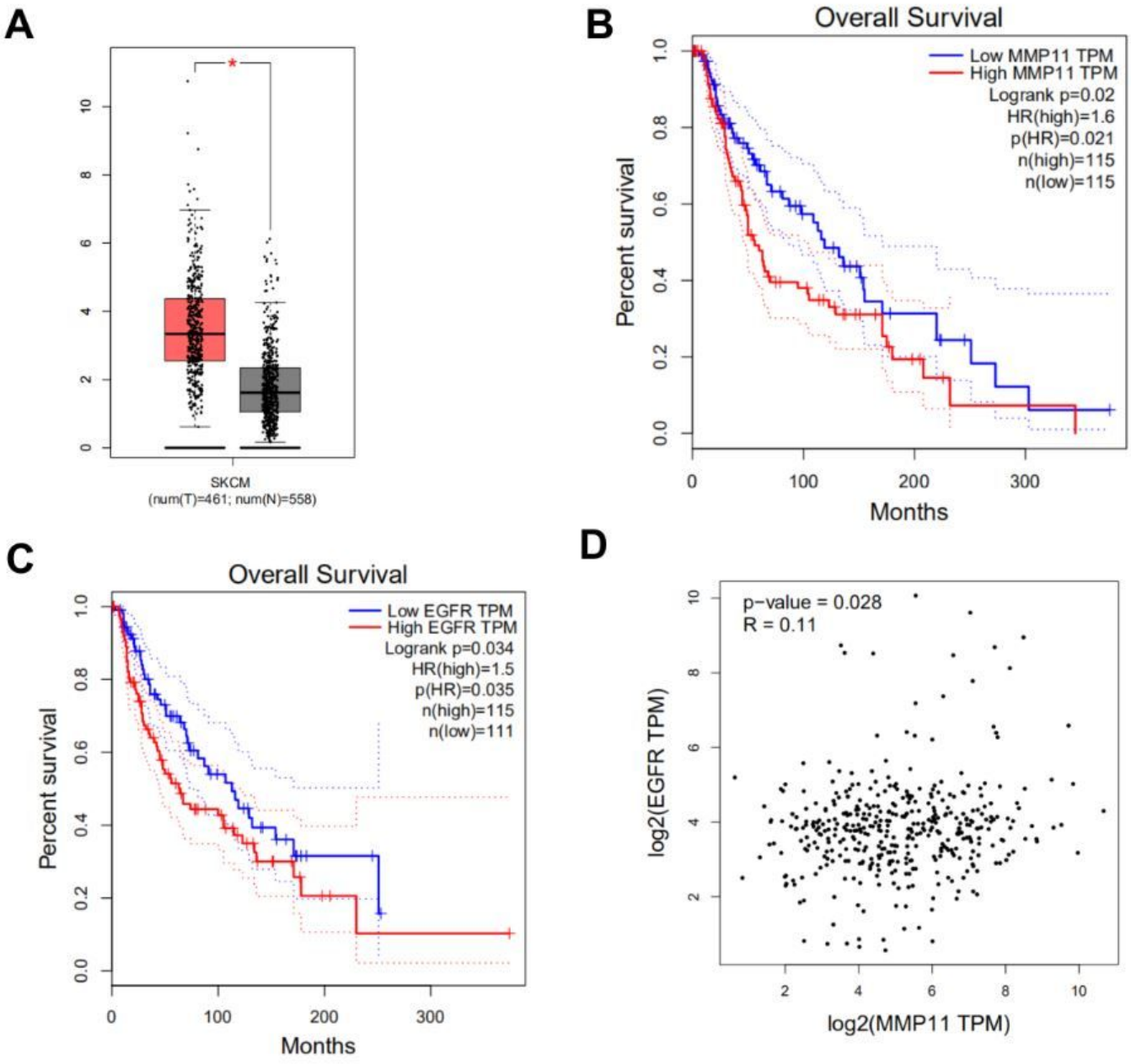

Figure 1

MMP11 presented higher expression in skin cancer and was correlated with lower survival rate in skin cancer patients. A. GEPIA online tool was utilized to analyze the differential expression of MMP11 in skin cancer tissues and normal tissues. B. Overall survival was analyzed on GEPIA in measuring the correlations between MMP11 expression and overall survival rate of skin cancer patients and between EGFR expression and overall survival rate. D. Pearson's correlation was performed to evaluate the association between the MMP11 expression and the expression of EGFR in skin cancer patients.

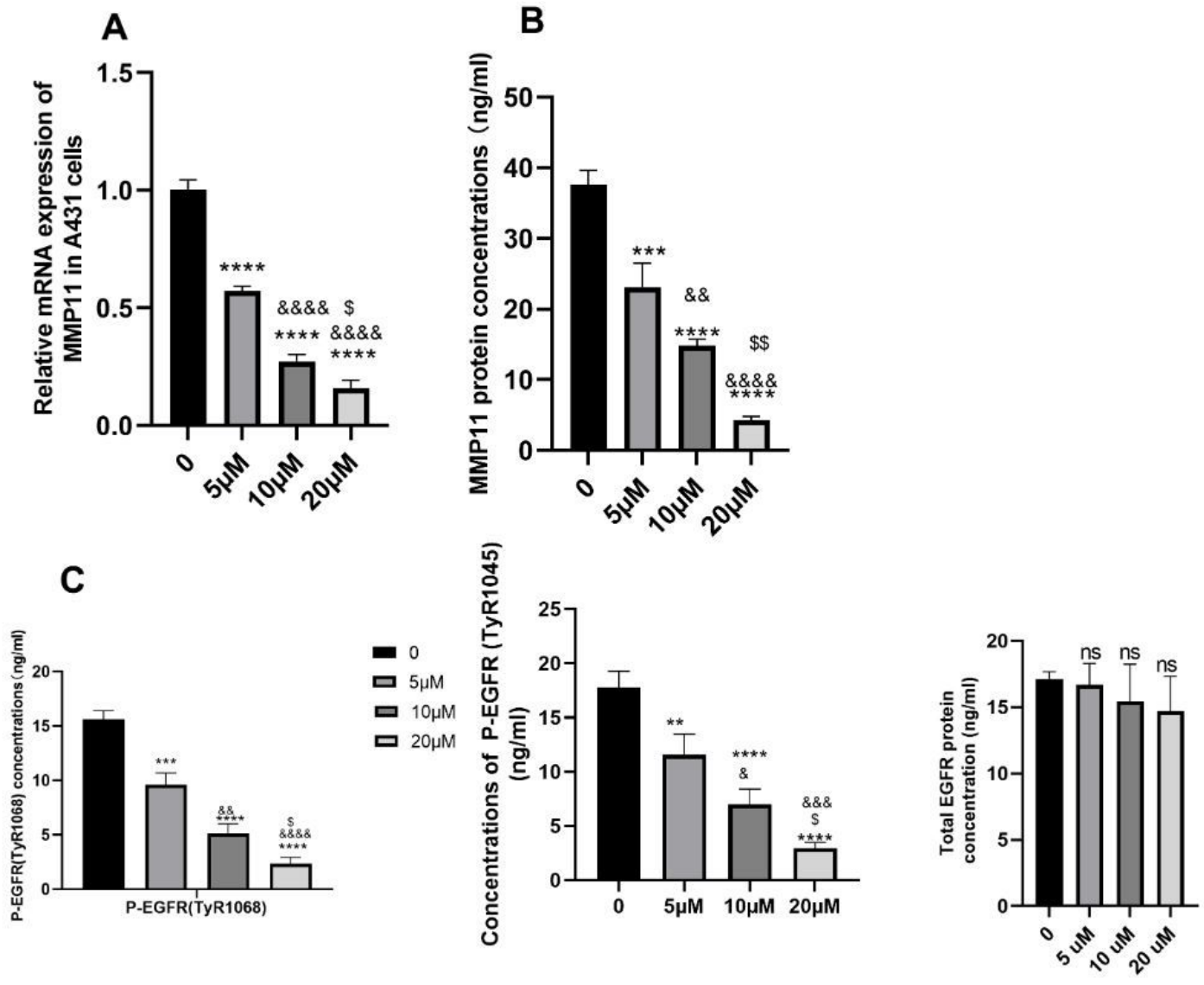

Figure 2

Carnosol inhibited MMP11 and inactivated the EGFR signaling pathway A．RT-PCR examined the mRNA expression of MMP11 in cells treated by 0, 5, 10, 20uM carnosol. B-F. Elisa method measured the protein concentrations of MMP11, phospho-EGFR (Tyr1086), phosphor-EGFR（Tyr1045）and total-EGFR proteins. All the experiments were repeated for three times. Results were compared within every other group using One-way ANOVA method along with post-hoc analysis using Bonferroni's correction. Vs 0uM group ****P<0.0001, ***P<0.001, **P<0.005, *P<0.05. Vs 5uM Group, &&&&P<0.0001, &&&P<0.001, &&P<0.01, &P<0.05. Vs10uM, $P<0.05.

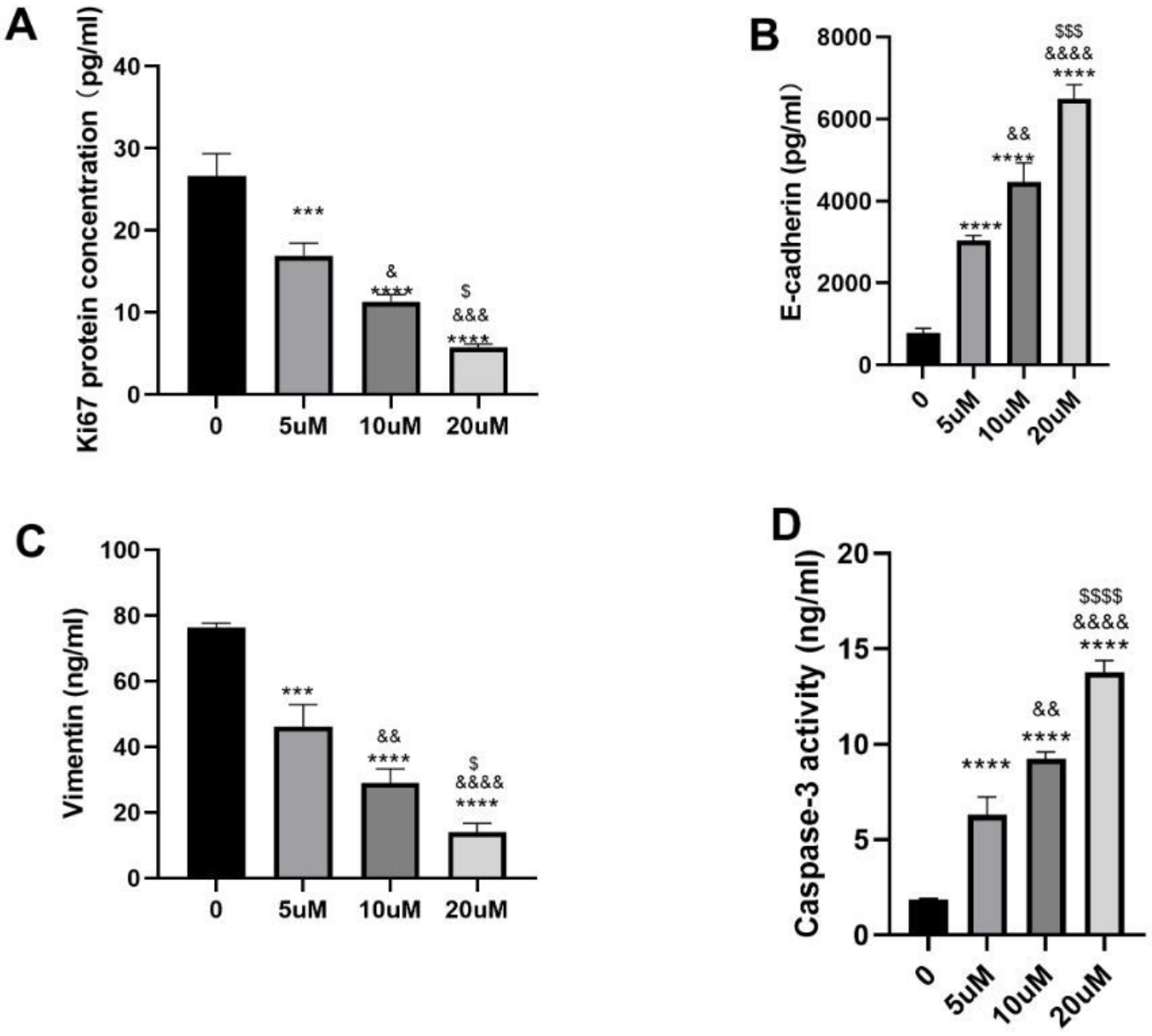

Figure 3

Carnosol promoted the caspase-3 activity and EMT process and inhibited Ki67 in skin cancer cells A-B Elisa methods analyzed Ki67, E-cadherin and Vimentin protein concentrations in the A431 cells treated by different concentrations of carnosol (5, 10, 20uM) with the non-treated group as a control. C. Caspase-3 activity was detected using the Caspase-3 activity assay kit. All the experiments were repeated for three times. Results were compared within every other group using One-way ANOVA method along with post-hoc analysis using Bonferroni's correction. Vs 0uM group, ****P<0.0001, ***P<0.001, **P<0.005, *P<0.05. Vs 5uM Group, &&&&P<0.0001, &&&P<0.001, &&P<0.01, &P<0.05. Vs10uM,

P<0.0001,

$$\$P < 0.001,$$

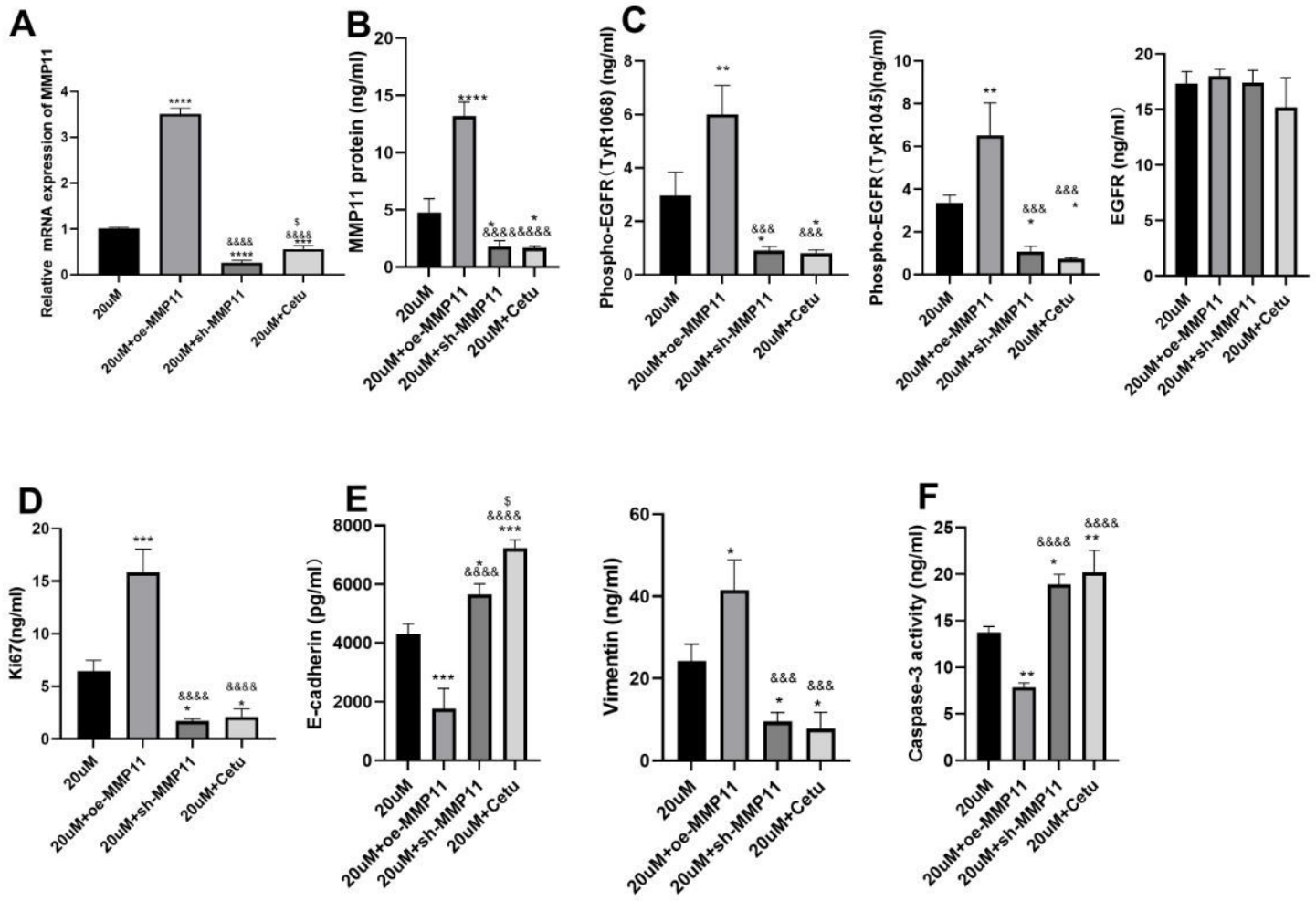

Figure 4

MMP11 downregulation inactivated EGFR signaling leading to the Ki67 inhibition, EMT prevention and higher Caspase-3 activity The A431 cells were transfected to upregulate or reduce the expression of MMP11 or cultured with EGFR inhibitor Cetuximab and then the cells were treated with 20uM carnosol, divided into 4 sub-groups, 20uM (as a control), 20uM+oe-MMP11, 20uM+sh-MMP11 and 20uM+Cetu. A.RT-PCR detected the mRNA expression of MMP11 in each group. B-E.Elisa methods were used to detect the concentration changes of proteins including MMP-11, phosphor-EGFR(TyR1068)、phospho-EGFR(TyR1045), total EGFR, Ki67, E-cadherin and Vimentin. F. Caspase-3 activity was detected using Caspase-3 assay kit in each group. All the experiments were repeated for three times. Results were compared within every other group using One-way ANOVA method along with post-hoc analysis using Bonferroni's correction. Vs 0uM group ****P<0.0001, ***P<0.001, **P<0.005, *P<0.05. Vs 5uM Group, &&&&P<0.0001, &&&P<0.001, &&P<0.01, &P<0.05. Vs10uM, $$P<0.01. $P<0.05.

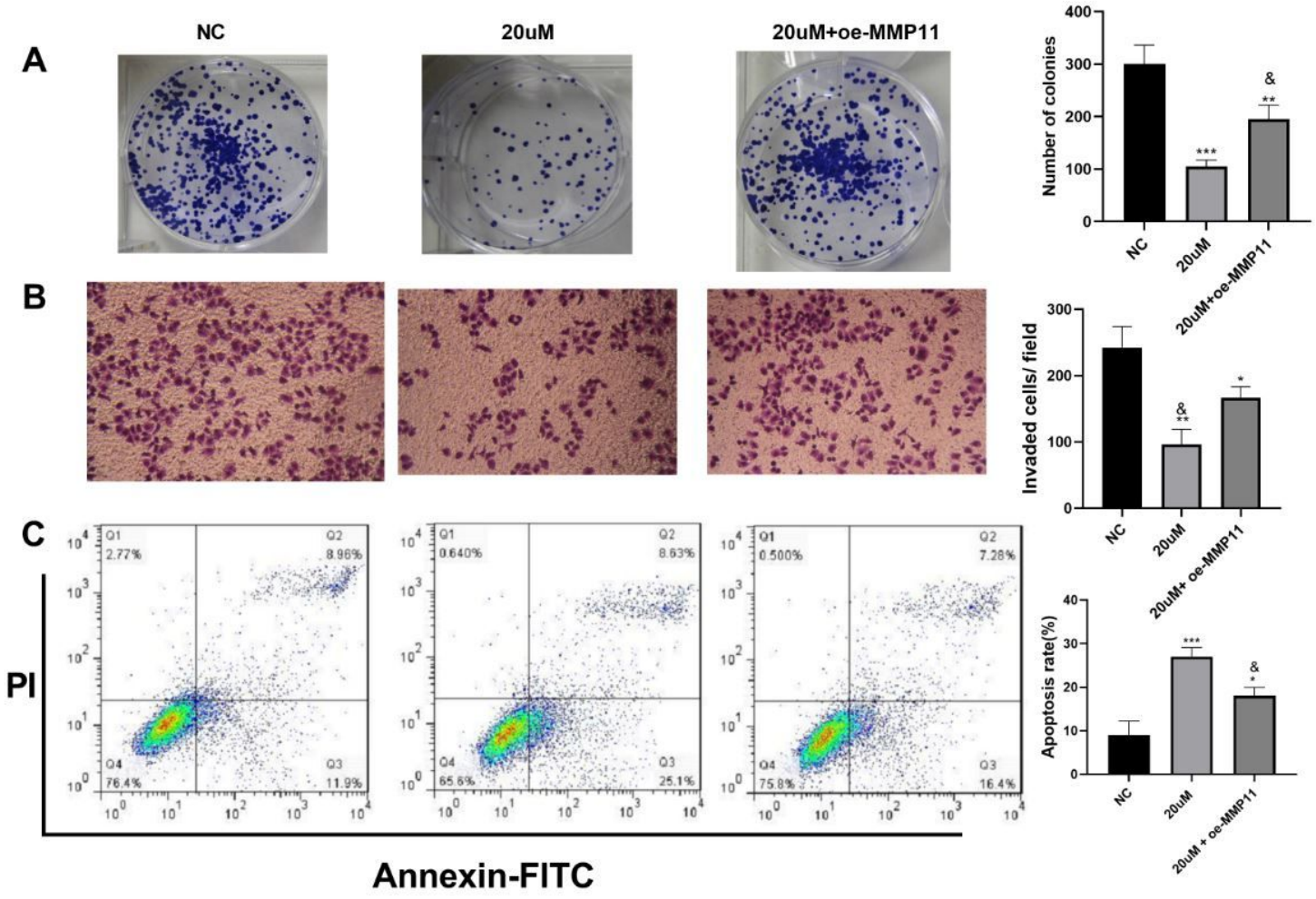

Figure 5

Down-regulation of MMP11 inhibited the cell proliferation and invasion and induced cell apoptosis Cells from the group that didn't undergo drug treatment or gene regulation, the one directly treated with 20uM carnosol and the one transfected with sh-MMP11 and then treated with carnosol were collected for functional analysis. A. Colony formation measured the colonies in each group. B. Transwell method was used to detect the invasion ability of cells from each group. C. Flow cytometry method observed the cell apoptosis in each group. All the experiments were repeated for three times. Results were compared within every other group using One-way ANOVA method along with post-hoc analysis using Bonferroni's correction. Vs NC group, ***P<0.001, **P<0.005, *P<0.05. Vs 20uM group, &P<0.05.