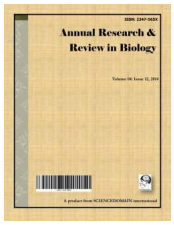

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All-Trans Retinoic Acid Downregulates Epidermal Growth Factor Receptor Mediated Regulation of Matrix Metalloproteinase-2 in B16F10 Murine Melanoma Cells

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Authors' contributions

The work was carried out by both the authors who have been listed according to their contributions. Both authors have read and approved the final manuscript.

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ABSTRACT

Aims and Study Design: Epidermal growth factor receptor (EGFR) regulates a number of cellular processes, including cell motility, proliferation, differentiation and survival. EGFR mediated signal transduction via mitogen activated protein kinase (MAPK) modulates expression and activity of matrix metalloproteinases (MMPs). Elevated expression and activity of MMP-2 strongly correlates with increased tumour invasiveness. As numerous studies indicate that all-trans retinoic acid (ATRA) has considerable anti-tumorigenic potential, the effect of ATRA on EGFR mediated regulation of MMP-2 via MAPK was studied in this paper using the highly metastatic murine melanoma cell line B16F10 as a model.
Methodology: B16F10 cells were cultured in the presence of ATRA (20 μM) for 6, 15 and 24 hrs. EGFR expression and phosphorylation and p38MAPK expression were assayed by Western blot. MMP-2 activity was assayed by gelatin zymography of culture supernatants. MMP-2 and TIMP-2 mRNA expression were assayed by RT-PCR. DNA fragmentation was used to assay the efficacy of ATRA in causing apoptosis.

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Results: Treatment of B16F10 murine melanoma cells with ATRA (20 µM) for 24 hrs led to an appreciable downregulation of EGFR phosphorylation and expression of EGFR and p38MAPK. Treatment of B16F10 cells with ATRA also inhibited MMP-2 activity and downregulated transcription of MMP-2 while transcription of TIMP-2 was upregulated. Treatment with ATRA did not show appreciable fragmentation of DNA.
Conclusion: Downregulation of EGFR expression and phosphorylation and EGFR mediated signal transduction through p38 MAPK could lead to downregulation of MMP-2 transcription. Downregulation of MMP-2 transcription and upregulation of transcription of the MMP inhibitor TIMP-2 could result in loss of MMP-2 activity. Loss of MMP-2 activity would render cells less metastatic. Our findings indicate that treatment with ATRA can inhibit EGFR mediated regulation of MMP-2 activity in B16F10 murine melanoma cells. Such inhibition could have therapeutic potential in clinical management of tumours.

Keywords: All-trans retinoic acid (ATRA); melanoma; epidermal growth factor receptor (EGFR); matrix metalloproteinase-2 (MMP-2); tissue inhibitor of metalloproteinases-2 (TIMP-2); mitogen activated protein kinase (MAPK).

1. INTRODUCTION

The retinoids, a class of chemical compounds that include vitamin A, its analogues and its metabolites, modulate several cellular processes, including proliferation, differentiation and apoptosis [1-4]. Retinoic acid is considered to be the biologically most active form of vitamin A and all-trans retinoic acid (ATRA) and its stereoisomer 9-cis retinoic acid are involved in the regulation of a number of biological processes. ATRA has been shown to suppress tumour formation in animals, inhibit proliferation of tumour cells *in vitro* and, in combination with chemotherapeutic drugs, is used for the treatment of acute promyelocytic leukemia (APL) [1-5]. The anti-tumorigenic effects of ATRA are usually attributed to induction of differentiation or apoptosis [1-3,6]. However, despite a number of experiments indicating its anti-tumorigenic potential, the effects of ATRA on solid tumours and the molecular mechanisms by which such effects are exerted remain largely to be elucidated in detail.

Epidermal growth factor receptor (EGFR), a 170 kDa transmembrane glycoprotein, is a member of the ErbB family of tyrosine kinase receptors. A number of ligands bind to and activate EGFR, including epidermal growth factor (EGF), transforming growth factor–α (TGF-α), betacellulin, heparin-binding EGF and epiregulin. [7-9]. The binding of ligands to EGFR leads to receptor dimerization, followed by internalization of the dimerized receptor and autophosphorylation of EGFR tyrosine kinase domains. Phosphorylation of EGFR stimulates activation of intracellular signal transduction cascades. The Ras-Raf/ mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) pathway and phosphatidylinositol 3' kinase (PI3K)/ Akt pathway are two major routes for EGFR mediated signalling [7-9]. EGFR mediated signalling plays important roles in regulation of gene expression, cell proliferation, cell migration, angiogenesis and apoptosis and has also been associated with tumour invasion and metastasis [7,8,10,11]. EGFR is overexpressed in a number of solid tumours and tumour cell lines, including breast cancers, ovarian cancers, squamous cell carcinomas of the head and neck and melanomas [7,8,12-14]. A number of reports indicate that EGFR is involved in progression and metastasis of melanomas and increased EGFR expression correlates with a worse prognosis and shortened survival [14,15]. Aberrant EGFR expression and phosphorylation has been shown to play a key role in tumour development by regulating malignant progression, metastatic potential, apoptosis and angiogenesis [7,8,12-14].

Numerous studies indicate that elevated expression and activity of matrix metalloproteinases (MMPs), a family of zinc dependant endopeptidases, strongly correlates with increased metastatic potential, tumour spread and poor prognosis in a number of cancers including melanomas and breast, lung, thyroid, oral, stomach and colon carcinomas [16-20]. MMPs play multiple roles in promoting tumour progression by regulating cell growth, survival, migration, invasion and angiogenesis [16-20]. Among the MMPs, MMP-2 (gelatinase A) is believed to be crucial for tumour invasion and metastasis. MMP-2 cleaves type IV collagen, leading to disruption of basement membrane

integrity, and is involved in the extensive extracellular matrix (ECM) degradation that occurs during invasion and metastasis [16-20]. The activity of MMPs *in vivo* is regulated by the tissue inhibitors of metalloproteinases (TIMPs), which are endogenous inhibitors of MMPs [16,18,19]. The N-terminal domains of TIMPs bind to the catalytic sites of active MMPs and inhibit MMP activity, downregulating proteolytic degradation of the ECM, tumour cell migration and invasiveness [16,18,19]. Interaction of EGFR with its ligands has been shown to regulate MMP expression in cancer cells [12,21,22]. EGFR regulates MMP expression and function in fibroblasts via MAPK and AP-1 [23] and EGFR stimulation of the ERK/MAPK pathway has been reported to activate several MMP genes, including MMP-1, MMP-3, MMP-7, MMP-9 and MT1-MMP in ovarian cancer [12].

In our present study, the effect of ATRA on EGFR mediated regulation of MMP-2 was studied using the highly metastatic murine melanoma cell line B16F10 as a model.

2. MATERIALS AND METHODS

2.1 Antibodies and Reagents

ATRA was purchased from Sigma-Aldrich (USA). Primary antibodies (anti-EGFR, anti-p-EGFR, anti-p38MAPK and anti-paxillin) and secondary antibodies (alkaline phosphatase coupled) were purchased from Santa Cruz (USA). NucleoSpin RNA XS kit and BluePrint RT-PCR Kit were purchased from Takara (Japan). Dulbecco's Modified Eagle's Medium (DMEM) and foetal bovine serum (FBS) were purchased from HiMedia (India). Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) were purchased from Bangalore Genei (India). Other fine chemicals were bought from Merck (USA) and Sigma-Aldrich (USA).

2.2 Cell Line

Highly metastatic murine melanoma cell line B16F10 was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured and maintained in DMEM containing 10% FBS at 37℃ in a CO_2 incubator (3% CO_2).

2.3 Preparation of ATRA Stock Solution and Treatment of Cells

ATRA was dissolved in DMSO to prepare a 5mM stock solution. B16F10 cells (300,000) cultured in petridishes were washed thrice with serum free culture medium (SFCM) and treated with ATRA (20 µM) for 6, 15 and 24 hrs. Control cells were cultured without ATRA in presence of equivalent amounts of DMSO (solvent for ATRA). Cells and culture supernatants were collected at the respective time points. Cells were extracted in extraction buffer (37.5 mM Tris, 75 mM NaCl, 0.5% Triton X-100, 1% protease inhibitors) by homogenization at 4℃. Protein contents of the resultant extracts and culture supernatants were estimated by modified Lowry's method [24].

2.4 Western Blot

Cell extracts (containing 80 µg protein) were suspended in Laemmli's buffer containing β-mercaptoethanol for 5 mins at 90℃, run on 7.5% or 10% SDS-PAGE as appropriate and proteins were transferred on to nitrocellulose membranes by Western blot. The membranes were blocked with 1% BSA and incubated with anti-EGFR, anti-p-EGFR, anti-p38MAPK or anti-paxillin antibodies (1:1000 dilution) for 1.5 hrs at 37℃, washed thrice with Tris buffered saline with Tween 20 (TBS-T), incubated with corresponding alkaline phosphatase coupled secondary antibodies (1:2000 dilution) for 1.5 hrs at 37℃ and washed thrice with TBS-T. Bands were visualized using NBT/BCIP substrate.

2.5 Gelatin Zymography

Culture supernatants (containing 20 µg protein) were mixed with sample buffer containing 2.5% SDS (without β-mercaptoethanol) and incubated at 37℃ for 30 min. Comparative gelatin zymography was performed on 10% SDS-PAGE co-polymerised with 0.1% gelatin. Gels were washed in 2.5% Triton X-100 for 30 min (to remove SDS), incubated for 40 hours in reaction buffer and stained with 0.25% Coomassie Brilliant Blue. Bands were clearly visualized by destaining the gel in water [25].

2.6 RT-PCR

RNA was extracted from control B16F10 cells and from B16F10 cells grown in the presence of ATRA (20 µM) for 6, 15 and 24 hrs using NucleoSpin RNA XS kit (Takara). Sequence of primers used for PCR were: MMP-2, forward 5'-CACCTACACCAAGAACTTCC-3' & reverse 5'-AACACAGCCTTCTCCTCCTG-3'; TIMP-2, forward 5'-CTCGCTGGACGTTGGAGGAA-3' &, reverse 5'-CACGCGCAAGAACCATCACT-3' [26]. β-actin primers were used as control to normalize for mRNA integrity and equal loading. RT-PCR was carried out using BluePrint RT-

PCR Kit (Takara). Following PCR, 20 µl of each PCR product was run on 1% agarose gel and bands were visualized under UV.

2.7 DNA Fragmentation Assay

Control and ATRA treated (20 µM for 24 hrs) cells were suspended in Tris-EDTA (TE) buffer, lysed at 4°C using lysis buffer, centrifuged and 0.2 M sodium acetate and absolute ethanol were added for evaluation of DNA laddering. After overnight incubation at –20°C, samples were centrifuged, pellets resuspended in TE buffer, subjected to RNAse and proteinase-K treatment, run on 1% agarose gel and visualized on a transilluminator [1].

3. RESULTS

3.1 Effect of ATRA on EGFR Expression and Phosphorylation

Western blot showed that treatment of B16F10 cells with 20 µM ATRA led to a partial decrease in EGFR expression after 6-15 hrs (Fig. 1A lanes 2-3) and an appreciable decrease in EGFR expression to virtually background levels after 24 hrs (Fig. 1A lane 4) compared to control B16F10 cells (Fig. 1A lane 1). Phosphorylation of EGFR was partially decreased upon treatment with 20 µM ATRA for 6 hrs (Fig. 1B lane 2) and appreciably decreased to virtually background levels upon treatment with 20 µM ATRA for 15-24 hrs (Fig. 1B lanes 3-4) in comparison to control B16F10 cells (Fig. 1B lane 1).

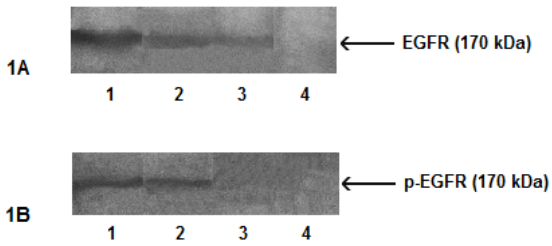

Fig. 1. Effect of ATRA on expression and phosphorylation of EGFR

Cell extracts of control B16F10 cells (lane 1) and B16F10 cells grown in presence of 20 µM ATRA for 6 hrs (lane 2), 15 hrs (lane 3) and 24 hrs (lane 4) were run on 7.5% SDS-PAGE and proteins were transferred onto nitrocellulose membranes by Western blot. Membranes were incubated with anti-EGFR antibodies (Fig. 1A) or anti-p-EGFR antibodies (Fig. 1B) followed by incubation with corresponding alkaline phosphatase coupled secondary antibodies. Bands were visualized using NBT/BCIP as substrate.

3.2 Effect of ATRA on Expression of p38MAPK and Paxillin

Western blot showed that treatment of B16F10 cells with 20 µM ATRA for 6-24 hrs reduced p38MAPK expression in a time dependant manner (Fig. 2A lanes 2-4) with p38MAPK expression being appreciably reduced after 15-24 hrs (lanes 3-4) in comparison to control B16F10 cells (Fig. 2A lane 1). However, treatment of B16F10 cells with 20 µM ATRA for 6-24 hrs did not appreciably affect the expression of paxillin (Fig. 2B lanes 1-4).

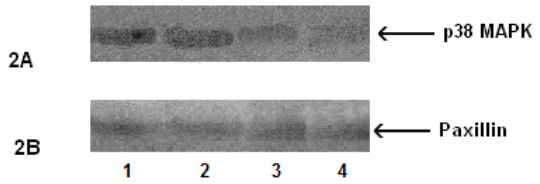

Fig. 2. Effect of ATRA on expression of p38MAPK and paxillin

Cell extracts of control B16F10 cells (lane 1) and B16F10 cells grown in presence of 20 µM ATRA for 6 hrs (lane 2), 15 hrs (lane 3) and 24 hrs (lane 4) were run on 10% SDS-PAGE and proteins were transferred onto nitrocellulose membranes by Western blot. Membranes were incubated with anti-p38MAPK antibodies (Fig. 2A) or anti-paxillin antibodies (Fig. 2B) followed by incubation with corresponding alkaline phosphatase coupled secondary antibodies. Bands were visualized using NBT/BCIP as substrate.

3.3 Effect of ATRA on Expression of MMP-2 and TIMP-2 mRNA

On treatment of B16F10 cells with 20 µM ATRA, RT-PCR showed a slight decrease in MMP-2 mRNA expression after 6 hrs (lane 2) compared to control B16F10 cells (lane 1) and an appreciable decrease in MMP-2 mRNA expression after 15-24 hrs (lanes 3-4). On treatment of B16F10 cells with ATRA, TIMP-2 mRNA expression showed a gradual increase (lanes 2-4) compared to control B16F10 cells (lane 1), with the increase being appreciable after 15-24 hrs of treatment (lanes 3-4). β actin was used as control to normalize for mRNA integrity and equal loading (lanes 1-4).

3.4 Effect of ATRA on MMP-2 Activity

Pro-MMP-2 (72 kDa) and active MMP-2 (68 kDa) activity could be detected in culture supernatants of control B16F10 cells (lane 1) by gelatin zymography. Pro- and active MMP-2 activity

were partially reduced on treatment of B16F10 cells with 20 μM ATRA for 6 hrs (lane 2). Treatment of B16F10 cells with 20 μM ATRA for 15-24 hrs led to a partial reduction of pro-MMP-2 (72 kDa) and an appreciable reduction of active MMP-2 (68 kDa) activity to almost background levels (lanes 3-4).

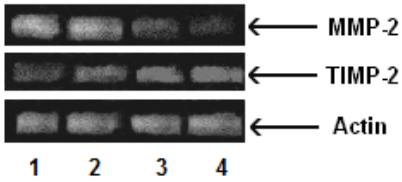

Fig. 3. Effect of ATRA on expression of MMP-2 and TIMP-2 mRNA

RNA was extracted from control B16F10 cells (lane 1) and B16F10 cells grown in the presence of ATRA (20 μM) for 6 hrs (lane 2), 15 hrs (lane 3) and 24 hrs (lane 4). RT-PCR was performed with equal amounts of total RNA using specific primers (MMP-2, TIMP-2) for PCR. 20 μl of each PCR product was run on 1% agarose gel and bands were visualized under UV. β-actin was used as control to normalize for mRNA integrity and equal loading.

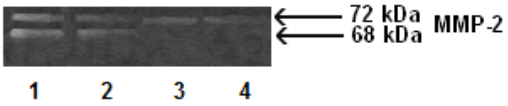

Fig. 4. Effect of ATRA on MMP-2 activity

Culture supernatants of control B16F10 cells (lane 1) and B16F10 cells grown in presence of 20 μM ATRA for 6 hrs (lane 2), 15 hrs (lane 3) and 24 hrs (lane 4) were subjected to zymography on 10% SDS-PAGE co-polymerised with 0.1% gelatin. After incubation in appropriate reaction buffers, the zymogram was stained with Coomassie Brilliant Blue. Bands were clearly visualized by destaining the gel in water.

3.5 Effect of ATRA on DNA Fragmentation

When DNA fragmentation assay was performed with control B16F10 cells (lane 1) and B16F10 cells treated with 20 μM ATRA for 24 hrs (lane 2), appreciable DNA laddering could not be detected even after 24 hrs of ATRA treatment. DNA ladder (Fermentas) was used as marker (lane M).

4. DISCUSSION

In our present study, treatment of B16F10 cells with ATRA (20 μM) led to an appreciable decrease in EGFR expression and phosphorylation. Binding of EGFR to its ligands promotes ligand-induced receptor dimerization and tyrosine phosphorylation, following which downstream signalling cascades are stimulated, leading to initiation of gene transcription and regulation of cellular processes including cell motility, proliferation, differentiation and survival [7-9]. A number of reports indicate that EGFR plays a critical role in promoting metastasis and tumour progression in melanomas [14,15]. Additionally, EGFR may activate other members of the ErbB family, including ErbB3, by formation of heterodimers following ligand interaction, thus promoting tumour growth and metastasis [27]. EGFR also increases melanoma cell motility, promoting increased invasion and metastasis [28]. Thus, downregulation of EGFR expression and phosphorylation upon treatment of B16F10 cells with ATRA could inhibit melanoma progression and metastasis and render melanomas less invasive.

Fig. 5. Effect of ATRA on DNA fragmentation

DNA fragmentation assay was performed with control B16F10 cells (lane 1) and B16F10 cells treated with 20 μM ATRA for 24 hrs (lane 2). Cell lysates were incubated overnight at –20°C with 0.2 M sodium acetate and absolute ethanol, subjected to RNAse and proteinase-K treatment, run on 1% agarose gel and visualized on a transilluminator. DNA ladder (Fermentas) was used as marker (lane M).

EGFR-MAPK signalling has been implicated in the progression of several types of cancers including melanomas [29-31]. Treatment of B16F10 cells with ATRA (20 μM) led to an appreciable reduction in p38MAPK expression. As the expression of paxillin was not appreciably reduced under similar conditions, this downregulation of expression is probably specific for p38MAPK. Downregulation of p38MAPK expression would hinder EGFR mediated signal

transduction. Inhibition of EGFR-dependant MAPK signalling has been reported to downregulate invasion and migration of fibrosarcomas and progression of melanomas [29,32]. Thus, downregulation of EGFR and p38MAPK expression upon treatment of B16F10 cells with ATRA would impair EGFR mediated signal transduction through p38MAPK and could inhibit metastasis and melanoma progression.

Increased expression of MMP-2 corresponds with increased metastatic potential and a worse prognosis in a number of cancers including melanomas [16-20]. Treatment of B16F10 cells with 20 µM ATRA led to an appreciable downregulation of MMP-2 activity in culture supernatants. In particular, active MMP-2 was reduced to virtually background levels after 15-24 hrs of ATRA treatment. MMP-2 secreted by cancer cells plays important roles in proteolytic degradation of the ECM and disruption of basement membrane integrity during tumour invasion and metastasis [16-20,33,34]. Several reports indicate that highly metastatic cells become less aggressive when MMP-2 activity is reduced [16-20,33]. Thus, downregulation of active MMP-2 might render tumour cells less invasive. An appreciable downregulation of MMP-2 expression at the mRNA level was observed after 15-24 hrs of ATRA treatment (20 µM). Transcription regulatory elements involved in the regulation of MMP expression may be modulated through MAPKs [12]. EGFR mediated signalling through MAPK has been reported to regulate MMP-2 expression [23,35]. Therefore, downregulation of EGFR and p38MAPK upon ATRA treatment could lead to the inhibition of MMP-2 gene transcription which, in turn, would downregulate MMP-2 expression and consequently lead to a reduction in MMP-2 activity. An appreciable increase in TIMP-2 mRNA after 15-24 hrs of ATRA treatment (20 µM) was also observed, indicating that treatment of B16F10 cells with ATRA could upregulate TIMP-2 gene transcription and consequently TIMP-2 expression. Although catalytic amounts of TIMP-2 are required for MMP-2 activation, TIMPs act as endogenous inhibitors of MMP activity [18,19,36]. TIMP-2 has been reported to inhibit *in vitro* invasiveness in highly invasive melanoma cells and to regulate MMP-2 activation in melanoma cell lines with low invasive cell lines expressing higher levels of TIMP-2 compared to higher invasive lines [33]. Thus an appreciable increase in TIMP-2 expression would inhibit MMP-2 activation and lower active MMP-2 levels, causing the observed downregulation of MMP-2 activity. The downregulation of MMP-2 activity could render melanoma cells less invasive.

Certain previous studies have indicated that the anti-tumorigenic potential of ATRA is due to the induction of apoptosis [1,37]. However, assays did not show appreciable fragmentation of DNA after treatment of B16F10 cells with ATRA (20 µM) for 24 hrs indicating that such treatment perhaps does not induce apoptotic changes in B16F10 melanoma cells. Thus, the observed anti-tumorigenic potential of ATRA and downregulation of MMP-2 activity in B16F10 cells are probably due to reasons other than solely induction of apoptosis.

5. CONCLUSION

Thus, treatment of highly metastatic B16F10 murine melanoma cells with ATRA (20 µM) appears to appreciably downregulate EGFR expression and phosphorylation and EGFR mediated signalling through p38MAPK. Treatment with ATRA also inhibits MMP-2 transcription and MMP-2 activity while upregulating transcription of TIMP-2. Downregulation of EGFR expression and phosphorylation and EGFR mediated signal transduction through p38 MAPK could cause downregulation of MMP-2 transcription and lead to loss in MMP-2 activity. An increase in TIMP-2 would also inhibit activation of MMP-2. The downregulation of MMP-2 activity would render cell less metastatic. Our findings indicate that treatment with ATRA can inhibit EGFR mediated regulation of MMP-2 activity in B16F10 murine melanoma cells and this could be one of the molecular mechanisms by which ATRA exerts its anti-tumorigenic effects on solid tumours. Such inhibition could have therapeutic potential in clinical management of tumours.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Orlandi M, Mantovani B, Ammar K, Avitabil E, Monte PD, Bartolinia G. Retinoids and cancer: Antitumoral effects of ATRA, 9-cis RA and the new retinoid IIF on the HL-60 leukemic cell line. Med Princ Pract. 2003;12:164-169.
2. Das S, Guha I, Chatterjee A, Banerji A. Anti-cancer potential of All-Trans Retinoic Acid (ATRA) - A review. Proceedings of the Zoological Society. 2013;66:1-7.
3. Liang C, Yang L, Guo S. All-trans retinoic acid inhibits migration, invasion and proliferation, and promotes apoptosis in glioma cells *in vitro*. Oncol Lett. 2015;9: 2833-2838.
4. Lo-Coco F, Ammatuna E, Montesinos P, Sanz MA. Acute promyelocytic leukemia: Recent advances in diagnosis and management. Semin Oncol. 2008;35:401-409.
5. Fang J, Chen SJ, Tong JH, Wang ZG, Chen GQ, Chen Z. Treatment of acute promyelocytic leukemia with ATRA and As_2O_3. Cancer Biol Ther. 2002;1:614-620.
6. Simeone AM, Tari AM. How retinoids regulate breast cancer cell proliferation and apoptosis. Cell Mol Life Science. 2004;61:1475-1484.
7. Herbst RS. Review of epidermal growth factor receptor biology. Int J Radiat Oncol Biol Phys. 2004;59:21–26.
8. Seshacharyulu P, Ponnusamy MP, Haridas D, Jain M, Ganti AK, Batra SK. Targeting the EGFR signaling pathway in cancer therapy. Expert Opin Ther Targets. 2012;16:15–31.
9. Dawson JP, Berger MB, Lin CC, Schlessinger J, Lemmon MA, Ferguson KM. Epidermal growth factor receptor dimerization and activation require ligand-induced conformational changes in the dimer interface. Mol Cell Biol. 2005;25: 7734–7742.
10. Baselga J, Albanell J. Epithelial growth factor receptor interacting agents. Hematol Oncol Clin North Am. 2002;16:1041-1063.
11. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2:127-137.
12. Hudson LG, Moss NM, Stack MS. EGF-receptor regulation of matrix metalloproteinases in epithelial ovarian carcinoma. Future Oncol. 2009;5:323–338.
13. Magkou C, Nakopoulou L, Zoubouli C, Karali K, Theohari I, Bakarakos P, et al. Expression of the epidermal growth factor receptor (EGFR) and the phosphorylated EGFR in invasive breast carcinomas. Breast Cancer Research. 2008;10:R49.
14. Boone B, Jacobs K, Ferdinande L, Taildeman J, Lambert J, Peeters M, et al. EGFR in melanoma: Clinical significance and potential therapeutic target. J Cutan Pathol. 2011;38:492-502.
15. Katunarić M, Jurišić D, Petković M, Grahovac M, Grahovac B, Zamolo G. EGFR and cyclin D1 in nodular melanoma: Correlation with pathohistological parameters and overall survival. Melanoma Res. 2014;24:584-91.
16. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nature Reviews, Cancer. 2002;2:161-174.
17. Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev. 2006;25:9–34.
18. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovascular Research. 2006;69:562–573.
19. Banerji A. The gelatinases and their role in tumour invasion. International Journal of Biology, Pharmacy and Allied Sciences. 2012;1:1003-1011.
20. Hofmann UB, Westphal JR, van Muijen GNP, Ruiter DJ. Matrix metalloproteinases in human melanoma. J Invest Dermatol. 2000;115:337-344.
21. Nutt JE, Durkan GC, Mellon JK, Lunec J. Matrix metalloproteinases (MMPs) in bladder cancer: The induction of MMP9 by epidermal growth factor and its detection in urine. BJU Int. 2003;91:99-104.
22. Cox G, Jones JL, O'Byrne KJ. MMP 9 and the epidermal growth factor receptor signal pathway in operable non-small cell lung cancer. Clin Cancer Res. 2000;6:2349-2355.
23. Kajanne R, Miettinen P, Mehlem A, Leivonen SK, Birrer M, Foschi M, et al. EGF-R regulates MMP function in fibroblasts through MAPK and AP-1 pathways. J Cell Physiol. 2007;212:489-497.
24. Hartree EF. Determination of protein: A modification of the lowry method that gives a linear photometric response. Anal Biochem. 1972;48:422-427.
25. Toth M, Fridman R. Assessment of gelatinases (MMP-2 and MMP-9) by

gelatin zymography. Methods Mol Med. 2001;57:163-174.
26. Wong H, Muzik H, Groft LL, Lafleur MA, Matouk C, Forsyth PA, et al. Monitoring MMP and TIMP mRNA expression by RT-PCR. In: Clark IM, editor. Matrix metalloproteinase protocols. 1st ed. Humana Press; 2001.
27. Ueno Y, Sakurai H, Tsunoda S, Choo MK, Matsuo M, Koizumi K, et al. Heregulin-induced activation of ErbB3 by EGFR tyrosine kinase activity promotes tumor growth and metastasis inmelanoma cells. Int J Cancer. 2008;123: 340-347.
28. Katunarić M, Zamolo G, Jonjić N. EGFR activated cell mobility - A link to melanoma ulceration. Med Hypotheses. 2015;85:498-499.
29. Shan X, Aziz F, Tian LL, Wang XQ, Yan Q, Liu JW. Ginsenoside Rg3-induced EGFR/MAPK pathway deactivation inhibits melanoma cell proliferation by decreasing FUT4/LeY expression. Int J Oncol. 2015; 46:1667-1676.
30. Sheu JJ, Lee CC, Hua CH, Li CI, Lai MT, Lee SC, et al. LRIG1 modulates aggressiveness of head and neck cancers by regulating EGFR-MAPK-SPHK1 signaling and extracellular matrix remodeling. Oncogene. 2014;33:1375-1384.
31. Mueller KL, Powell K, Madden JM, Eblen ST, Boerner JL. EGFR tyrosine 845 phosphorylation-dependent proliferation and transformation of breast cancer cells require activation of p38 MAPK. Transl Oncol. 2012;5:327-334.
32. Hwang YP, Yun HJ, Choi JH, Han EH, Kim HG, Song GY, et al. Suppression of EGF-induced tumor cell migration and matrix metalloproteinase-9 expression by capsaicin via the inhibition of EGFR-mediated FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 signaling. Mol Nutr Food Res. 2011;55:594-605.
33. Kurschat P, Zigrino P, Nischt R, Breitkopf K, Steurer P, Klein CE, et al. Tissue inhibitor of matrix metalloproteinase-2 regulates matrix metalloproteinase-2 activation by modulation of membrane-type 1 matrix metalloproteinase activity in high and low invasive melanoma cell lines. J Biol Chem. 1999;274:21056-21062.
34. Kamiya N, Kishimoto T, Suzuki H, Sekita N, Nagai Y, Oosumi N, et al. Increased in situ gelatinolytic activity in renal cell tumour tissues correlates with tumour size and vessel invasion. Int J Cancer. 2003;106: 480-485.
35. Razfar A, Grandis JR. Growth factor receptor signalling and metastasis of oral cancer. In: Myers J, editor. Oral Cancer Metastasis. Springer; 2010.
36. Remillard TC, Bratslavsky G, Jensen-Taubman S, Stetler-Stevenson WG, Bourboulia D. Molecular mechanisms of tissue inhibitor of metalloproteinase 2 in the tumor microenvironment. Mol Cell Ther. 2014;2:17.
37. Mangiarotti R, Danova M, Alberici R, Pellicciari C. All-trans retinoic acid (ATRA)-induced apoptosis is preceded by G1 arrest in human MCF-7 breast cancer cells. Br J Cancer. 1998;77:186-191.

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