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ORIGINAL CONTRIBUTIONS

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EFFECTS OF IL-6, IL-10 AND TGF-β ON THE EXPRESSION OF SURVIVIN AND APOPTOSIS IN NASOPHARYNGEAL CARCINOMA TW01 CELLS

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The *aim* of this study is to investigate whether IL-6, IL-10 and TGF-β are able to confer resistance to apoptosis in nasopharyngeal carcinoma cells by upregulating the expression of survivin. *Methods:* The human nasopharyngeal carcinoma cell line TW01 (WHO NPC Type I) was cultured in DMEM-F12 Ham medium containing 10% FBS in a humidified atmosphere of 5% CO_2 and 37°C and treated with different concentrations of IL-6, IL-10 and TGF-β. Survivin mRNA expression was measured by real-time quantitative PCR and Western blot. Apoptosis was determined based on the assay for caspase-3 activity. *Results:* Of all the cytokines tested, only TGF-β (10 pg/mL) induced the over-expression of survivin at a significant level and this correlated with resistance to apoptosis ($p \leqslant 0.05$). To confirm if survivin is responsible for resistance to apoptosis, YM155 which is a survivin inhibitor was used and the results showed that YM155 abrogated the protective effect of TGF-β. Interestingly, IL-10 did not significantly alter the expression of survivin. *Conclusions:* We conclude that TGF-β up-regulates the expression of survivin leading to the resistance to apoptosis in NPC TW01 cells.

***Key Words*: apoptosis, interleukin 6, interleukin 10, nasopharyngeal carcinoma, survivin, transforming growth factor β (TGF-β).**

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells that line and covers the surface of the nasopharynx. It develops most often within the lateral nasopharyngeal recess or fossa of Rosenmuller [1]. NPC occurs more frequently in males as compared to females with incidence rates commonly two to three times higher in males as compared to his female counterparts. According to world area, incidence rates are highest in South-Eastern Asia, namely Hong Kong and Guangdong, and in other parts of Southern Asia namely, the Philippines and Thailand [2, 3]. Globally, the three highest national incidence rates are estimated in Malaysia, Indonesia, and Singapore, where rates are high among the Cantonese Chinese and Malay populations [4]. The prevalence of this malignancy are considerably lower in most populations Americas, Europe, Africa, Central and Eastern Asia.

The World Health Organization (WHO) divides NPC into three histological types based on their degree of differentiation with the WHO Type II being more prevalent in Asia and is strongly associated with Epstein — Barr virus (EBV) [5]. NPC has viral, genetic and environmental components to its etiology [6].

Analysis of NPC biopsies revealed the expression of a panel of cytokines: interleukin (IL)-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, TNF-α and TGF-β [7]. These cytokines are functionally important in shaping the NPC microenvironment. Differential blood levels of IL-6, IL-10 and TGF-β have been reported in patients with NPC as compared to healthy controls [8]. Thus, it is fascinating to know if these cytokines are involved in the promotion of malignant phenotype through one of the cardinal features of cancer that is, resistance to apoptosis.

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Abbreviations used: IL-6 – interleikin-6; IL-10 – interleukin-10; NPC – nasopharyngeal carcinoma; TGF-β - transforming growth factor beta.

Protection from apoptosis can be achieved through up-regulation of anti-apoptotic mediators such as survivin [9]. The expression of survivin in NPC cells was reported to be triggered by the Epstein — Barr virus latent membrane protein 1 (EBV LMP1) [10].

Survivin, also known as Baculoviral IAP Repeat-Containing Protein 5 (*BIRC5*), is a member of the inhibitors of apoptosis (IAP)/BIRP gene family [11]. Survivin or *BIRC5* is known to prevent cells from undergoing apoptosis by inhibiting caspases and promotes cell growth through stabilizing microtubules during cell mitosis [12]. In cancer cells, elevated Survivin is commonly associated with enhanced proliferative index [13, 14], reduced levels of apoptosis [15, 16], resistance to chemotherapy [17, 18], and increased rate of tumor recurrence [19].

The purpose of the present study was to investigate the effects of IL-6, IL-10 and TGF-β on the expression levels of survivin and subsequently its protective effect against apoptosis in the nasopharyngeal TW01 cells.

MATERIALS AND METHODS

Antibodies and reagents. In the study, antibodies (Ab) to survivin (Cell Signalling Technology, INC, USA), anti-rabbit IgG, HRP-linked Ab (Cell Signalling Technology, INC, USA) and survivin inhibitor, YM155 (Selleck Chemicals, USA) were used.

Cell culture. The human NPC cell line TW01 (WHO NPC Type I) was maintained in monolayer cultures in Dulbecco's Modified Eagle's medium (Gibco, California, USA) containing 10% fetal bovine serum (Gibco, California, USA) in a humidified atmosphere of 5% CO_2 and 37°C.

Cytokine treatment. Cytokines: IL-6, IL-10 and TGF-β with concentrations of 10 pg/mL, 100 pg/mL and 1000 pg/mL respectively were used. Duration of treatment was 3 h prior to any tests carried out on the cells.

RNA extraction and RNA quantification by spectral absorbance. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Germany) as per manufacturer's instructions. Total RNA was quantified using absorption of light at 260 and 280 nm (A260/A280) using the Infinite® 200 PRO NanoQuant (Tecan, Switzerland).

Reverse-transcriptase polymerase chain reaction (RT-PCR). Survivin mRNA expression was measured by RT-PCR. The first-strand cDNA was generated in a 20 μL RT reaction containing 1 μg of total RNA as template, random primers from the TaqMan® Gene Expression Assays (Applied Biosystems, California, USA), and TaqMan® RNA-to-C_T™ *1-Step* Kit reverse transcriptase (Applied Biosystems, Foster City, CA). The cDNA product was subjected to 40 cycles of amplification with appropriate primers using the iQ5 iCycler™ Real-time PCR system (Bio-Rad, USA). Cycling conditions were denaturation at 95°C for 15 s, annealing at 60°C for 1 min and extension at 60°C for 1 min. Amplification reaction for each sample and standard was performed in triplicates. The survivin primer used was *BIRC5* (assay ID Hs_00153353_m1) purchased from Applied Biosystems, California, USA. GAPDH expression was used as house-keeping gene; therefore, amplification signals for survivin mRNA were normalized to the respective amplification signals of GAPDH mRNA. The delta-delta CT ($2^{-\Delta\Delta CT}$) method was used for the quantification of mRNA levels.

Western blotting. Western blot was used to determine the expression of survivin in cells treated with TGF-β of 10 pg/mL concentration, cells treated with TGF-β of 10 pg/mL concentration together with survivin inhibitor, YM155 and non-treated cells. The protein concentration of cells was determined using the Bradford assay method. Survivin was detected using rabbit anti-survivin IgG followed by detection with chemiluminescent agent.

Apoptosis assay. Caspase-3 activity in cell lysates was measured using the Caspase-3 Colourimetric Assay kit™ (Sigma, USA) according to the manufacturer's protocols. Briefly, the caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-ValAsp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm (εmM = 10.5). The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm.

Statistical methods. The Wilcoxon rank-sum test was used to assess apoptotic index (AI) between TW01 treated cells and TW01 non-treated cells. Statistical analysis was performed using the SPSS 17.0 software. Generally, a p-value $\leqslant 0.05$ was considered to imply statistical significance.

RESULTS

Apoptotic index in NPC cells pre-treated with cytokines. The response of TW01 cells treated with different cytokines of different concentrations to apoptotic stimulus was assessed base on the enzymatic activity of caspase-3 [21]. Apoptosis was stimulated with staurosporine (1 μg/mL) from *Streptomyces sp*. (Sigma Aldrich, USA). Fig. 1 illustrates the apoptotic index of TW01 treated with different cytokines. The control consisted of cells that were not treated with cytokines. The apoptotic index was calculated from the ratio of induced cells divided by the non-induced cells. An average value from readings of triplicates was used.

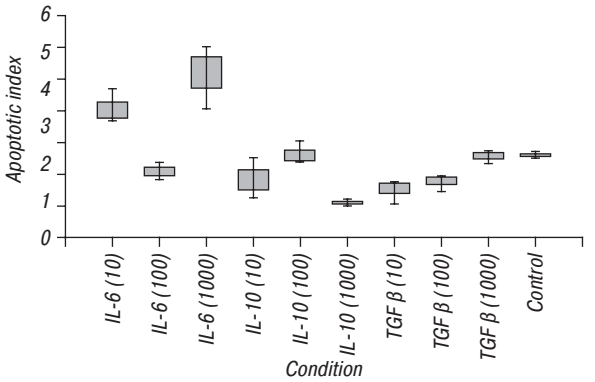

Fig. 1. Apoptotic index of TW01 cells treated with cytokines at concentrations: 10 pg/mL, 100 pg/mL and 1000 pg/mL respectively. The level of apoptosis correlates with the level of hydrolyzed pNA measured at 405 nm. Each boxplot represents the average value for assays conducted in triplicates**.** The symbol '*' indicates significant p-value $\leqslant 0.05$

TW01 cells that were treated with IL-6 (100 pg/mL), IL-10 (1000 pg/mL), TGF-β (10 pg/mL) and TGF-β (100 pg/mL) resulted in significantly lower apoptotic indices ($p \leqslant 0.05$), while IL-6 (10 pg/mL and 1000 pg/mL) generated significantly higher apoptotic indices. This comparison was made with the "control" which were cells not treated with any cytokine. There are cells which showed no significant findings: cells treated with IL-10 at 10 pg/mL ($p = 0.127$) and 100 pg/mL ($p = 0.513$) concentration and also TGF-b at 1000 pg/mL ($p = 0.827$).

Over-expression of survivin in TGF-β (10 pg/mL) treated cells. Over-expression of survivin in response to TGF-β (10 pg/mL) treatment was demonstrated in the results obtained from real-time quantitive-PCR. This correlated with the results from the apoptosis assay in which cells treated TGF-β had a lower apoptotic index as compared to the others.

Fig. 2 illustrates the gene expression of survivin in all TW01 cells treated with different cytokines of different concentrations. The $2^{-\Delta\Delta CT}$ method was used to quantify the mRNA levels. GAPDH expression was used as the house-keeping gene, whereby amplification signals for survivin mRNA were normalized to the respective amplification signals of GAPDH mRNA. Interestingly, survivin mRNA expression was increased in TW01 cells treated with TGF-β (10 pg/mL). The normalized fold expression of TGF-β (10 pg/mL) was 11.31 (SD ± 2.98). The differential expression of survivin in all the other treated cells was not sigbnificant. An inverse correlation between survivin expression and apoptotic index was noted in a study conducted by Rodel et al. [22], high survivin expression was associated with low median apoptotic index. Conversely, low survivin expression was associated with a high median apoptotic index .

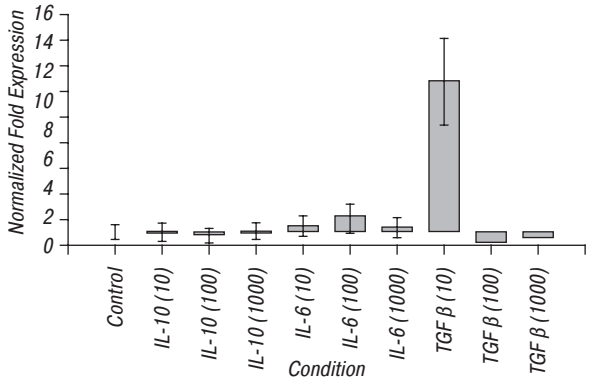

Fig. 2. Normalized fold expression of survivin mRNA levels in TW01 treated cells. Total mRNA of TW01 cells were extracted. Level of survivin mRNA was determined by real-time PCR (RT-PCR). The delta-delta CT ($2^{-\Delta\Delta CT}$) method was used for the quantification of mRNA levels. Survivin mRNA levels in cells treated with TGF-β (10 pg/mL) was elevated to 11.31 with a standard deviation value of 2.98

Survivin inhibitor reverses resistance to apoptosis effect. Protection from apoptosis can be achieved through the up-regulation of anti-apoptotic mediators such as proteins from the INK4 family [23] and survivin [9]. In order to ascertain that survivin is the anti-apoptotic mediator responsible for the protection of cells against apoptosis, a survivin inhibitor, YM155 was incubated together with TGF-β (10 pg/mL) together with staurosporine for three hours prior to the apoptosis assay. The final concentration of YM155 used was 500 nmol/L. As observed, cells treated only with TGF-β (10 pg/mL) had a low apoptotic index (AI) while in those treated with YM155 the apoptotic index was seen to increase as survivin was inhibited in comparison to the control which was neither treated with TGF-β (10 pg/mL) nor YM155.

Expression of survivin analyzed via Western blotting. Western blotting was performed in order to investigate the corresponding expression of survivin at the protein level. In agreement with results from RT-PCR (Fig. 2), the protein levels of survivin were higher in cells treated with TGF-β (10 pg/mL) and significantly reduced in the presence of YM155 (Fig. 3).

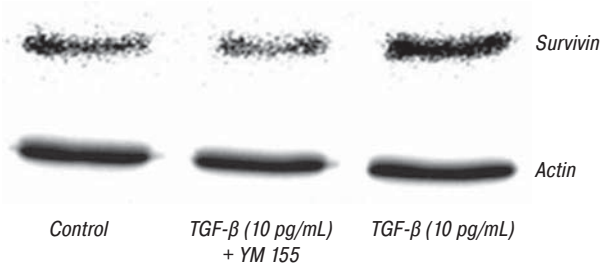

Fig. 3. Western blotting analysis of the expression of Survivin at the protein level. TW01 cells were subjected to 3 forms of treatment i.e. those treated with TGF-β (10 pg/mL), those treated with TGF-β (10 pg/mL) + YM155 and those not treated, named control in the diagram. Western blotting was then performed to analyze the expression of survivin at the protein level. Actin expression served as the control

DISCUSSION

The ability of cancer cells to by-pass apoptosis and continue to proliferate is one of the fundamental hallmarks of cancer development and progression [24]. Effector caspases are caspases that execute apoptosis [25, 26]. In normal conditions, caspase activity is held in check by the IAP protein family, namely survivin, XIAP, cIAP2 etc and c-FLIP [27]. Although these proteins are not directly involved in the apoptotic signaling pathway, cell death can be averted via the suppression of initiator and effector caspase activity. It has been reported that the IAPs: survivin and cIAP1 are over expressed in several malignancies [28].

This leads us to the objective of this study which is to investigate the effects of IL-6, IL-10, and TGF-β on the expression of survivin and its protective effect against apoptosis. Several studies have been done on cytokines: IL-1, IL-2, IL-3, IL-5 and IL-8 and their relations to NPC [7, 29, 30]. There are also some studies done on our cytokines of interest, however, to date, no investigation had been carried out investigate the effects of IL-6, IL-10, and TGF-β of 3 different concentrations respectively on the expression of survivin.

The concentration of IL-6, IL-10, and TGF-β chosen for this study was based on a previous study conducted by Tan et al. [8]. In that study, levels of IL-6, IL-10, and TGF-β were found to differ amongst NPC patients pre- and post-treatment. Results from that investigation showed that high levels of IL-6 (164 ± 37 pg/mL) were seen in untreated NPC patients indicating that high IL-6 levels played a role in tumor progression. As for IL-10, there were no variations in the cytokine levels of plasma in pre- and post-treated NPC patients. In untreated patients, lower levels of TGF-β (6368 ± 682 pg/mL) were recorded, indicating that lower levels of TGF-β played a role in tumor progression. Therefore, it was decided for each cytokine, the concentrations used would be 10 pg/mL, 100 pg/mL and 1000 pg/mL.

In our study, it was found that apoptotic indices of cells treated with different cytokines were concentration-dependent. IL-6 is a growth factor that regulates cell proliferation, metabolism and survival [31] and is known to play pivotal roles in cell differentiation and tumor progression [32]. Nevertheless, its involvement in cancer is quite dubious, as IL-6 can play both tumor-suppressive roles as well as tumor-progressive roles, depending on the type of cancer involved [33, 34]. As observed in Fig. 1, IL-6 at concentrations of 10 pg/mL and 1000 pg/mL resulted in apoptotic indices that were significantly higher as compared to the non-treated cells ($p \leqslant 0.05$). On the other hand, the apoptotic index of cells treated with IL-6 (100 pg/mL) was significantly lower ($p \leqslant 0.05$). This showed that in concentrations of 10 pg/mL and 1000 pg/mL, IL-6 did not confer protection against apoptosis as high apoptotic indices were observed, nonetheless, IL-6 in concentrations of 100 pg/mL conferred protection against apoptosis.

This phenomenon can also be observed in IL-10 treated cells, where at certain concentrations, the apoptotic index was low while at other concentrations, the apoptotic index was high. At concentrations of 10 pg/mL and 100 pg/mL, apoptotic indices were non-significant ($p > 0.05$) while at the concentration of 1000 pg/mL, a significantly lower ($p \leqslant 0.05$) than the control apoptotic

index was observed.This can be explained in an animal study by Stuhler et al. [35] showing that IL-10 can either promote or inhibit the progression of tumors.

The question to whether TGF-β plays a tumor-suppressive or tumor-promoting role is still unknown. However, based on this study, the apoptotic index in TW01 cells was correlated positive to increasing concentrations of TGF-β (Fig. 1). It can be concluded that TGF-β (10 pg/mL) and TGF-β (100 pg/mL) protected the cells from apoptosis with a significantly lower apoptotic index as compared to the control ($p \leqslant 0.05$) but not at TGF-β 1000 pg/mL.

Numerous reports have examined the role of survivin in cancer. Survivin, a dimericbaculovirus inhibitor of apoptosis is a newly discovered IAP which plays an important role in both inhibition of apoptosis and cell division regulation [36]. Survivin plays an important role in the suppression of apoptosis by either directly or indirectly inhibiting the activity of caspases [27]. Caspases are cell death proteases that induce apoptosis. There are studies indicating that survivin directly binds to caspase-3, inhibiting its activity *in vitro* [37–39].

In the present study, we found that TGF-β of 10 pg/mL concentration up regulated the expression of survivin. The results from RT-PCR showed an astounding 11.31 normalized fold increase in the expression level (Fig. 2). As for the other cells treated with IL-6 and IL-10 at different concentrations, no significant increase in survivin expression was observed. The findings in our study were in line with that of Rodel et al. as the apoptotic index in cells treated with TGF-β of 10 pg/mL concentration was amongst the lowest.

To determine if survivin was solely responsible for the resistance to apoptosis in cells treated with TGF-β (10 pg/mL), a co-treatment with survivin inhibitor, YM155 was performed. YM155 is an inhibitor of the survivin expression [40]. As evident in Fig. 2, the protein levels of survivin were high in cells treated with TGF-β (10 pg/mL) and significantly reduced in the presence of YM155 and this correlated to the abrogation of resistance to apoptosis (Fig. 4).

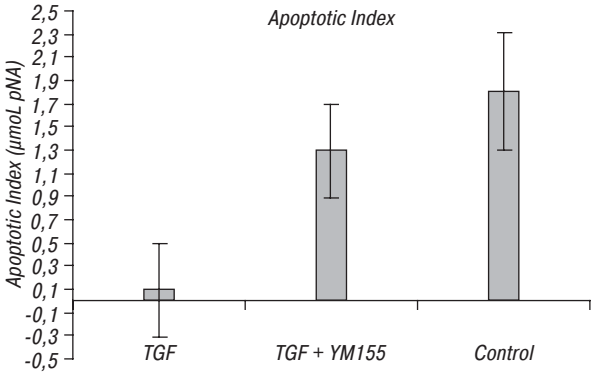

Fig. 4. Apoptotic index of TW01 cells treated with TGF-β (10 pg/mL), YM155 and non-treated cells. Bar chart reveals that cells treated only with TGF-β (10 pg/mL) had a low apoptotic index (AI) while in those treated with YM155; the apoptotic index was seen to increase as survivin is inhibited in comparison to the control which was neither treated with TGF-β (10 pg/mL) nor YM155. Each bar represents the average value for assays conducted in triplicates

We conclude that TGF-β conferred protection to apoptosis in NPC TW01 cells by up-regulating the expression of survivin. IL-6 and IL-10 did not significantly alter the expression of survivin, and thus did not confer protection against apoptosis.

COMPETING INTERESTS

The authors report no conflicts of interest with people or organizations that could inappropriately influence the work. The authors did not receive any outside assistance writing this manuscript.

AUTHORS' CONTRIBUTIONS

TEL: conceived, designed and coordinated the study. YW: conducted the experiments and prepared the manuscript. SY: contributed with study design. All authors read and approved the final manuscript.

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