Characterization of tumorspheres generated from nasopharyngeal carcinoma cell line, TW06 and chemoresistance to docetaxel and oxaliplatin

Iris Goh Wen Li,1 Yip Wai Kien,1 Seow Heng Fong*,1
1Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor

ABSTRACT
In this study, tumorspheres were generated from TW06 nasopharyngeal carcinoma cell line and examined their expression of putative cancer stem-like cell surface markers and drug sensitivity. The rate of tumorsphere expansion from dissociated late passage TW06 tumorspheres (≥ passage 15) was higher than that from parental cells and dissociated 10-day-old (passage 0) tumorspheres. The expression of CD24 surface marker was lost in the generation of tumorspheres and the loss was reversible after differentiating the tumorspheres in monolayer culture conditions. Drug sensitivity assay showed that late passage tumorspheres were resistant to docetaxel and oxaliplatin treatment. Our data suggest that serially passaged tumorspheres possess the characteristics of CSCs that render them a suitable preclinical in vitro model for evaluating anticancer drug efficacy and elucidating the underlying mechanisms of drug resistance.

Keywords: Tumorspheres, Cancer stem cells, Nasopharyngeal carcinoma, Chemoresistance.

INTRODUCTION
Tumor recurrence and metastasis are two of the major obstacles in cancer treatment which results in mortality. Cancer stem-like cells (CSCs) are widely linked to tumorigenesis and metastasis.1-3 In addition, these cells have also been proposed to be linked to chemoresistance and also resistance to radiotherapy.4-8 According to the CSC model, tumor is initiated by a subpopulation of cancer cells termed as cancer stem-like cells. These CSCs have intrinsic properties that are identical to normal stem cells which includes longevity and self-renewing ability. Normal adult tissues have a small portion of stem cells that play a role in the replacement of terminally differentiated cells. During self-renewal, these stem cells generate an identical stem cell and also a progenitor cell which will further give rise to a number of differentiated cells. Similarly, these cancer stem cells have the ability to initiate tumor in immune-deficient mice. CSCs were first identified in leukemia and later found in a wide variety of solid tumors. One of the methods that is frequently used as a way to maintain these CSCs in vitro is to culture them in anchorage-independent conditions as tumorspheres.9 This culture method is originally established from a neural cell activity assay.10 This method has been adapted into many other studies that are linked to CSCs.

Nasopharyngeal carcinoma occurs more frequently in regions of South East Asia.11 Most of the mortality in NPC patients is believed to be due to distant metastasis and local recurrence of the cancer.12 Chemotherapies have been developed based on the ability of these chemotherapeutic agents to cause regression of tumor. Cisplatin combined with 5-fluorouracil has been widely used as a standard regimen for metastatic NPC.13-15 However, cisplatin-based chemotherapy are often associated with increased and acute toxicities. Newer agents such as taxanes, gemcitabine and capecitabine exert more effective antineoplastic activities in both NPC and other head and neck cancers.16-18 Docetaxel is a member of the taxane drug class which shows activity against a variety of solid tumors including breast, lung and squamous cell head and neck cancers. The ability of these drugs in NPC has been studied in combination with platinum drugs in both metastatic/recurrent and locally advance cancer.19,20 Oxaliplatin (OXA), on the other hand, blocks DNA replication and transcription by forming intrastrand cross-links in DNA. Resistance towards chemotherapeutic agent which is one of the characteristics of CSC, is thought to be one of the main causes of cancer recurrence. Although many other theories have attempted to explain chemoresistance, the CSC theory has attracted much interest. Since CSCs are believed to be only less than 10% of the total tumor population, tumor regression by chemotherapeutic drug is expected to be mainly due to the elimination of the non-CSC population. This allows CSCs to remain after chemotherapy and they are able to regenerate the tumor causing tumor recurrence. CSCs can also be progenitor cells in the bulk tumor by going through self-renewal and cell division which causes metastasis.

*Corresponding author: Prof. Dr. Seow Heng Fong
shf@upm.edu.my

Malaysian Journal of Medicine and Health Sciences Vol. 11 (2) June 2015
Knowledge concerning the changes that cause chemoresistance in CSC are limited. The effects of chemotherapeutic agents on CSC, the changes that these drugs induce in CSC, the association between CSC and proteins with drug-resistance, and also the relationship between CSCs and anti-apoptotic genes provides a better understanding on the characteristics of CSC. This will facilitate more effective design of future therapies which target CSCs. Although there are already many studies on CSCs and its properties, few reports are currently available on tumorspheres generated from nasopharyngeal carcinoma cell lines.

The objectives of this study were to compare the expression of putative CSC surface markers in tumorspheres from TW06 versus its parental cells in monolayer culture and to determine the drug sensitivity of the tumorspheres towards common chemotherapeutic agents.

**MATERIALS AND METHODS**

**Cell line**

The human nasopharyngeal carcinoma cell line, TW06, was provided by Prof. Chin-Tarng Lin (National Taiwan University, Taipei, Taiwan). The cells were grown and maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2g/L sodium bicarbonate, 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified incubator with 5% CO₂. All these reagents were purchased from Thermo Fisher Scientific Inc., Waltham, MA (Life Technologies, Gibco, USA), except for sodium bicarbonate (Sigma-Aldrich, St. Louis, MO). Subculture was done every 3-4 days.

**Tumorsphere and monolayer cultures**

Tumorspheres were generated by plating 3x10⁴ TW06 cells in 35-mm-diameter non-tissue culture-treated petri dish pre-coated with poly(2-hydroxyethyl methacrylate) (poly-HEMA) (Sigma-Aldrich). The cells were grown in serum-free DMEM/F-12 medium (Gibco) containing 1.24% methylcellulose (Sigma-Aldrich), supplemented with 0.5% bovine serum albumin (BSA) (Amresco, Solon, OH), B27 (1:50 dilution)(Gibco), 10 µg/mL insulin (Sigma-Aldrich), 5.5 µg/mL transferrin (Sigma-Aldrich), 0.48 µg/mL hydrocortisone (Sigma-Aldrich), 5 ng/mL selenium (Sigma-Aldrich), 4 µg/mL heparin (Sigma-Aldrich), 20 ng/mL basic fibroblast growth factor (bFGF)(Gibco) and 20 ng/mL epidermal growth factor (EGF)(Gibco). The cells were incubated at 37 °C in a humidifier incubator with 5% CO₂. Tumorspheres were passaged every 10 days by dissociating the intact floating spheres with Accutase (Gibco) and replating the single cells in new dishes containing fresh medium.

In monolayer culture of TW06 cells for experiments, the cells were grown in standard tissue culture dishes or plates containing DMEM/F-12 medium with 10% FBS at 37 °C in a humidified incubator with 5% CO₂.

**Cell surface phenotyping by flow cytometry**

The expression of CD44, CD24 and EpCAM on cell surface was assessed using flow cytometric analysis. Tumorspheres and cell monolayer were dissociated using Accutase and resuspended in PBS containing 0.5% BSA (PBS/BSA). Phycoerythrin (PE)-conjugated mouse anti-human CD24 (clone ML5; BD Biosciences, San Jose, CA), allophycocyanin (APC)-conjugated mouse anti-human CD44 (clone G44-26; BD Biosciences), and Alexa Fluor 488-conjugated mouse anti-human EpCAM (clone VU1D9; Cell Signaling Technology, Danvers, MA) monoclonal antibodies were added to the cell suspension at a dilution recommended by the manufacturers, followed by incubation for 30 min at 4 °C in the dark. Alexa Fluor 488-conjugated mouse IgG1 (BD Biosciences), PE-conjugated mouse IgG2a (BD Biosciences), and APC-conjugated mouse IgG2b (Pierce, Thermo Scientific, Thermo Fisher Scientific Inc., MA, USA) isotype control antibodies were used for background staining. Flow cytometry was performed using FACSCalibur™ instrument equipped with CellQuest software (BD Biosciences). Approximate 10,000 cells were acquired and the percentages for different cell populations were determined using CellQuest software.

**Cell viability assay**

Drug sensitivity of parental TW06 cell monolayer was assessed by the methyl-thiazolyl-tetrazolium (MTT) assay. The cells were plated in 96-well tissue culture plates at 500 cells per well. After 24 h to allow cell attachment, the cells were treated with increasing concentrations of 5-fluorouracil, oxaliplatin, gemcitabine or docetaxel (all from Selleck Chemicals, Houston, TX) in 100 µL of medium per well. After 3 d of treatment at 37 °C, 10 µL of MTT solution (5 mg/mL) (Amresco, Solon, OH) was added to all the wells and the plates were further incubated for 4 h. The formazan
product in each well was dissolved by 200 µL of DMSO after the removal of the media. The absorbance was measured at 570 nm wavelength using a microplate spectrophotometer (Dynex Technologies, Chantilly, VA). Cell viability was expressed as percentage of absorbance relative to the control cells. The drug concentration required to decrease the cell viability by 50% (IC$_{50}$) was determined by interpolation from the dose-response curves. All treatments and controls were done in triplicate wells.

**Drug sensitivity assay for tumorspheres**

Tumorspheres and cell monolayer were dissociated with Accutase and plated into the poly-HEMA-coated 96-well plates at a density of 5,000 cells per well, with or without drugs in DMEM/F-12 medium containing methylcellulose and supplements (similar to the tumorsphere culture conditions). Photomicrographs were captured at a magnification of 40X on day 0 (day of plating and treatment), day 3, and day 9 using Olympus IX51 inverted microscope equipped with Olympus XC50 camera (Olympus Corporation, Tokyo, Japan). Tumorsphere size was evaluated by using the area parameter in the Image-Pro Plus software (Media Cybernetics, Rockville, MD). Three independent experiments were performed in triplicate wells.

**Statistical Analysis**

Statistical analysis was performed using GraphPad InStat software for Windows (version 3.05; GraphPad Software, San Diego, CA). A two-sided P<0.05 was considered statistically significant.

**RESULTS**

**Immunophenotyping of TW06 tumorspheres**

Tumorspheres were grown from a nasopharyngeal carcinoma cell line, TW06 according to the methods as described by Dontu et al. [9] with modifications. Tumorspheres generated after 10 days of culture (defined as passage 0) showed loose cell aggregates while serially passaged (≥ passage 15, defined as late passage) tumorspheres appeared to be more compact and in regular shape (Figure 1).

![Figure 1](image-url)

We investigated the expression of three putative CSC surface markers, namely, CD24, CD44 and EpCAM on cells from the monolayer cell culture, 10-day-old tumorspheres (passage 0) and late passage tumorspheres. Flow cytometric analysis showed that parental TW06 monolayer cells mainly (~90%) consisted of CD24$^+$CD44$^+$EpCAM$^+$ population, while passage 0 and late passage tumorspheres were largely (~87%) composed of CD24$^+$CD44$^+$EpCAM$^+$ cells (Table 1). This indicates loss of CD24 expression on tumorspheres. Although there were statistically significant differences in the CD24$^+$CD44$^+$EpCAM and CD24$^+$CD44$^+$EpCAM populations between parental cell monolayer, passage 0 tumorspheres, and late passage tumorspheres, the 1-5% differences were not drastic.
Table 1. Comparison of the expression of CD24, CD44 and EpCAM between TW06 monolayer culture and tumorspheres at different passages.

<table>
<thead>
<tr>
<th>Population</th>
<th>Parental cell monolayer (%)</th>
<th>Passage 0 (%)</th>
<th>Late passage (%)</th>
<th>Differentiated latepassage(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>0.59±0.41</td>
<td>5.80±0.92***</td>
<td>1.14±0.20</td>
<td>4.74</td>
</tr>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>0.09±0.08</td>
<td>0.00±0.01</td>
<td>0.03±0.02</td>
<td>0.28</td>
</tr>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>0.01±0.01</td>
<td>1.23±0.49**</td>
<td>0.03±0.03</td>
<td>0.41</td>
</tr>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>0.02±0.03</td>
<td>0.00±0.00</td>
<td>0.00±0.01</td>
<td>0.18</td>
</tr>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>0.58±0.30</td>
<td>2.38±0.53</td>
<td>6.37±4.70</td>
<td>1.31</td>
</tr>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>1.56±1.38</td>
<td>0.16±0.05</td>
<td>3.31±2.49</td>
<td>0.63</td>
</tr>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>6.27±1.19</td>
<td>87.84±3.01***</td>
<td>86.14±3.87***</td>
<td>16.16</td>
</tr>
<tr>
<td>CD24^−CD44^-EpCAM^-</td>
<td>90.36±1.13</td>
<td>0.90±0.38***</td>
<td>2.13±0.52</td>
<td>76.37</td>
</tr>
</tbody>
</table>

Note: Data represents the means ± SD of three independent experiments except for data which are from one experiment. Statistical analysis was performed by One-way ANOVA followed by Tukey–Kramer multiple comparisons test (**P<0.01, ***P<0.001 vs. corresponding parental cell monolayer; ##P<0.01, ###P<0.001 vs. corresponding passage 0 tumorspheres).

In order to show whether the late passage tumorspheres have the ability to undergo differentiation toward their parental phenotype, the tumorspheres were dissociated and cultured in a condition similar to that in culturing parental cell monolayer. After growing for 3 days, the monolayer of cells was dissociated from the surface of petri dish using Accutase and harvested for immunophenotyping. We observed a decrease in the CD24^−CD44^-EpCAM^- population and an increase in the CD24^−CD44^-EpCAM^- population during differentiation (Table 1), indicating that the late passage tumorspheres have the ability to re-express the immunophenotype of parental monolayer cells.

Growth-inhibitory effect of chemotherapeutic agents on TW06 cell monolayer and tumorspheres.

The effects of four chemotherapeutic drugs [5-fluorouracil (5-FU), oxaliplatin (OXA), gemcitabine (GCB) and docetaxel (DTX)] at various concentrations were first examined on the growth of parental TW06 cells in adherent monolayer culture. The drug concentration that decreased the viability of cells by 50% (IC_50) was obtained from the dose-response curve and presented in Table 2.
Characterization of tumourspheres generated from nasopharyngeal carcinoma cell line, TW06 and chemoresistance to docetaxel and oxaliplatin

Table 2. IC$_{50}$ value of the chemotherapeutic agents tested in monolayer culture of TW06 cells.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Fluorouracil</td>
<td>6.5 µM</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>15 nM</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.2 nM</td>
</tr>
</tbody>
</table>

Note: Viable cell densities were assessed using MTT assay after 3 days of drug treatment at various concentrations. IC$_{50}$ values were determined from the generated dose-response curves. All treatments and controls were done in triplicate wells.

We next evaluated the inhibitory effect of these drugs at their respective IC$_{50}$ on the tumoursphere formation from parental adherent cells and dissociated passage 0 and late passage tumourspheres. After 9 days of treatment, we observed marked differences in tumoursphere size between treated and non-treated tumourspheres (Figure 2). These differences were not obviously seen on day 6 of treatments. We further analyzed the data by calculating the expansion rate of tumourspheres between day 3 and day 9 (Table 3). In growth condition without drug treatments, late passage tumourspheres expanded ~4-fold faster than tumourspheres from passage 0 and parental cells (Table 3). 5-FU (6.5 mM) treatment significantly inhibited the tumoursphere expansion from the parental cells and the dissociated tumourspheres at passage 0 and late passage (Figure 2 and Table 3). Late passage tumourspheres, but not passage 0 tumourspheres and parental cells, were sensitive to the inhibitory effect of GCB (0.3 mM) on tumoursphere expansion (Figure 2 and Table 3). However, OXA (15 nM) and DTX (0.2 nM) were not effective in suppressing tumoursphere formation in the parental cells and the dissociated passage 0 and late passage tumourspheres (Figure 2 and Table 3).
Figure 2. Effect of 5-FU (6.5 mM), OXA (15 nM), GCB (0.3 mM) and DTX (0.2 nM) chemotherapeutic drugs on tumorsphere expansion from parental cells and dissociated passage 0 and late passage tumorspheres. Passage 0 tumorspheres were generated from parental cells in 10 days. Late passage tumorspheres were generated through serial passaging of tumorspheres (passage ~ 25-27). Tumorspheres and parental cell monolayer were dissociated into single cells and plated in tumorspheres culture conditions with or without treatment. Images of the tumorspheres were
captured on day 0 (day of plating and treatment), day 3 and day 9. The sizes of tumorspheres were quantified using Image-Pro Plus software and expressed relatively to tumorsphere size on day 0 (set as 1). Each bar represents the mean ± SD of three independent experiments performed in triplicates. The differences of relative tumorsphere size between treated and non-treated tumorspheres on day 9 were analyzed using unpaired t-test (*P<0.05, **P<0.01, ***P<0.001).

Table 3. Effect of chemotherapeutic agents on expansion rate of tumorspheres

<table>
<thead>
<tr>
<th>Drug, concentration</th>
<th>Parental cells</th>
<th>Passage 0</th>
<th>Late passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.61 ± 1.63***</td>
<td>4.51 ± 1.66***</td>
<td>17.57 ± 0.93</td>
</tr>
<tr>
<td>5-FU, 6.5 mM</td>
<td>1.08 ± 0.58*</td>
<td>1.47 ± 0.68*</td>
<td>1.51 ± 0.65***</td>
</tr>
<tr>
<td>OXA, 15 nM</td>
<td>4.45 ± 0.92</td>
<td>5.51 ± 0.67</td>
<td>17.67 ± 2.23</td>
</tr>
<tr>
<td>GCB, 0.3 mM</td>
<td>6.60 ± 0.77</td>
<td>3.29 ± 0.91</td>
<td>11.93 ± 1.67 **</td>
</tr>
<tr>
<td>DTX, 0.2 nM</td>
<td>2.39 ± 1.36</td>
<td>2.11 ± 0.94</td>
<td>16.88 ± 3.44</td>
</tr>
<tr>
<td>DTX, 0.5 nM</td>
<td>-0.17 ± 0.40**</td>
<td>-0.18 ± 0.29*</td>
<td>7.85 ± 1.53 ***</td>
</tr>
<tr>
<td>DTX, 1 nM</td>
<td>-0.43 ± 1.26*</td>
<td>-0.48 ± 0.99*</td>
<td>0.25 ± 0.30 ***</td>
</tr>
<tr>
<td>DTX, 2 nM</td>
<td>-0.42 ± 0.25*</td>
<td>-0.40 ± 0.21*</td>
<td>0.00 ± 0.17***</td>
</tr>
</tbody>
</table>

Note: The expansion rate of tumorspheres were calculated by using the formula, , where \( V_1 \) and \( V_2 \) denote the relative size of tumorspheres in an earlier timepoint, \( T_1 \)= day 3 and a later timepoint, \( T_2 \)=day 9, respectively. Data represent the means ± SD of three independent experiments, each performed in duplicate. Statistical analysis was performed by unpaired t-test (*P<0.05, **P<0.01, ***P<0.001 vs. corresponding control) or One-way ANOVA followed by Tukey–Kramer multiple comparisons test (+++P<0.001 vs. late passage tumorspheres). 5-FU, 5-fluorouracil; OXA, oxaliplatin; GCB, gemcitabine; DTX, docetaxel.

Although not statistically significant, the DTX (0.2 nM) decreased tumorsphere expansion rate by ~2-fold in passage 0 tumorspheres and parental cells, as compared to controls, whereas the tumorsphere expansion rate in late passage tumorspheres was not affected (Table 3). We noticed an outlier in one of the repeated experiments that might be the cause of the statistical insignificance. Therefore, we conducted additional experiments using higher concentrations of DTX, 0.5 nM, 1 nM, and 2 nM. All these DTX concentrations prominently inhibited tumor formation in the parental cells and the dissociated passage 0 and late passage tumorspheres (Figure 3). In these treatments, parental cells and tumorspheres at passage 0 showed nearly zero or negative rates of tumorsphere expansion (Table 3), indicating the occurrence of cytostasis or cell death in the tumorspheres. Although this effect was also observed in 1 and 2 nM DTX-treated late passage tumorspheres, the 0.5 nM DTX decreased tumorsphere expansion rate only by ~2-fold in late passage tumorspheres, as compared to the control (Table 3). This indicates that the 0.5 nM DTX-treated late passage tumorspheres were able to expand but at a slower rate and, therefore, resistant to DTX. The experiment for higher doses of OXA will be carried out in future.
Figure 3. Effect of DTX (0.5, 1 and 2 nM) on tumorsphere expansion from parental cells and dissociated passage 0 and late passage tumorspheres. Passage 0 tumorspheres were generated from parental cells in 10 days. Late passage tumorspheres were generated through serial passaging of tumorspheres (passage ~ 25-27). Tumorspheres and parental cell monolayer were dissociated into single cells and plated in tumorsphere culture conditions with or without treatment. Images of the tumorspheres were captured on day 0 (day of plating and treatment), day 3 and day 9. The
Characterization of tumorspheres generated from nasopharyngeal carcinoma cell line, TW06 and chemoresistance to docetaxel and oxaliplatin

sizes of tumorspheres were quantified using Image-Pro Plus software and expressed relatively to tumorsphere size on day 0 (set as 1). Each bar represents the mean ± SD of three independent experiments performed in triplicates. The differences of relative tumorsphere size between treated and non-treated tumorspheres on day 9 were analyzed using unpaired t-test (*P<0.05, **P<0.01, ***P<0.001).

DISCUSSION

Culturing cells in low adherent culture allows them to form cell aggregates termed tumorspheres. Tumorsphere culture has been very popular in the study of CSCs as it has been reported to enrich the CSC population in many cell lines such as breast, liver, colon and ovarian cancer cell line. Under serum-free condition, the CSCs can be maintained in an undifferentiated state. These tumorspheres were maintained in serum-free media with the addition of various growth factors. In this study, the CSC enrichment by tumorsphere culture of a nasopharyngeal carcinoma cell line, TW06 was carried out. The cell line was able to form viable tumorspheres after 10 days of culture in non-anchorage condition. The CSC hypothesis states that tumorigenesis is initiated by cells with stem cell-like characteristics which have acquired a proliferative potential and have ability to self-renew. Our experiment showed that the late passage tumorspheres of TW06 showed the highest expansion rate between day 3 and day 9 in culture as compared to the parental cells and passage 0 tumorspheres (Table 3). This observation was consistent with what some authors reported where CSC had shown rapid proliferation rate as compared to non-CSC.

Using cell surface markers is ideal for isolation and identification of CSCs, if more specific markers were identified. Currently, inspecific markers have been identified in various tumors. As for nasopharyngeal carcinoma, Yang et al. (2014) had reported that CD24+ cells isolated from TW02 and TW04 nasopharyngeal carcinoma cell lines have CSC-like properties such as increased expression of stem cell genes, enhanced proliferation and sphere formation, and also the ability to induce tumor in NOD/SCID mice. In another study, CD44+ cells isolated from nasopharyngeal carcinoma cell lines, SUNE-1, C666-1 have CSC-like properties. These studies suggested that CD24 and CD44 may be regarded as a marker of CSCs of nasopharyngeal carcinoma. However, our study showed loss of CD24+ population during/in the generation of tumorspheres from TW06 cells which are ~92% positive for CD24 (Table 1). The CD24+ population was increased after differentiation (Table 2). This data is in concordance with an earlier finding by Hermann et al. who found that CD133+ subpopulation from human pancreatic cell line which have CSC-like properties were able to differentiate into CD133 tumor cells which were non-tumorigenic. The identification of CSCs within human nasopharyngeal carcinoma has important implications for treatment. In many cancers, CSCs are thought to be the reason behind cancer metastasis and recurrence after clinical remission. In a study by Yang et al., the CSC-like CD24+ cells isolated from nasopharyngeal carcinoma cell lines, TW04 and TW02 showed enhanced resistance towards DTX and cisplatin. Evidence of the resistance of human gastric cancer to standard therapies was shown in a study of tumorspheres generated from GC-27, MGC-803 and MKN-45 human gastric cell lines, where the tumorspheres were more resistant to OXA and mitomycin compared with adherent cells. Todaro et al. demonstrated resistant to cell death induced by OXA and 5-FU in CD133+ colon cancer stem cells. Although no differences can be seen in terms of surface markers between passage 0 tumorspheres and late passage tumorspheres, our study has found that only the late passage tumorspheres of TW06 had developed resistance towards DTX and OXA (Figure 2, Figure 3 and Table 3). DTX is a member of the taxane drug class, like paclitaxel. These drugs causes apoptotic cell death by inhibiting microtubule depolymerisation which prevents the completion of cell cycle. On the other hand, OXA causes genotoxic stress and apoptosis by damaging the DNA in the form of single-strand and double-strand DNA break which induce cell cycle arrest for cell fate determination. The exact mechanism behind the chemoresistance of these tumorspheres to DTX and OXA is still unclear. The link to chemoresistance first emerged when Notch activation can be seen in multiple colon cancer cell lines treated with OXA. Notch is also known to coordinate with Sonic Hedgehog (SHH) pathway, contributing to the chemoresistance of CD133+ glioma CSCs. On its own, abnormal activation of the SHH pathway has been observed in a many different CSC models and inhibition of the SHH pathway is known to be able to sensitize CSCs to chemotherapeutic drugs in a variety of tumor types which includes gastric cancer, pancreatic cancer, ovarian cancer and prostate cancer. Studies have also suggested that the WNT/b-catenin pathway also plays a role in the chemoresistance to 5-FU and doxorubicin.

One possible mechanism for the WNT pathway meditated chemoresistance is the upregulation of ABC transporter pumps. This can be clearly seen in an experiment involving e-kit+ ovarian CSCs. Hence, it is important to evaluate the effect of inhibition/activation of these signalling pathways towards chemoresistance to OXA and DTX, which will be done in our further study. Further studies are also needed to evaluate the effect of DTX and OXA on the cell cycle status of the tumorspheres.
CONCLUSIONS

In conclusion, the late passage tumorspheres of TW06 NPC cell line has higher expansion rate than its parental cells. Also, the generation of these tumorspheres causes a loss of CD24 expression which is reversible after differentiating the tumorspheres in monolayer culture conditions. Data also suggest that late passage tumorsphere of TW06 NPC cell line consist of enriched numbers of CSCs, which potentially makes them less susceptible to the actions of both DTX and OXA. Further knowledge of these NPC tumorspheres and the mechanism of their resistant to DTX and OXA may provide important information that leads to the development of novel therapeutic strategy for NPC treatment.

ACKNOWLEDGEMENT

This study was supported by grant FRGS/1/2014/SKK01/UPM/01/1 from the Malaysian Ministry of Higher Education.

REFERENCES


