CHAPTER V

DISCUSSION AND CONCLUSION

The aim of this study was to investigate the possible effects of TCS on the aggressive population of H460 cells. TCS has been widely used for over 30 years in health care products as well as in medical devices. It is readily and completely absorbed following oral administration. TCS has been identified in urine, plasma, and breast milk of humans with wide range of levels depending upon the individual daily intake, the concentration in products as well as route and frequency of administration (2, 3). The low and high concentrations of TCS in human plasma was reported at 0.02 and 20 μg/ml (0.069 and 69 μM), respectively (4). About 30-35% of total TCS in human plasma was found as unconjugated form during the first 6 h after oral administration (5). Previous studies reported the possible effect of TCS in induction of hepatocellular adenoma and carcinoma formations in rodent model (105). Recently, Ma et al. (2013) found that TCS reduced global DNA methylation (GDM) in HepG2 cells which was proposed as the possible mechanism of TCS promoting tumor in rodents (98). Since global DNA hypomethylation is associated with aberrant gene expression, loss of imprinting, chromosome instability and anomalies, it has been shown to play a key role in controlling EMT and cancer metastasis (101). Thus, TCS induced global DNA hypomethylation raises a question whether TCS can promote cancer metastasis in the carcinogenesis safety aspect of this compound.

This study included 3 main parts: the effect of TCS on anoikis of H460 cells, the EMT-inducing effect of TCS on AR H460 cells and the effect of TCS-induced EMT

on cancer aggressiveness including tumorigenicity as well as migration and invasion of AR H460 cells. Before investigate the possible effect of TCS on H460 cells, cytotoxic effect of the compound on the cells was first characterized using MTT assay and nuclear staining assay. This study found that TCS at 0-7.5 μM was not cytotoxic to H460 cells in attachment condition. Comparing to other cell lines, Ma et al. (2013) demonstrated that TCS possessed non-cytotoxic effect on HepG2 cells at the concentrations ranging from 0-10 μM (98). This finding suggested that human lung cancer cells (H460 cells) were slightly more susceptible to the toxicity of TCS than human hepatocellular carcinoma cells (HepG2 cells). Therefore this concentration range of TCS (0-7.5 μM) was used for the subsequent experiments.

Overcoming anoikis after detachment from primary site has been considered as an important characteristic of cancer cells which have capability to form metastases (16, 17, 19, 20). Since TCS was found in human plasma following TCS exposure, the effect of TCS on anoikis process of cancer cells in detached condition was investigated using anoikis assay. This study found that TCS possessed no significant effect on cell anoikis of H460 cells after cells were detached. The result suggested that TCS at non-toxic concentrations had no effect on anoikis process of human lung cancer cells after detachment from primary site.

To investigate the effect of TCS on circulating tumor cells (CTCs) in systemic circulation, AR cells were employed as a model for studying CTCs according to several studies (102, 103, 106). In this study, AR cells formed multicellular aggregation in suspended condition. The expression of E-cadherin determined by western blotting revealed that AR cells still expressed high level of E-cadherin which is consistent with previous studies (102, 106). In several clinical reports, it was also found that sub-

population of CTCs was clinically found as multicellular aggregates with epithelial marker expression (91, 107).

The cytotoxic effect of TCS on AR cells was investigated using PrestoBlue assay and the results showed that TCS at 0-7.5 μM also caused no significant toxic effect on AR cells in detached condition. This result was consistent with the results from anoikis assay that TCS had no effect on cell viability after detachment. Therefore this concentration range of TCS was also used for the experiments of AR H460 cells.

However, this study found that treatment of the AR cells with TCS significantly decreased cell-cell adhesion of AR cells in detached condition. Loss of cell-cell adhesion is considered as the dominant EMT phenotype and caused by cadherin switching, the downregulation of E-cadherin and the increasing in the N-cadherin expression (8, 64, 73). The results from western blotting revealed that TCS mediated cadherin switching by decreasing E-cadherin level and increasing N-cadherin level (Figure 30 and 31, respectively). These results suggested that loss of cell-cell interaction in response to TCS treatment in AR cells was mediated by cadherin switching.

Importantly, TCS-treated cells possessed less epithelial phenotype and more mesenchymal phenotype indicated by the downregulation of E-cadherin expression and the upregulation of N-cadherin expression, respectively. Additionally, TCS-treated cells possessed mesenchymal phenotype confirmed by the increased expression of vimentin which is a type-III intermediate filament that highly expressed in mesenchymal cells (17, 69). TCS also enhanced expression of snail and slug which are the transcription factors that regulate cadherin switching and drive cells into EMT

process (12, 63, 71, 73). These results suggested that TCS promoted AR cells to undergo EMT as shown by the loss of cell-cell adhesion which is the dominant EMT phenotype as well as the increase of cadherin switching and EMT markers expression.

The possible mechanism of TCS to induce snail and slug expression in this case may be, at least in part, due to such effect of TCS on global DNA hypomethylation that found in the study of Ma et al. (2013) (98). TCS was shown to decrease the level of GDM in cancer cells (98). Global DNA hypomethylation is associated with aberrant activation of many genes and related to EMT process and stem-like phenotype of cancer cells resulting in enhanced cancer aggressiveness (101). The aggressiveness of CTCs has been clinically shown to be enhanced by EMT process and found in many types of cancers (16, 22). Therefore TCS was possible to promote aggressiveness of AR cells such as tumorigenicity and cell motility via EMT-inducing effect.

Tumorigenicity of cancer cells has been shown to be essential for cancer metastasis. EMT has been reported as a potential mechanism to promote tumorigenic ability which is a hallmark of cells with stem-like properties (85, 86, 108, 109). The results from this study showed that EMT induction in response to TCS significantly increased colony formation of AR cells while decreased proliferation rate of the cells (Figure 32 and 33). These results suggested that TCS promoted tumorigenic ability of AR cells mediated by EMT-inducing effect. Moreover, these results might refer to the possibility of TCS to induce stem-like properties of AR cells. Such results were consistent with the previous study reporting that the cancer cells undergoing EMT possess ability to survival in detached condition with decreased rate of growth (12, 13, 110, 111). The possible explanation on this phenomenon is that

EMT-related transcription factors are responsible for the attenuation of cell proliferation through the impairment of cell cycle progression (110, 111).

High ability to migrate and invade is the important hallmark of aggressive cancer cells (112, 113). EMT is recognized as an important factor facilitating cell motility (13, 37, 39). In this study, EMT induction in response to TCS promoted cell polarity, the front-rear polarity, which is normally found in migrating cells. Additionally, TCS-treated cells exhibited filopodia which stimulate cell migration at the border of cells. Moreover, TCS-mediated EMT enhanced migratory and invasive ability of AR H460 cells in transwell assay. These results suggested that TCS promoted cell motility of AR cells via EMT process. The results were consistent with the previous studies that EMT has been reported to cause a dramatic reorganization of actin cytoskeleton, an increase of filopodia formation and the concomitant formation of membrane protrusions and then result in the high migratory and invasive properties of cells (39, 88, 89).

Several signaling molecules play enhancing role in cell motility. The expression of migratory-related proteins including FAK, Akt and the downstream effectors including Rac1 and RhoA were investigated using western blotting. FAK plays the important role in dynamic turnover of focal adhesion of cells, increases filopodia formation and then modulates cell migration. FAK activation was reported to lead the phosphorylation of Akt resulting in cell movement (49, 53). FAK/Akt phosphorylation also activated the GTP-binding of several Rho-family GTPases such as Rac1 and RhoA. Rac1 is essential to stimulate the formation of membrane ruffling, lamellipodia and focal-complex formation and RhoA induces the formation of stress fibres and focal adhesion (45, 53). This study found that TCS-induced EMT

significantly promoted FAK/Akt activation as well as Rac1 activation. However, TCS possessed no effect on the level of RhoA-GTP. These results were consistent with previous reports that N-cadherin and slug were able to activate Akt pathway involving in cell survival and migration (8, 114). In addition, N-cadherin increased steady-state levels of activated Rac1 resulting in the increase of actin remodeling (8). Moreover, when cells undergo EMT, free p120-catenin in cytoplasm was recruited to N-cadherin on cell surface resulting in the activation of Rac1 and the subsequent inhibition of RhoA activation (39).

In summary, this study highlighted the effect of TCS to induce EMT process in AR cells (Figure 45). TCS-treated cells exhibited loss of cell-cell adhesion which is the hallmark of EMT phenotype. Importantly, TCS promoted cadherin switching and increased vimentin, snail, and slug which reflect mesenchymal phenotype of cells. In addition, TCS-mediated EMT promoted tumorigenicity of AR cells in anchorage-independent condition. Moreover, the study found that such TCS-mediated EMT enhanced ability of the cells to migrate and invade through FAK/Akt and Rac1-dependent mechanisms. Consequently, such findings show the potential effect of TCS to promote EMT in CTCs which may result in cancer aggressiveness. This study provides the new toxicological information of TCS leading to the careful use of TCS in cancer patients.

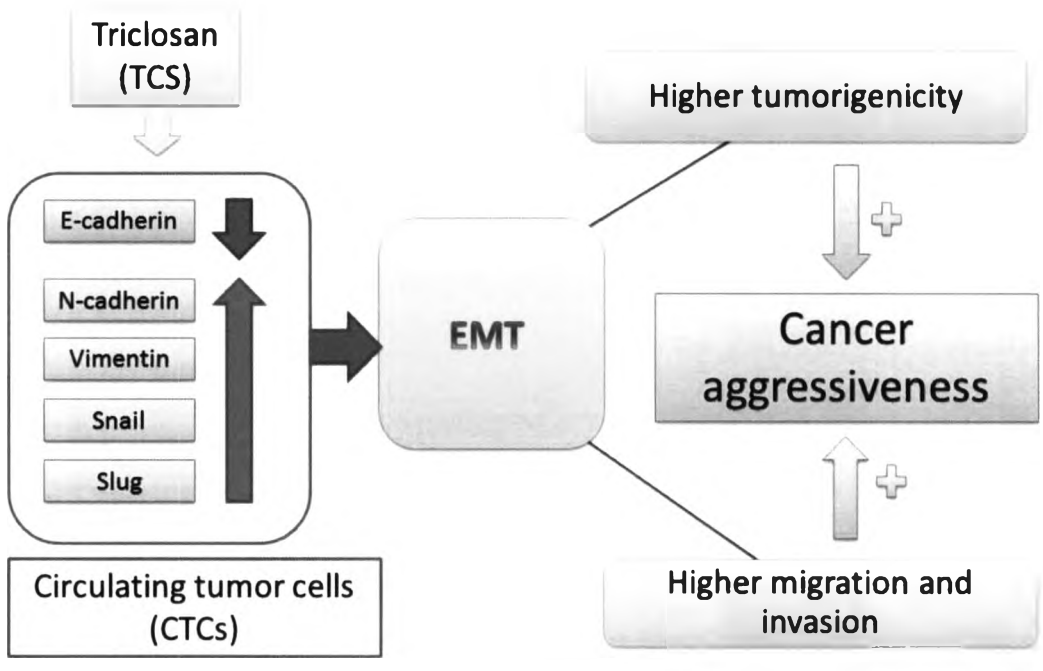

Figure 45 The conclude effects of triclosan (TCS) to induce EMT process in circulating tumor cells (CTCs). TCS was able to induce EMT process in circulating tumor cells and also promoted tumorigenicity as well as migration and invasion of cells which may result in increased cancer aggressiveness.