ANTICANCER RESEARCH *38*: 5079-5086 (2018)
doi:10.21873/anticanres.12827

Trichomonas Vaginalis Inhibits HeLa Cell Growth Through Modulation of Critical Molecules for Cell Proliferation and Apoptosis

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Abstract. *Background/Aim: Cervical cancer is one of the deadliest gynecological cancers in USA. The role of Trichomonas Vaginalis (T. Vag) in the etiology or pathogenesis of cervical cancer is still poorly understood and controversial. Materials and Methods: Clonogenic assay, PCNA staining, TUNEL staining and caspase-3 activity assay were used to investigate the direct in vitro effect of T. Vag on human cervical cancer by using HeLa cells. We further investigated the potential molecular mechanisms using RT-PCR and immunohistochemical staining. Results: We found that culture supernatant of T. Vag inhibited growth of HeLa cervical cancer cells and this correlated with up-regulation of p15. We also found that culture supernatant of T. Vag induced apoptosis of HeLa cells and this correlated with up-regulation of Fas, TRAIL and TRAILR1. Conclusion: Culture supernatant of T. Vag inhibits growth of HeLa cervical cancer cells by inhibition of proliferation and promotion of apoptosis. Our study might be helpful to address the association between the development of cervical cancer and infection of T. Vag.*

Cervical cancer is a malignancy arising from the cervix that could invade surrounding tissues and metastasize to other organs. It is one of the deadliest gynecological cancers in the United States and worldwide. In the United States in 2016, there are about 13,000 estimated new cases and over 4,000 estimated deaths due to cervical cancer (1). Worldwide, there are over 500,000 new cases and 274,000 deaths reported each year (1). The prognosis for cervical cancer is very challenging. The five year survival rate for women with cervical cancer is less than 50% in developing countries, and about 66% in developed countries (2). To improve the prognosis of cervical cancer, it is critical to understand the etiology and the pathogenesis of the disease. Unfortunately, little progress has been made in recent years regarding the etiology or pathogenesis of cervical cancer. *Trichomonas vaginalis* (*T. Vag*) is an anaerobic, flagellated extracellular protozoan parasite (3). It is well-known that it is the most common non-viral sexually transmitted pathogen worldwide, with an incidence of 275 million cases annually (4). In the Unites States alone, 8-10 million estimated new infections occur annually (5). Despite the fact that only 50% of infected women will show clinical symptoms, the clinical significance of this infection lies in the fact that it increases susceptibility to HIV, infertility, premature labor, and possibly a higher incidence of cervical cancer (6).

T. Vag has been suspected to be associated with the pathogenesis of cervical cancer for more than twenty years (7-10). However, increasing evidence seems to suggest that *T. Vag* might not be associated with the development of cervical cancer (11-13). Consistent with these findings, our previous study has also shown that culture supernatant of *T. Vag* inhibits growth of prostate cancer cells through up-regulation of the anti-proliferative molecule p21 and down-regulation of the anti-apoptotic molecule Bcl-2 (14). Interestingly, one study even suggests that *T. Vag* can damage normal genital epithelial cells and induce apoptosis

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Key Words: Trichomonas Vaginalis, cervical cancer, apoptosis, proliferation, p15, TRAILR1.

of Siha cervical cancer cells through the Bcl-2 family (15). Obviously, the association between *T. Vag* and cervical cancer is not fully understood and further studies are needed to clarify their relationship. This study was designed and performed to investigate the direct effect of *T. Vag* on HeLa cell growth by using the culture supernatant of *T. Vag*.

Materials and Methods

Cervical cancer cell culture. Human cervical cancer cell line, HeLa, was obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa cervical cancer cells were cultured in DMEM medium in the presence of 10% FBS as well as 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). HeLa cervical cancer cells were cultured at 37˚C in 5% CO_2 humidified incubator. HeLa cancer cells were used for the designed experiment when they reached 70% confluence.

T. Vag cell culture. *Trichomonas vaginalis* G3 (ATCC PRA-98) was obtained from the American Type Culture Collection. The culture method has been described in detail in our previous study (14).

Collection of T. Vag conditioned growth medium. Following 48 h of growth, cultures reached an average density of 3.5×10^6 cells/ml. Conditioned media of cultured *T. Vag* were obtained by centrifugation. Post-centrifugation, conditioned medium without any cell debris was immediately frozen using liquid nitrogen and stored at –80˚C. Control media, which had not been inoculated with *T. Vag*, was processed in an identical manner.

Treatment of cervical cancer cells with T. Vag conditioned growth medium. 70% confluent HeLa cells were treated for 3 days with conditioned growth medium or control medium alone. The dilution ratio for *T. Vag* conditioned growth medium used in this study is based on our previous study (14). A dilution of 1:15 for *T. Vag* conditioned growth medium was used in this study.

Immunohistochemistry (IHC). IHC staining for PCNA, p15 and TRAILR1 was performed as previously described (16-20). Average staining intensity for proteins within the area covered by cells was measured using ImageJ software. Results are expressed as the average integrated immunostaining intensity of 3 slides ±SEM relative to that in control cells.

Clonogenic survival assay. Three days after *T. Vag*. conditioned growth medium treatment, cancer cells were detached and counted in a hemocytometer. Clonogenic survival assay was performed as described previously (16-20). The number of colonies was counted and expressed as a percentage of total colonies in control.

RT-PCR. HeLa cancer cells were washed with PBS and homogenized in TRIzol (Invitrogen). RNA was extracted and its concentration was determined by Nanodrop, then 1 μg RNA was reverse transcribed. GAPDH was used as a housekeeping gene. Primer sequences used have been described previously (16).

TUNEL staining. Apoptosis was determined by TUNEL assay using an ApopTag kit (Chemicon, El Segundo, CA, USA) according to the manufacturer's instructions which has been described in detail in our previous studies (16-20). To quantify the number of apoptotic cells, all cells in 5-6 randomly selected high-power fields (magnification: ×400) were manually counted. TUNEL+ cells were expressed as a percentage of total cells.

Measurement of caspase-3 activity. As we have described in our previous studies (16-20), cellular caspase-3 activity of HeLa cells was measured using a Caspase-3/CPP32 Colorimetric Assay kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

Statistics. All experiments were repeated at least two times. Comparisons between 2 groups were performed using a 2-tailed unpaired Student's *t*-test. A p-value<0.05 was considered significant.

Results

Proliferation of HeLa cervical cancer cells was inhibited by culture supernatant of T. Vag. HeLa cell line is the most commonly used cell line in cervical cancer research. In this study, to investigate the direct effect of *T. Vag* supernatant on proliferation of cervical cancer. 70% confluent HeLa cells were treated with culture supernatant of *T. Vag* or control medium alone for 3 days and cell survival was evaluated by the traditional clonogenic survival assay. As shown in Figure 1A, the percentage of colonies of HeLa cells after *T. Vag* treatment was significantly lower than that in control treated cells with medium alone (Figure 1A, $p<0.05$). To further confirm this finding, IHC for PCNA, which is a marker of cell proliferation, was also used to evaluate the direct effect of the culture supernatant of *T. Vag* on HeLa cells. Consistent with the finding revealed by the clonogenic survival assay, immunohistostaining intensity for PCNA in cells after *T. Vag* treatment was significantly weaker than that in control (Figure 1B and C). Taken together, these results indicate that proliferation of HeLa cells was inhibited by the culture supernatant of *T. Vag*.

Culture supernatant of T. Vag up-regulated expression of anti-proliferative molecule p15 in HeLa cells. Cell cycle is mainly determined by the balance between pro- and anti-proliferative molecules. When pro-proliferative molecules dominate, cells start to proliferate. Whereas, when anti-proliferative molecules dominate, cell cycle arrest is observed. To investigate the possible molecular mechanisms by which proliferation of HeLa cells was inhibited by culture supernatant of *T. Vag*, mRNA expression of important pro- and anti-proliferative molecules was first determined by RT-PCR in both groups (Figure 2). The mRNA expression of these molecules was all comparable in both groups ($p>0.05$) except that of p15 whereby its mRNA expression level was significantly higher in *T. Vag* group than that in the control group (Figure 2, $p<0.05$). This finding was further supported by IHC staining for anti-proliferative protein p15. The staining intensity for p15 was much stronger in *T. Vag* group than in the control group (Figure 3). Thus, these data

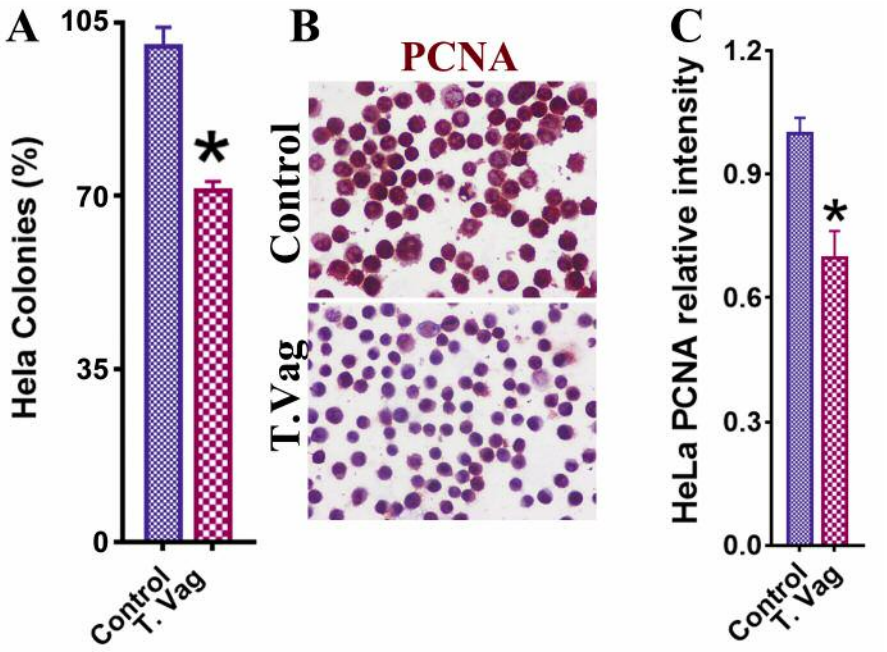

Figure 1. *Proliferation of HeLa cervical cancer cells was inhibited by culture supernatant of T. Vag. A: The clonogenic survival data for HeLa cells treated with T. Vag conditioned growth medium are shown. The number of colonies was counted and expressed as a percentage of total colonies in control (medium alone). B-C: Shown are IHC results for PCNA and staining intensity of HeLa cells treated with culture supernatant of T. Vag or medium alone. A significant difference in the percentage of colonies or staining intensity in each group compared to that in control is indicated by the asterisk ($p<0.05$). Original magnification in B: ×400.*

strongly indicated that up-regulation of p15 correlated with the inhibitory effect of *T. Vag* on proliferation of HeLa cells.

Culture supernatant of T. Vag induced apoptosis of HeLa cells. In our previous study (14), we found that the growth inhibitory effect of *T. Vag* on prostate cancer cells was also attributed to the apoptosis inducing effect by *T. Vag*. To address this possibility, 70% confluent HeLa cells were treated with culture supernatant of *T. Vag* or control medium alone for 3 days and apoptosis was evaluated by using two different methods, TUNEL staining (Figure 4A) and caspase-3 activity kit (Figure 4C). We found that the number of $TUNEL^+$ cells in the group treated with culture supernatant of *T. Vag* was much higher than that in the control group and this difference was significant (Figure 4B, $p<0.05$). To confirm this finding, apoptosis was evaluated by using caspase-3 activity kit, which showed that the relative caspase-3 activity in the group treated with culture supernatant of *T. Vag* was also significantly higher than that in the control group (Figure 4C, $p<0.05$). Clearly, these data indicated that *T. Vag* induced apoptosis of HeLa cells.

Culture supernatant of T. Vag up-regulated the expression of pro-apoptotic molecules in HeLa cells. We have previously shown that apoptosis is the result of an imbalance of pro- and anti-apoptotic molecules which favors the direction of apoptosis (14). To further investigate the potential molecular mechanisms by which *T. Vag* induced apoptosis of HeLa cells, mRNA expression of important pro- and anti-apoptotic molecules in HeLa cells was determined by RT-PCR. As shown in Figure 5, the mRNA expression of these molecules was comparable in both groups ($p>0.05$) except for Fas, TRAIL, TRAILR1 and FLIP. Their mRNA expression level was significantly higher in *T. Vag* group than that in the control group (Figure 5, $p<0.05$). In fact, the seemingly contradictory up-regulation of the anti-apoptotic molecule FLIP was not a surprise since a similar result had been observed in our previous studies (16, 21-23). Due to limited slides available, we randomly chose to perform IHC for TRAILR1 to confirm the data revealed by RT-PCR at a protein level. Results from IHC staining for TRAILR1 further supported the finding by RT-PCR (Figure 6). These results indicated up-regulation of pro-apoptotic molecule TRAILR1 as well as Fas and TRAIL correlated with the increased apoptosis in HeLa cells induced by *T. Vag*.

Discussion

This study focused on whether *T. Vag*. has any direct effect on proliferation and/or apoptosis of the HeLa cell line. We

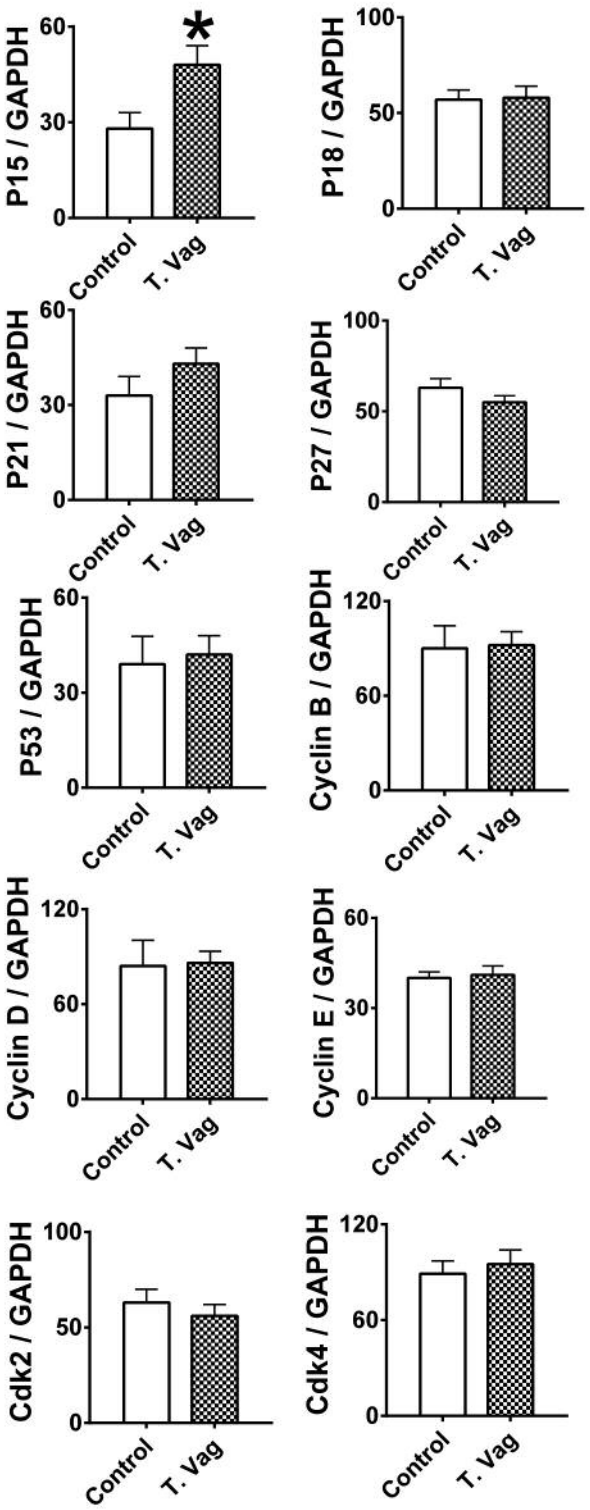

Figure 2. *Effect of culture supernatant of T. Vag on expression of pro- and anti-proliferative molecules evaluated by RT-PCR. Results are expressed as the mean ratio of pro- and anti-proliferative molecule densitometric Units/GAPDH + SEM (×100). A significant difference in mRNA expression between HeLa cells treated with culture supernatant of T. Vag and those in control is indicated by the asterisk (p<0.05).*

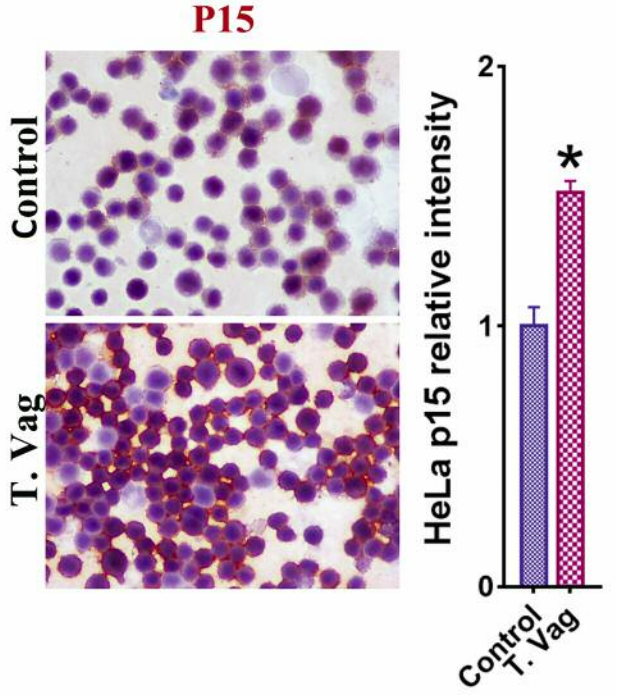

Figure 3. *Effect of culture supernatant of T. Vag on the expression of p15 evaluated by IHC. Representative pictures of IHC and the relative staining intensity are shown. Results are expressed as the average integrated staining intensity relative to that in control. A significant difference in staining intensity between cells treated with culture supernatant of T. Vag and those in control is indicated by the asterisk (p<0.05). Original magnification: ×400.*

found that culture supernatant of *T. Vag* inhibited the growth of cervical cancer cells and its potential mechanism might be correlated with up-regulation of p15, TRAILR1 as well as Fas and TRAIL. This study extends our previous study showing that *T. Vag* inhibited growth of prostate cancer cells and suggests that the growth inhibitory effect of *T. Vag* on cancer cells is not only limited to a type of cancer. To the best of our knowledge, our study is the first to directly demonstrate that there is a negative association between growth of HeLa cells and infection of *T. Vag*.

It has been more than 20 years since the first study proposed that there might be an association between *T. Vag* and cervical cancer (7). In the years that followed, some other studies supported this proposition. These studies suggested that *T. Vag* was related to a higher risk for the development of cervical cancer of a higher pathological grade and a more invasive type (7, 8, 11). The presence of *T. Vag* was even proposed to be a predictor for cervical cancer (7, 8, 11). These studies have been challenged by a study published in 2013 which proposed that *T. Vag* was not associated with promotion of HSIL and cervical cancer at all (13). Interestingly, the data

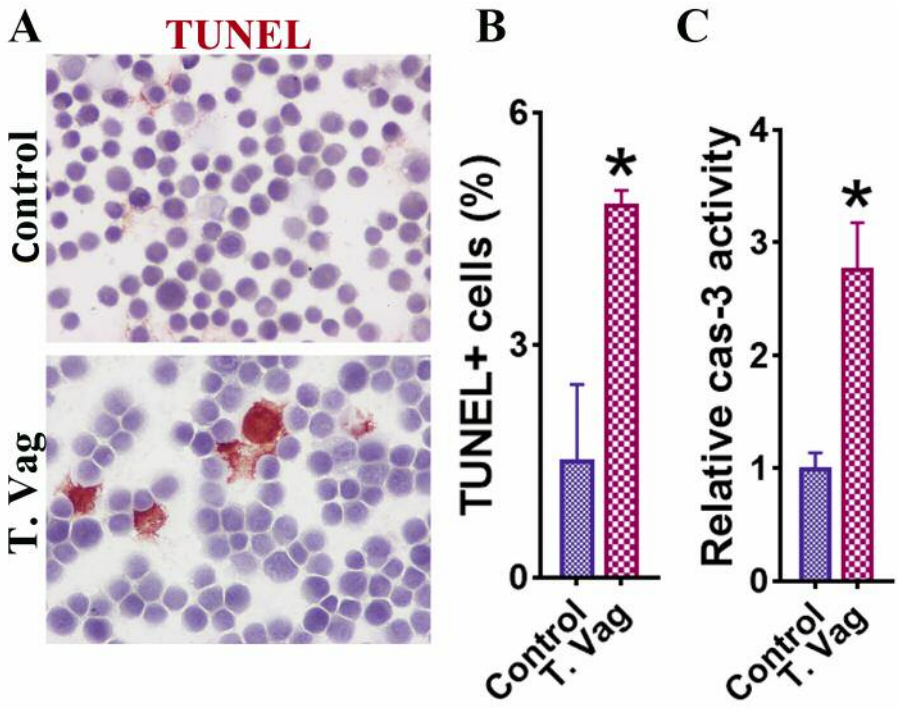

Figure 4. *Culture supernatant of T. Vag induced apoptosis of HeLa cells. A and B: Representative TUNEL staining and TUNEL+ cell counting are shown. C: Shown is cellular caspase-3 activity. Results are expressed as mean activity relative to control + SEM. A significant difference in the percentage of TUNEL+ cells or relative caspase-3 activity in cells treated with culture supernatant of T. Vag and those in control is indicated by the asterisk ($p<0.05$). Original magnification: A: ×400.*

presented here also challenge the concept that there might be a positive association between *T. Vag* and cervical cancer. In this study, we showed that culture supernatant of *T. Vag* inhibited the growth of HeLa cells *in vitro*, suggesting that *T. Vag* might inhibit growth of cervical cancer. In fact, our study is consistent with a study showing that *T. Vag* promoted apoptosis of cervical cancer cells by using Siha cells (15).

Cell proliferation is a process resulting in an increased number of cells. Cell proliferation is elaborately regulated by the balance between pro- and anti-apoptotic molecules (24-27). One of the hallmarks of neoplasia is uncontrolled proliferation (28). Cyclin B, D, E and cyclin-dependent kinase 2 and 4 (cdk2 and cdk4) are regarded as pro-proliferative molecules because they promote cell proliferation (29). Whereas, p15, p18, p21, p27 and p53 are traditionally regarded as anti-proliferative molecules because they inhibit cell proliferation (24, 25, 30). In this study, we found that the anti-proliferative effect of the cultured supernatant of *T. Vag* correlated with increased expression of p15. It is well-known that p15 is a critical anti-proliferative molecule encoded by CDKN2B that is frequently mutated and deleted in a variety of tumors such as acute lymphoblastic leukemia and melanoma (31-33). p15 is a cyclin-dependent kinase inhibitor, also known as p15Ink4b, which can form a complex with cdk4 or cdk6, and prevents the activation of the CDK kinases by cyclin D to inhibit cells from progressing from G1 to S phase (31, 34). As a result, up-regulation of this molecule would arrest cell cycle and subsequently result in cell growth inhibition. As shown in our study, *T. Vag* significantly increased p15 which was evidenced at both mRNA and protein levels. Thus, it is reasonable that the growth and proliferation of HeLa cervical cancer cells are inhibited in the presence of culture supernatant of *T. Vag*. In this study, among the the pro- and anti- proliferative molecules examined, only the expression level of p15was altered by following treatment with the culture supernatant of *T. Vag*, suggesting that p15 is a key target of *T. Vag* in HeLa cells.

The number of cell in tissues is not only determined by cell proliferation, but also by cell apoptosis (28). Apoptosis is mediated by the sequential activation of caspases and caspase-3 is a critical caspase activated in apoptosis (35, 36). Similar to TUNEL staining which detects the break of DNA, caspase-3 activity has also been regarded as a marker for apoptosis. In this study, besides TUNEL staining, an increased activity for caspase-3 was detected in HeLa cells after treatment with the culture supernatant of *T. Vag*. This strongly indicates the contribution of apoptosis to constrain the growth of HeLa

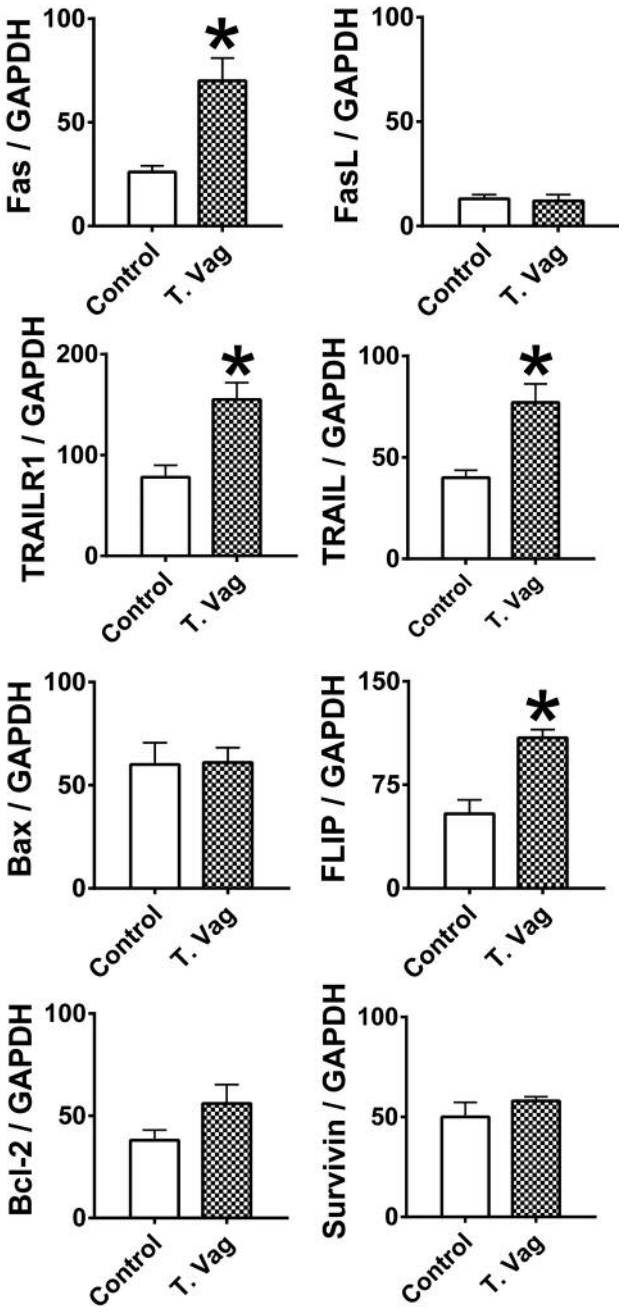

Figure 5. *Effect of culture supernatant of T. Vag on expression of pro- and anti-apoptotic molecules evaluated by RT-PCR Results are expressed as the mean ratio of pro- and anti-apoptotic molecule densitometric Units/GAPDH + SEM (×100). A significant difference in mRNA expression between cells treated with culture supernatant of T. Vag and those in control is indicated by the asterisk (p<0.05).*

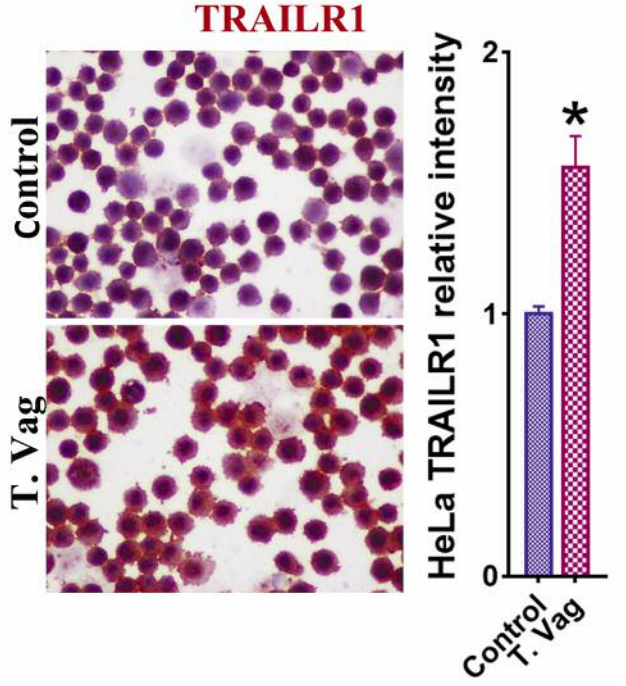

Figure 6. *Effect of culture supernatant of T. Vag on expression of TRAILR1 evaluated by IHC. Representative pictures of IHC and relative staining intensity are shown. Results are expressed as the average integrated staining intensity relative to that in control cells. A significant difference in staining intensity between cells treated with culture supernatant of T. Vag and those in control is indicated by the asterisk (p<0.05). Original magnification: ×400.*

cancer cells. Apoptosis is also a fine-tuned process orchestrated by the balance between pro- and anti-apoptotic molecules. Traditionally, Fas, FasL, TRAILR1, TRAIL and Bax are categorized as pro-apoptotic molecules because they promote cell apoptosis, whereas, FLIP, Bcl-2 and survivin are traditionally categorized as anti-apoptotic molecules because they inhibit apoptosis (18). Apoptosis could be triggered by two pathways, the extrinsic and intrinsic pathway. Both Fas/FasL and TRAIL/TRAILR signals induce apoptosis through classic extrinsic apoptotic pathway, that eventually leads to the activation of sequential caspases and induction of apoptosis (37). In this study, we showed a significant increase in the expression level of pro-apoptotic molecules TRAILR1, TRAIL as well as Fas in the *T. Vag* group. This suggests that *T. Vag* induces apoptosis of HeLa cervical cancer cells, not just by targeting one pro-apoptotic molecule, but by targeting multiple pro-apoptotic molecules. It has been shown that TRAIL/TRAILR pathway is one of the critical pathways for cancer cells to undergo apoptosis (37). TRAIL/TRAILR signal pathway is also targeted by cytokines when they induce apoptosis. We have reported that IL-9 and IL-32 promoted apoptosis by up-regulation of TRAILR1 (21, 22). In our previous study (14), culture supernatant of *T. Vag* promoted apoptosis through down-regulation of anti-apoptotic molecule Bcl-2, but not through modulation of other pro- or anti-apoptotic molecules. Furthermore, *T. Vag*. has been shown to induce apoptosis in SiHa cells through intrinsic pathway by

the mechanisms of the dissociation of Bcl-xL/Bim and Mcl-1/Bim complexes (15). All of these strongly suggest that the molecular mechanism by which *T. Vag* promotes apoptosis is complicated and might be cell line specific.

In this study, the expression of FLIP was found to be up-regulated by the culture supernatant of *T. Vag*. In fact, this is not a surprise to us at all since this seemingly contradictory finding has also been noticed in our previous studies (16, 21-23). The detailed possible mechanisms for this are still under investigation. We attribute this to an adaptive response of cancer cells to cell injury so that further damage to cancer cells could be minimized. Nevertheless, despite the fact that higher expression of FLIP induced by *T. Vag* in HeLa cancer cells may favor the survival of cancer cells, the overwhelming higher expression of Fas, TRAIL as well as TRAILR1 is predominating. This eventually results in the direction of apoptosis for HeLa cancer cells after treatment with supernatant of *T. Vag*.

Conclusion

In summary, *T. Vag* inhibits the growth of HeLa cells by modulation of p15, TRAILR1, TRAIL and Fas, all of which are critical molecules in cell proliferation or apoptosis.

Conflicts of Interest

The Authors have no conflicts of interest regarding this study.

Acknowledgements

This study was supported by grants for Yujiang Fang (Iowa Science Foundation Grant ISF 16-8, IOER 05-14-01, IOER 112-3749 and IOER 112-3104).

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Received July 5, 2018
Revised July 23, 2018
Accepted July 24, 2018