UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND CHEMORESISTANCE OF HT29 (COLON CANCER) AND TW06 (NASOPHARYNGEAL CANCER) TUMORSPHERES

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CHARACTERIZATION AND CHEMORESISTANCE OF HT29 (COLON CANCER) AND TW06 (NASOPHARYNGEAL CANCER) TUMORSPHERES

By

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November 2014

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The cancer stem cell (CSC) hypothesis states that these subpopulation of cells are responsible for the initiation of tumor and its recurrence. It has been shown that these cancer stem cells can be enriched *in-vitro* as tumorspheres under serum-free conditions. The objectives of this study is to establish and characterize the tumorspheres generated from a colon adenocarcinoma cell line (HT29) and a nasopharyngeal carcinoma cell line (TW06), and also to evaluate the chemoresistance properties of these tumorspheres of these cells. Here, two cell lines were grown as tumorspheres *in vitro* in serum-free DMEM/F12-supplemented medium with methylcellulose and plated on poly(2-hydroxyethyl methacrylate)-coated plates. Flow cytometric studies were performed to evaluate the surface markers expression of the tumospheres. Reverse-transcription-qPCR was also conducted to evaluate the expression level of cancer stem cell-related genes. Also, chemotoxicity assay was carried out to evaluate the chemoresistant properties of these tumorspheres to chemotherapeutic agents. In this study, it was found that both TW06-passage 0 and late passage tumorspheres had higher percentage of $CD44^+/CD24^-/EpCAM^+$ population compared to parental adherent cells. HT29 only show differences in terms of CD133 expression where only HT29-late passage tumorspheres were found to have higher $CD133^+$ population. In addition, the expression of both SOX2 and ALDH1A3 was significantly lower in the passage 0 and late passage TW06 tumorspheres while the expression of Bmi1 is much higher in the late passage TW06 tumorsphere. A lower expression of the Bmi1 gene was found in the late passage tumorsphere of HT29. Chemotoxicity assay result showed that the TW06-late passage tumorspheres was resistant towards docetaxel at concentrations at or below 2.5 times of IC_{50} but not at higher concentrations. As for the HT29 tumorspheres, it was found that a few tumorspheres from passage 0 was able to survive oxaliplatin treatment and its size appeared to be much larger as compared to the untreated tumorspheres. The DTX resistant tumorspheres of TW06 (TW06-DTX-R) and OXA resistant tumorspheres of HT29 (HT29-OXA-R) were isolated and then cultured with DTX and OXA respectively for

further evaluation of their chemoresistant properties. Both cell cycle assay and apoptosis assay further confirmed that TW06-DTX-R generated from TW06-late passage tumorspheres showed resistance towards docetaxel and HT29-OXA-R generated from HT29-parental tumorspheres were resistant to the oxaliplatin treatment. Finally, this study concluded that serially passaged tumorspheres may be enriched in cancer stem cells and were towards specific chemotherapeutic agents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PENCIRIAN DAN RINTANGAN UBAT KEMOTERAPI TERHADAP SFERA-SFERA TUMOR HT29 (KANSER KOLON) DAN TW06 (KANSER NASOFARINKS).

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Hipotesis sel stem kanser menyatakan bahawa subpopulasi sel inilah yang bertanggungjawab untuk permulaan dan perulangan sesuatu tumor. Ianya dipercayai bahawa sel stem kanser dapat diperkayai in-vitro sebagai sfera tumor di bawah keadaan bebas serum. Objektif kajian ini adalah untuk mencirikan sfera-sfera tumor dari sel adenokarsinoma kolon (HT29) dan sel karsinoma nasofarinks (TW06), dan juga untuk menilaikan sifat-sifat rintangan terhadap ubat-ubat kemoterapi yang ditunjukkan oleh sfera tumor sel-sel tersebut. Kedua-dua sel tersebut telah ditumbuh sebagai sfera-sfera tumor in-vitro dalam medium DMEM/F12 yang ditambah dengan menggunakan metilselulosa sebagai dasarnya. Sitometri aliran telah dijalankan untuk menilai ekspresi penanda permukaan sfera-sfera tumor. Selain daripada itu, qPCR juga dijalankan untuk menilai ekspresi gen yang berkaitan dengan sel-sel stem kanser. Cerakin ketoksikan ubat kemoterapi juga dijalankan bagi menentukan reaksi sfera-sfera tumor kepada beberapa ejen kemoterapi yang biasa digunakan. Dalam kajian ini, ia didapati bahawa populasi sel $CD44^+/CD24^-/EpCAM^+$ di kedua-dua sfera-sfera tumor pasaj 0 dan pasaj lewat sel TW06 adalah jauh lebih tinggi berbanding dengan sel lekat induknya. HT29 hanya mempunyai perbezaan dari segi expressi CD133 di mana sfera-sfera tumor lewat pasaj mempunyai populasi $CD133^+$ adalah lebih tinggi. Di samping itu, ekspresi kedua-dua gen SOX2 dan ALDH1A3 adalah jauh lebih rendah dalam sfera-sfera tumor pasaj 0 dan pasaj lewat sel TW06 manakala ekspresi gen Bmi1 adalah lebih tinggi di dalam sfera-sfera tumor pasaj lewat tumorsphere sel TW06. Ekspresi yang lebih rendah bagi gen Bmi1 dapat dilihat dalam sfera-sfera tumor pasaj lewat HT29. Cerakin ketosikan ubat kemoterapi menunjukan bahawa sfera-sfera tumor TW06 pasaj lewat memperolehi rintangan terhadap dos DTX hingga 2.5 kali ganda dos IC_{50} tetapi bukan pada dos-dos DTX yang lebih tinggi. Bagi sfera-sfera tumor HT29, keputusan cerakin ketoksikan ubat kemoterapi tidak menunjukkan berbezaan signifikan antara reaksi sfera-sfera tumor pasaj 0 tumorspheres, pasaj awal dan pasaj lewat terhadap oxaliplatin (OXA). Sfera-sfera tumor TW06 yand mempunyai rintangan terhadap DTX (TW06-DTX-R)

dan sfera-sfera tumor HT29 yang mempunyai rintangan terhadap OXA (HT29-OXA-R) diasingkan untuk terus ditumbuh dalam keadaan dirawat bagi mengkaji ciri-ciri rintangan tersebut. Cerakin kitaran sel-sel dan cerakin apoptosis kemudian mengesahkan bahawa TW06-DTX-R mempunyai rintangan terhadap docetaxel dan HT29-OXA-R mempunyai rintangan terhadap oxaliplatin. Akhirnya, kajian in menyimpulkan bahawa sel stem kanser dapat diperkayai daripada sfera-sfera tumor yang telah dipasaj secara bersiri. Selain daripada itu, sfera-sfera tumor juga mempunyai ciri-ciri rintangan terhadap ubat kemoterapi.

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LIST OF ABBREVIATIONS

5-FU	5-fluouracil
ABC	ATP binding cassette
ABCG	ATP binding cassette, sub-family G
AKT	Protein kinase B
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
APC	Adenomatosis polyposis coli
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad-3-related
Bcl-2	B-cell lymphoma 2
Bcr/Abl	Breakpoint cluster region/Abelson oncogene locis
bFGF	Basic fibroblast growth factor
Bmi1	B lymphoma Mo-MLV insertion region 1 homolog
BRCA1	Breast cancer 1, early onset
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
CHK	Checkpoint kinase
CRC	Colorectal cancer
CSC	Cancer stem cell
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTX	Docetaxel
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ESA	Epithelial-specific antigen
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCB	Gemcitabine
HMLE	Human mammary epithelial
HOXA9	Homeo box A9
HSC	Hematopoietic stem cell
HT29-OXA-R	Oxaliplatin resistant HT29

IC	Inhibitory concentration
IL-4	Interleukin-4
LSC	Leukemic stem cell
MCL	Myloid cell leukemia
MDR1	Multidrug resistance protein 1
MTT	Methylthiazolyl-tetrazolium
NF-KB	Nuclear factor-KB
NOD/SCID	Non-obese diabetic/Severe combined immunodeficiency
NPC	Nasopharyngeal carcinoma
Oct4	Octamer-binding transcription factor 4
OXA	Oxaliplatin
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
polyHEMA	Poly(2-hydroxyethyl methacrylate)
POU5F1	POU domain, class 5, transcription factor 1
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SHH	Sonic Hedgehog pathway
siRNA	Small interfering ribonucleic acid
Sox2	SRY (sex determining region Y)-box 2
SSEA	Stage-specific embryonic antigen
TIC	Tumor initiating cell
TW06-DTX-R	Docetaxel resistant TW06
WNT	Wingless-type MMTV integration site family

CHAPTER 1

INTRODUCTION

Tumor recurrence and metastasis are two of the major obstacles in cancer treatment which result in making most cancer incurable. Cancer stem cells (CSCs) are widely linked to tumorigenesis and metastasis (Charafe-Jauffret et al., 2010; F. Li et al., 2007; Wicha, 2006). In addition, these cells have also been proposed to be linked to chemoresistance and also resistance to radiotherapy (Debeb et al., 2009; Diehn et al., 2009; Li et al., 2008; Phillips et al., 2006; Woodward et al., 2007). According to the CSC model, tumor is initiated by a subpopulation of cancer cells termed as cancer stem cells. These CSCs have the intrinsic properties that is identical to normal stem cells which include longevity and self-renewing ability. Normal adult tissues have a small portion of stem cells that plays a role in the replacement of terminally differentiated cells. During self-renewal, these stem cells generate two daughter cells which are identical to its parental cell. Similarly, these cancer stem cells have the ability of initiate tumor in immune-deficient mice. CSCs were first identified in leukemia and later found in a wide variety of solid tumors. The presence of CSCs may be the reason behind the cancer recurrence and chemoresistance towards many chemotherapeutic drugs. 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 (Dontu et al., 2003). This culture method was originally established from a neural cell activity assay (Reynolds et al., 1992). This method is soon adapted into many other studies that are linked to CSCs.

Currently in Asia, colorectal cancer is the third most common malignant disease in both men and women (Sung et al., 2005). According to a case review done on CRC patients at Universiti Kebangsaan Medical Centre (UKMMC), it was found that 26% of the patients developed metastasis after surgery with adjuvant therapy (Rashid et al., 2009). On the other hand, nasopharyngeal carcinoma occurs more frequently occur in regions of Southern China and South East Asia (Ayadi et al., 2010; Toumi et al., 2010). Most of this mortality in NPC patients is believed to be due to distant metastasis and local recurrence of the cancer (Lo et al., 2004). Resistance towards chemotherapeutic agent which is one of the characteristic of CSC, is thought to be one of the main cause of cancer recurrence. Many other theories have attempted to explain chemoresistance, but this CSC theory has attracted much interest. Chemotherapies are developed based on the ability of these chemotherapeutic agents to cause regression in the tumor in animal models. Since CSCs are believed to be only less than 10% of the total tumor population, this 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 be also progenitor cells in the bulk tumor by going through self-renewal and cell division which causes metastasis. Knowledge concerning the changes that causes chemoresistance in CSC are limited. The effects of chemotherapeutic agents on CSC, the changes that these drugs cause induce in CSC, the association between CSC and proteins connected to drug-resistance, and also the relationship between CSCs and anti-apoptosis genes may enable researchers to find more effective therapy which targets CSCs.

The hypothesis of this study is that the tumorspheres are enriched in CSCs which has resistance towards chemotherapeutic agents and express putative CSC surface markers and CSC-related genes.

This work aimed to achieve the following objectives:

1) To compare the expression of CSC surface markers and genes in tumorspheres from HT29 (colon adenocarcinoma) and TW06 (nasopharyngeal carcinoma) versus parental adherent cells
2) To determine the chemoreactivity of the tumorspheres towards common chemotherapeutic agents
3) To evaluate the effect of the drugs on the cell cycle status and apoptotic effects on resistant tumorspheres

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