ANTICANCER RESEARCH *41*: 3343-3348 (2021)
doi:10.21873/anticanres.15122

Raspberry Extract With Potential Antitumor Activity Against Cervical Cancer

NELSON SHAM[1*], CHENGLU QIN[2,3*], ZIWEN ZHU[3], CHASE G. REDINGTON[3], HUAPING XIAO[1], QIAN BAI[3], MARK R. WAKEFIELD[3], LEON KOU[1] and YUJIANG FANG[1,3]

[1]*Department of Microbiology, Immunology & Pathology, Des Moines University, Des Moines, IA, U.S.A.;*
[2]*Department of Obstetrics and Gynecology, Luohu District People's Hospital, Shenzhen, Guangdong, P.R. China;*
[3]*Department of Surgery, University of Missouri School of Medicine, Columbia, MO, U.S.A.*

Abstract. *Background: Cervical cancer (CC) is one of the leading causes of mortality worldwide. Previously, we reported that blueberry extract constrains the growth of CC. Raspberry is a widely consumed fruit that exhibits antitumor properties against several cancer types but little is known about its direct effect on CC. This study was designed to investigate the potential antitumor effect of raspberry extract (RE) on CC cells and to elucidate the possible mechanisms behind it. Materials and Methods: Clonogenic survival assay and caspase-3 activity kits were used to evaluate the effects of RE on cell survival, proliferation, and apoptosis of a widely used CC cell line, HeLa. Possible molecular mechanisms were investigated using reverse transcription-polymerase chain reaction. Results: The percentage of colonies and optic density value of HeLa cells decreased in the presence of RE in comparison to controls. Relative caspase-3 activity in cancer cells increased in the presence of RE in comparison to controls. The antitumor effect displayed on HeLa cells by RE was associated with the increased expression of antiproliferative molecule P53 and the increased expression of pro-apoptotic molecule tumor necrosis factor receptor superfamily member 6 (FAS). Conclusion: RE displays anticancer activity against CC HeLa cells. The mechanism behind this is by up-regulation of anti-proliferative molecule P53 and pro-apoptotic molecule FAS.*

*These Authors contributed equally to this study.

Correspondence to: Dr. Yujiang Fang, Department of Microbiology, Immunology & Pathology, Des Moines University College of Osteopathic Medicine, Des Moines, IA 50312, U.S.A. Tel: +1 5152711435, Fax: +1 5152711543, e-mail: yujiang.fang@dmu.edu

Key Words: Raspberry, apoptosis, proliferation.

Among women worldwide, cervical cancer (CC) is the fourth most common cancer (1). Historically, CC was one of the deadliest disease for U.S. women, but the implementation of Papanicolaou and human papillomavirus (HPV) testing as standard screening tools has remarkedly reduced the mortality rate of CC (2). Almost all CC can be linked to HPV infection. HPV16 and -18 accounted for at least 70% of CC. HPV vaccines, such as the 9-valent vaccine which protects against five additional HPV strains, have proven to be very effective in preventing invasive CC (3).

Nevertheless, CC remains a global threat especially in developing countries where standard screening and vaccinations are not readily available. Currently, treatments recommended by the World Health Organization for pre-cancerous lesions as secondary prevention include cryotherapy, thermal ablation, and loop electrosurgical excision procedure (4). For patients with recurrent or advanced-stage CC, treatments are limited to surgical excision, radiotherapy, and systemic chemotherapy. Treatments with immunotherapy have gained momentum in recent years. However, given the complex nature between HPV-infected cells and the chronic inflammatory microenvironment that is needed to promote malignant progression, more investigation is needed in this field.

Raspberry extract (RE) consists of many components. Studies have found that the potential antitumor property of many berries, in part, lie in the phytochemicals called polyphenols (5). RE is especially rich in a class of polyphenol called ellagitannin. Ellagitannin has been shown to be a prominent antiproliferative agent through its high antioxidant property. It has the ability to counteract, reduce, and repair damage from oxidative stress and inflammation (6-9). Several studies have shown the link between RE and its antiproliferative effect on CC (8-10). We have also reported the role of blueberry extract as a potential radiosensitizer for treating CC (11). To our knowledge, no studies have shown the underlying mechanisms responsible for the antitumor properties of RE against CC. This study

was designed to investigate the direct role of RE on CC cell line HeLa and to elucidate its potential mechanisms.

Materials and Methods

Tumor cell line. The human CC cell line HeLa was acquired from the American Type Culture Collection, Manassas, VA, USA. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). The cultures were grown in a humidified incubator at 37˚C with 5% CO_2. The cell cultures were then grown to 70% confluence before being subjected to experimental regimens.

Treatment with RE. The 70% confluent HeLa cells were treated with 50 μg/ml of RE (NutriVitaSHOP, Lake Forest, CA, USA) or medium alone as control for 72 h. Treatment concentration and duration were based on our previous pilot studies (7, 11-14).

Clonogenic survival assay. Clonogenic survival assay was performed after RE treatment as previously outlined (14-16). The number of colonies were tallied and expressed as the percentage of total colonies in RE-treated cells compared to those in medium alone.

Quick Cell proliferation assay. Quick Cell proliferation assay kit (BioVision, Milpitas, CA, USA) was utilized to examine cell proliferation. In this assay, the proliferation of viable cells is directly proportional to the activity of cellular mitochondrial dehydrogenase. The increase in activity results in increased production of formazan dye by these cells, which can be quantified by spectrophotometry. This procedure is detailed in our previous studies (7, 11-14).

Reverse transcription-polymerase chain reaction (RT-PCR). RE-treated and control HeLa cells were washed with phosphate buffered saline (PBS) and homogenized in TRIzol (Invitrogen). RNA was extracted and 1 μg of RNA was reverse transcribed as described in previous studies (7, 11-14). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), P18, P21, P27, P53, cyclins B, D and E, cyclin-dependent kinase 2 (*CDK2*), *CDK4*, *FAS*, FAS ligand (*FASL*), tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*), TRAIL receptor 1 (*TRAILR1*), and BCL2 apoptosis regulator-associated X protein (*BAX*), along with key anti-apoptotic molecules FLICE-like inhibitory protein (*FLIP*), BCL2 and survivin primers used have been previously described (7, 11-14).To ensure equal amounts of RNA had been amplified, *GAPDH* was utilized as a control.

Measurement of caspase-3 activity. Caspase-3/CPP32 colorimetric assay kit (BioVision) was used to measure the activity of apoptotic marker caspase-3 in HeLa cells, as previously outlined (7, 11-14).

Statistical analysis. Experiments were repeated three times each. Data analysis was performed using an unpaired, two-tailed Student's *t*-test. Statistical significance was considered with a *p*-value of <0.05.

Results

Effect of RE on inhibition of cervical cancer cell growth and proliferation. As outlined in the Materials and Methods section, HeLa cells at 70% confluence were treated with either 50 μg/ml of RE or medium alone for 72 h. Assessment of cell survival using clonogenic survival assay demonstrated a significant reduction in the number of surviving HeLa colonies in RE-treated cells as compared to the controls with medium alone (Figure 1A, $p<0.05$). Optic density values obtained using a Quick Cell proliferation assay to quantify proliferation further supported these findings (Figure 1B, $p<0.05$). These results together presented a strong indication that RE constrains the growth and survival of HeLa cells.

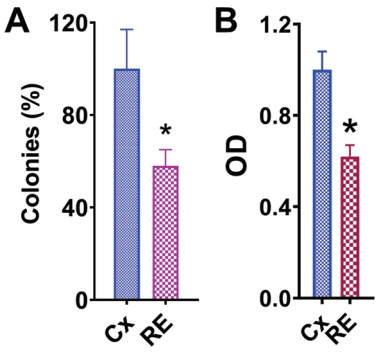

Figure 1. *Effect of raspberry extract (RE) on HeLa cervical cancer cell survival. A: Clonogenic survival assay of HeLa cells treated with RE (50 μg/ml) or medium alone. Colony numbers were counted and expressed as a total percentage of colonies in the controls (Cx). B: Representative result evaluated with a cell proliferation kit. Results are expressed as the mean+standard error of the mean, and are representative of two independent experiments. *Significantly different at p<0.05 versus the control.*

Effect of RE increased expression of antiproliferative molecule P53 and pro-proliferative molecule cyclin D in CC cells. Next, we explored how RE inhibited HeLa cell growth and proliferation. Using RT-PCR, mRNA expression of anti-proliferative molecules (*P18, P21, P27,* and *P53*) and pro-proliferative molecules (cyclin B, cyclin D, cyclin E, *CDK2,* and *CDK4*) in RE-treated and control HeLa cells was determined (Figure 2). mRNA expression of antiproliferative *P53* was significantly increased in RE treated HeLa cells compared to control HeLa cells (Figure 2, $p<0.05$). However, mRNA expression of pro-proliferative cyclin D was also significantly increased (Figure 2, $p<0.05$). The results demonstrate that the up-regulation of *P53* correlated with the inhibitory effect of RE on the proliferation and growth of HeLa CC cells.

RE induces apoptosis of CC cells. To investigate how RE affected apoptosis of HeLa cells, we examined the relative caspase-3 activity in RE-treated and control HeLa cells using a caspase-3 activity kit. The relative caspase-3 activity was

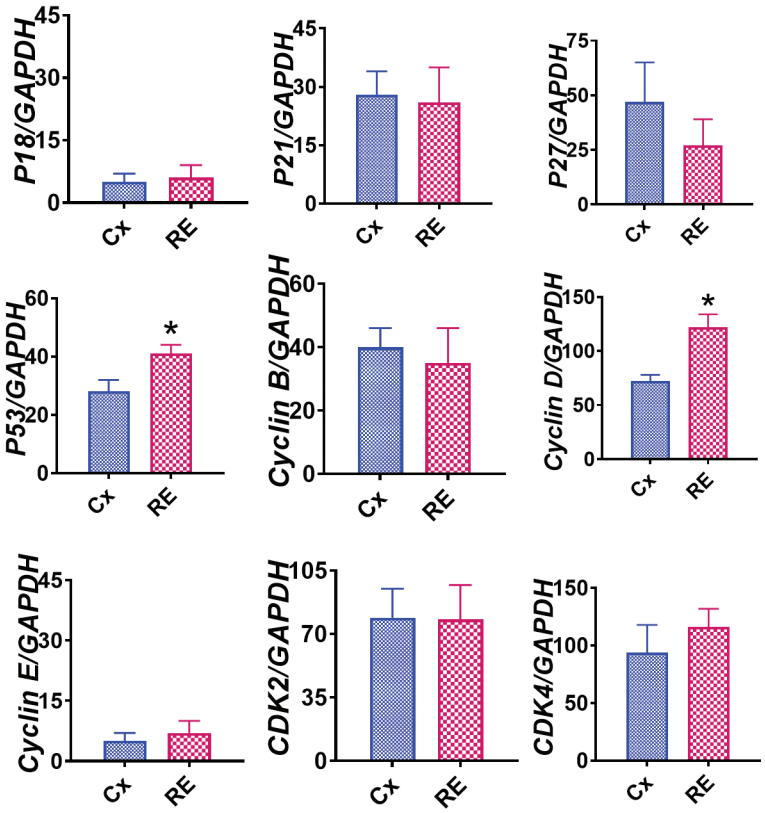

Figure 2. *Effect of 50 μg/ml raspberry extract (RE) on expression of pro- and anti-proliferative molecules P18, P21, P27, P53, cyclin B, cyclin D, cyclin E, cyclin-dependent kinase 2 (CDK2) and CDK4 in HeLa cells as evaluated using reverse transcription-polymerase chain reaction. The results are expressed as the ratio (mean+standard error of the mean) of molecule densitometric units relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (×100), and are representative of three independent experiments. *Significantly different at p<0.05 versus the control (Cx).*

significantly higher in RE-treated HeLa cells as compared to controls (Figure 3, $p<0.05$). These results demonstrated that RE induces apoptosis of HeLa cells, which might be another explanation for the inhibitory effects of RE on the survival of HeLa cells.

RE increases the expression of pro-apoptotic molecule FAS in CC cells. We further investigated the molecular mechanisms behind RE-induced apoptosis in HeLa cells. Using RT-PCR, mRNA expression of pro-apoptotic molecules (*FAS*, *FASL, TRAILR1, TRAIL,* and *BAX*) and anti-apoptotic molecules (*FLIP, BCL2,* and survivin) in RE-treated and control HeLa cells was determined (Figure 4). mRNA expression of pro-apoptotic molecule *FAS* was significantly increased in RE-treated HeLa cells compared to controls (Figure 4, $p<0.05$). The expression of the other molecules studied was not significantly modulated. The results suggest that the pathway RE utilized to induce apoptosis in HeLa cells involved up-regulation of pro-apoptotic molecule FAS.

Discussion

This study demonstrated that RE inhibited the survival of HeLa CC cells by up-regulating both anti-proliferative molecule P53 and pro-apoptotic molecule FAS. While this is not the first study to reveal antitumor effects of RE on CC cells, to our knowledge, this is the first study to decode the underlying molecular mechanisms behind this phenomenon.

The cell cycle consists of a sequence of events that prepare the cell for growth and division. Progression of the cell cycle relies on many positive and negative regulators which ensure the proper production of daughter cells (17). P53 is one such regulator that acts as a critical tumor suppressor. P53 prevents the progression of the cell cycle in response to DNA damage such as mismatches or single-stranded DNA (17). In our study, we have shown RE significantly up-regulated P53 in HeLa cells. CC is strongly linked to infection by high-risked HPV. Interestingly, it is well established that the HPV oncoprotein E6 has the power to associate with and neutralize P53 (18). E6 interacts with

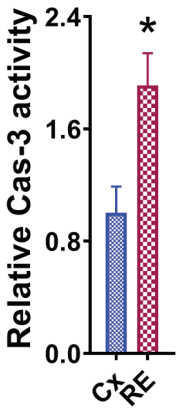

Figure 3. *Relative caspase-3 activity was measured in triplicate in HeLa cells treated with and without 50 μg/ml raspberry extract (RE) as described in the Materials and Methods section. The results are expressed as caspase-3 activity (mean+standard error of the mean) relative to the control (Cx). *Significantly different at p<0.05 versus the control.*

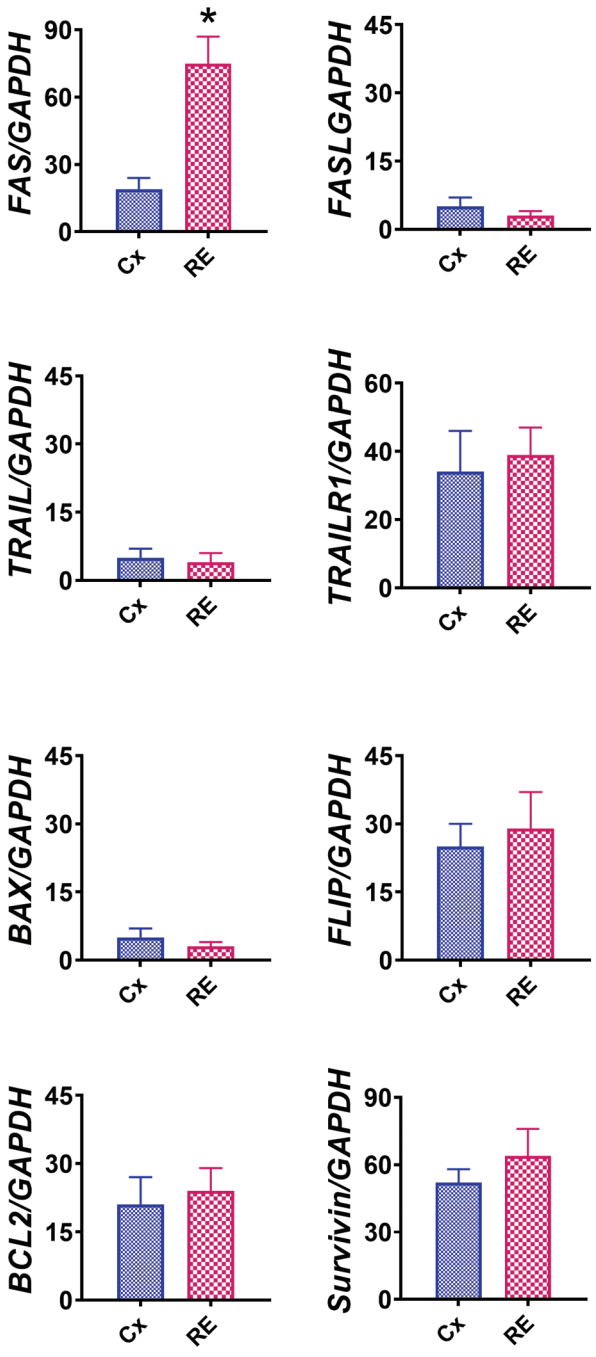

Figure 4. *Effect of 50 μg/ml raspberry extract (RE) on the expression of pro-apoptotic molecules FAS, FAS ligand (FASL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), TRAIL receptor 1 (TRAILR1), and BCL2 apoptosis regulator-associated X protein (BAX) along with key anti-apoptotic molecules FLICE-like inhibitory protein (FLIP) and BCL2 in HeLa cells as evaluated by reverse transcription-polymerase chain reaction (RT-PCR). according to the Materials and Methods section. The results are expressed as the ratio (mean+standard error of the mean) of molecule densitometric units relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (×100), and are representative of three independent experiments. *Significantly different at p<0.05 versus the control (Cx).*

E6 associated protein (E6AP), which is a ubiquitin protein ligase. The dimeric complex of E6 and E6AP binds P53, E6AP then catalyzes multi-ubiquitination of P53, which leads to its degradation (19). In line with the interaction between E6 and P53 is the fact that the *P53* gene is wild-type in most HPV-positive CC; however, its expression is significantly reduced (15, 19, 20). Therefore, it is reasonable to suggest that treatments aiming to increase P53 protein expression, such as RE, may play a role in the treatment of HPV-positive CC. The up-regulation of *P53* by RE may aid in the active efforts to explore the use of *P53* induction and restoration of wild-type in the treatment of CC (16).

As seen by the increase of both relative caspase-3 activity and pro-apoptotic expression of *FAS* gene in RE-treated HeLa cells, induction of apoptosis is another important mechanism behind the antitumor activity of RE on CC cells. FAS, also known as FAS receptor, CD95 and apoptosis antigen 1, is a death receptor of the extrinsic apoptosis pathway (21). Once bound by FASL, it initiates a cascade of events that ultimately lead to programmed cell death. Agonists of FAS have been investigated as a potential chemotherapy due to its powerful and straightforward capacity to induce apoptosis in tumor cells. However, the problem of systemic toxicity, especially to hepatocytes with unspecific FAS activation, needs to be overcome. RE-induced *FAS* up-regulation might be of interest to researchers in the future development of chemotherapy targeting the FAS apoptotic pathway of CC cells. We also reported up-regulation of the pro-proliferative molecule cyclin D in RE-treated HeLa CC cells. In fact, this is not a novel phenomenon. Studies have shown that overexpression of

cyclin D leads to apoptosis of both normal and cancer cells (22-24). It is reasonable to deduce that RE induces apoptosis of HeLa cells in part through overexpression of cyclin D.

Current treatment options for CC *in situ* include conization and hysterectomy. Increasing needs for radical hysterectomy, radical trachelectomy, radiation therapy, and chemotherapy are seen in patients with advanced stage CC or recurrent cases. We previously demonstrated blueberry extract to be a potential radiosensitizer for treatment of CC. Blueberry extract sensitized CC cell line SiHa to radiation therapy by inhibition of proliferation and promotion of apoptosis (11, 25, 26). Similarly to blueberry extract, RE as a radiosensitizer for the treatment of CC holds promising potential, but more investigation is needed.

In summary, RE was shown to exhibit antitumor effects on CC *in vitro* by inhibiting proliferation and inducing apoptosis. The results of our study highlight the potential use of RE as an additional treatment modality for CC. Future clinical studies may lead to the utilization of RE in the treatment of CC and other tumors.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

Authors' Contributions

YF conceived and designed this study. All Authors performed experiments. YF and ZZ analyzed and interpreted the data. NS and QC wrote the draft and carried out critical revision. All Authors approved the final version of the article.

Acknowledgements

This work was supported by grants (IOER 112-3749) from Des Moines University for Yujiang Fang.

References

1 Siegel RL, Miller KD, Fuchs HE and Jemal A: Cancer statistics, 2021. CA Cancer J Clin *71(1)*: 7-33, 2021. PMID: 33433946. DOI: 10.3322/caac.21654

2 Mustafa RA, Santesso N, Khatib R, Mustafa AA, Wiercioch W, Kehar R, Gandhi S, Chen Y, Cheung A, Hopkins J, Ma B, Lloyd N, Wu D, Broutet N and Schünemann HJ: Systematic reviews and meta-analyses of the accuracy of HPV tests, visual inspection with acetic acid, cytology, and colposcopy. Int J Gynaecol Obstet *132(3)*: 259-265, 2016. PMID: 26851054. DOI: 10.1016/j.ijgo.2015.07.024

3 Lei J, Ploner A, Elfström KM, Wang J, Roth A, Fang F, Sundström K, Dillner J and Sparén P: HPV vaccination and the risk of invasive cervical cancer. N Engl J Med *383(14)*: 1340-1348, 2020. PMID: 32997908. DOI: 10.1056/NEJMoa1917338

4 Santesso N, Mustafa RA, Wiercioch W, Kehar R, Gandhi S, Chen Y, Cheung A, Hopkins J, Khatib R, Ma B, Mustafa AA, Lloyd N, Wu D, Broutet N and Schünemann HJ: Systematic reviews and meta-analyses of benefits and harms of cryotherapy, LEEP, and cold knife conization to treat cervical intraepithelial neoplasia. Int J Gynaecol Obstet *132(3)*: 266-271, 2016. PMID: 26643302. DOI: 10.1016/j.ijgo.2015.07.026

5 Seeram NP: Berry fruits for cancer prevention: current status and future prospects. J Agric Food Chem *56(3)*: 630-635, 2008. PMID: 18211019. DOI: 10.1021/jf072504n

6 Ross HA, McDougall GJ and Stewart D: Antiproliferative activity is predominantly associated with ellagitannins in raspberry extracts. Phytochemistry *68(2)*: 218-228, 2007. PMID: 17126865. DOI: 10.1016/j.phytochem.2006.10.014

7 Schroeder AC, Xiao H, Zhu Z, Li Q, Bai Q, Wakefield MR, Mann JD and Fang Y: A potential role for green tea as a radiation sensitizer for prostate cancer. Pathol Oncol Res *25(1)*: 263-268, 2019. PMID: 29101735. DOI: 10.1007/s12253-017-0358-4

8 Kumar D, Basu S, Parija L, Rout D, Manna S, Dandapat J and Debata PR: Curcumin and Ellagic acid synergistically induce ROS generation, DNA damage, p53 accumulation and apoptosis in HeLa cervical carcinoma cells. Biomed Pharmacother *81*: 31-37, 2016. PMID: 27261574. DOI: 10.1016/j.biopha.2016.03.037

9 McDougall GJ, Ross HA, Ikeji M and Stewart D: Berry extracts exert different antiproliferative effects against cervical and colon cancer cells grown in vitro. J Agric Food Chem *56(9)*: 3016-3023, 2008. PMID: 18412361. DOI: 10.1021/jf073469n

10 Zhang Z, Knobloch TJ, Seamon LG, Stoner GD, Cohn DE, Paskett ED, Fowler JM and Weghorst CM: A black raspberry extract inhibits proliferation and regulates apoptosis in cervical cancer cells. Gynecol Oncol *123(2)*: 401-406, 2011. PMID: 21831414. DOI: 10.1016/j.ygyno.2011.07.023

11 Davidson KT, Zhu Z, Bai Q, Xiao H, Wakefield MR and Fang Y: Blueberry as a potential radiosensitizer for treating cervical cancer. Pathol Oncol Res *25(1)*: 81-88, 2019. PMID: 28963664. DOI: 10.1007/s12253-017-0319-y

12 Kou L, Zhu Z, Redington C, Bai Q, Wakefield M, Lequio M and Fang Y: Potential use of kiwifruit extract for treatment of melanoma. Med Oncol *38(3)*: 25, 2021. PMID: 33586074. DOI: 10.1007/s12032-021-01465-2

13 Xiao H, Alisic H, Reiman BT, Deng Z, Zhu Z, Givens NT, Bai Q, Tait A, Wakefield MR and Fang Y: IL-39 reduces proliferation and promotes apoptosis of bladder cancer by altering the activity of cyclin E and Fas. Anticancer Res *41(5)*: 2239-2245, 2021. PMID: 33952450. DOI: 10.21873/anticanres.15000

14 Zhu Z, Davidson KT, Brittingham A, Wakefield MR, Bai Q, Xiao H and Fang Y: Trichomonas vaginalis: a possible foe to prostate cancer. Med Oncol *33(10)*: 115, 2016. PMID: 27613161. DOI: 10.1007/s12032-016-0832-y

15 Wrede D, Tidy JA, Crook T, Lane D and Vousden KH: Expression of RB and p53 proteins in HPV-positive and HPV-negative cervical carcinoma cell lines. Mol Carcinog *4(3)*: 171-175, 1991. PMID: 1648361. DOI: 10.1002/mc.2940040302

16 Zhao X, Sun W, Ren Y and Lu Z: Therapeutic potential of p53 reactivation in cervical cancer. Crit Rev Oncol Hematol *157*: 103182, 2021. PMID: 33276182. DOI: 10.1016/j.critrevonc.2020.103182

17 Wenzel ES and Singh ATK: Cell-cycle checkpoints and aneuploidy on the path to cancer. In Vivo *32(1)*: 1-5, 2018. PMID: 29275292. DOI: 10.21873/invivo.11197

18 Wang X, Huang X and Zhang Y: Involvement of human papillomaviruses in cervical cancer. Front Microbiol *9*: 2896, 2018. PMID: 30546351. DOI: 10.3389/fmicb.2018.02896

19 Tommasino M, Accardi R, Caldeira S, Dong W, Malanchi I, Smet A and Zehbe I: The role of TP53 in Cervical carcinogenesis. Hum Mutat *21(3)*: 307-312, 2003. PMID: 12619117. DOI: 10.1002/humu.10178

20 Koncar RF, Feldman R, Bahassi EM and Hashemi Sadraei N: Comparative molecular profiling of HPV-induced squamous cell carcinomas. Cancer Med *6(7)*: 1673-1685, 2017. PMID: 28556593. DOI: 10.1002/cam4.1108

21 Nagata S: Fas ligand-induced apoptosis. Annu Rev Genet *33*: 29-55, 1999. PMID: 10690403. DOI: 10.1146/annurev.genet.33.1.29

22 Katayama K, Dobashi Y, Kitagawa M, Kawai M, Kadoya Y and Kameya T: Cdk4/cyclin D1 kinase, a universal and critical regulator of apoptosis. Anticancer Res *23(1A)*: 235-243, 2003. PMID: 12680219.

23 Zhang D, Li X, Chen C, Li Y, Zhao L, Jing Y, Liu W, Wang X, Zhang Y, Xia H, Chang Y, Gao X, Yan J and Ying H: Attenuation of p38-mediated miR-1/133 expression facilitates myoblast proliferation during the early stage of muscle regeneration. PLoS One *7(7)*: e41478, 2012. PMID: 22911796. DOI: 10.1371/journal.pone.0041478

24 Zhu Z, Zhang D, Lee H, Menon AA, Wu J, Hu K and Jin Y: Macrophage-derived apoptotic bodies promote the proliferation of the recipient cells *via* shuttling microRNA-221/222. J Leukoc Biol *101(6)*: 1349-1359, 2017. PMID: 28274991. DOI: 10.1189/jlb.3A1116-483R

25 Davidson KT, Zhu Z, Balabanov D, Zhao L, Wakefield MR, Bai Q and Fang Y: Beyond conventional medicine – a look at blueberry, a cancer-fighting superfruit. Pathol Oncol Res *24(4)*: 733-738, 2018. PMID: 29285736. DOI: 10.1007/s12253-017-0376-2

26 Davidson KT, Zhu Z and Fang Y: Phytochemicals in the fight against cancer. Pathol Oncol Res *22(4)*: 655-660, 2016. PMID: 26857640. DOI: 10.1007/s12253-016-0045-x

Received June 4, 2021
Revised June 14, 2021
Accepted June 15, 2021