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Establishment and Analysis of the 3-dimensional (3D) Spheroids Generated from the Nasopharyngeal Carcinoma Cell Line HK1

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Abstract: Spheroids have been shown to recapitulate the tumour in vivo with properties such as the tumour microenvironment, concentration gradients, and tumour phenotype. As such, it can serve as a platform for determining the growth and invasion behaviour pattern of the cancer cells as well as be utilised for drug sensitivity assays; capable of exhibiting results that are closer to what is observed in vivo compared to two-dimensional (2D) cell culture assays. This study focused on establishing a three-dimensional (3D) cell culture model using the Nasopharyngeal Carcinoma (NPC) cell line, HK1 and analysing its growth and invasion phenotypes. The spheroids will also serve as a model to elucidate their sensitivity to the chemotherapeutic drug, Flavopiridol. The liquid overlay method was employed to generate the spheroids which was embedded in bovine collagen I matrix for growth and invasion phenotypes observation. The HK1 cells formed compact spheroids within 72 hours. Our observation from the 3 days experiments revealed that the spheroids gradually grew and invaded into the collagen matrix, showing that the HK1 spheroids are capable of growth and invasion. Progressing from these experiments, the HK1 spheroids were employed to perform a drug sensitivity assay using the chemotherapeutic drug, Flavopiridol. The drug had a dose-dependent inhibition on spheroid growth and invasion.

Keywords: 3-dimensional Spheroid, Nasopharyngeal Carcinoma, HK1 Cell Line

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a cancer that affects the nasopharynx. NPC affects the nasopharynx but the common region whereby NPC develops is the posterior and superior region in the pharyngeal recess (Fossa of Rosenmüller) (Hoe 1989). Based on the Malaysian National Cancer Registry 2007, NPC is the fourth most common cancer in Malaysia and third most common cancer affecting men (Omar & Ibrahim Tamin 2011). The endemic proportion of NPC in Malaysia calls upon research into this area especially in terms NPC therapeutics. We intend to elucidate the drug sensitivity of NPC against several chemotherapeutic drugs and small molecule inhibitors, however to do so, we need to establish the 3D model which is a model that mimics the tumour architecture and phenotype as previously shown in melanoma studies (Lucas *et al.* 2012).

The xenograft model on the other hand, is an animal model with complex extracellular matrix and living components that allows for a more physiological environment compared to the artificial 2D platform but its complexity becomes a disadvantage especially when the multiple variables makes it difficult for researchers to evaluate the effect of particular variables on the tumour phenotypes (Smalley *et al.* 2006; Smalley *et al.* 2008) and the fundamental basis of the model being animal origin could offer different results than as seen with the preclinical tests.

The 3D model offers a solution in terms of mimicking the tumour architecture with the versatility of the artificial component and the tumour in vivo with accurate phenotypes (Smalley *et al.* 2006; Smalley *et al.* 2008). The 3D model also functions as a platform that describes the growth and invasion characteristics of a tumour. Thus there is critical need to actually have drug sensitivity assays performed on 3D models first as a pre-screening step for data and approximations before proceeding with xenograft studies for a more cost effective, time efficient and ethically accepted approach in drug sensitivity studies (Beaumont *et al.* 2014).

MATERIALS AND METHODS

Cells and Cell Culture

The HK1, EBV-negative (Huang *et al.* 1980) NPC cell line was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS), 10 U/mL of penicillin and 10 µg/mL streptomycin (Gibco) and cultured at 37°C in a 5% CO_2 humidified incubator. The cell line was authenticated using AmpFISTR profiling as described (Daker *et al.* 2012). Experiments were performed within 2 passages of the foundation stocks.

Generation of the 3D Spheroids from the NPC Cell Line HK1

NPC spheroids were generated using the liquid overlay method as described (Smalley *et al.* 2006; Smalley *et al.* 2008). Once the spheroids were embedded into the bovine collagen I matrix, spheroids were either monitored for growth and invasion or treated with chemotherapeutic drug Flavopiridol (Selleckchem) at the concentrations indicated. Spheroids were stained with Calcein-AM (Molecular Probes) and Ethidium homodimer I (Molecular Probes) once experiment was terminated. Spheroids growth and invasion were quantified using the ImageJ (NIH, Bethesda, MD) and Axiovision LE software (Carl Zeiss Microscopy, GmbH, Germany).

RESULTS AND DISCUSSION

Establishment and Analysis of the 3D Spheroids Generated from the HK1 Cell Line

To employ the 3D spheroids to analyse the growth, invasion and drug sensitivity, we first established the spheroids from the NPC cell line HK1. We utilised the cervical cancer cell lines HeLa and CaSki as positive controls as these two cell lines were previously shown to form spheroid (data not shown).The HK1 spheroids formed compact and uniform spheroids within 72 hours [Fig. 1(A)]. The cadherin interactions with the ECM and itself, plays an important role in forming the compact spheroid in the biochemical basis (Lin *et al.* 2006). After generating the model, the spheroids were embedded into the bovine collagen I matrix to determine their growth and invasion phenotypes. This takes into consideration of the need for cell–cell contact between the tumour cells and a scaffold (Smalley *et al.* 2006; Smalley *et al.* 2008; Lucas *et al.* 2012). The HK1 spheroids grew and gradually invaded into the collagen matrix over 3 days [Fig. 1(B), (C), and (D)]. Calcein-AM stained the invading cells green and the dead core was stained red by Ethidium homodimer I [Fig. 1(B)].

Spheroid Growth and Invasion Inhibition with Flavopiridol

The 3D spheroid assay was employed to determine the effect of Flavopiridol on spheroid growth and invasion. Compared to standard endpoint drug sensitivity assays in 2D culture, this assay allows us to study the dynamic effects of the drug (Vinci *et al.* 2012). Furthermore, the assay can demonstrate if a single agent is sufficient to cause inhibition of the growth and invasion of the spheroids (Vinci *et al.* 2012). Spheroids were treated with Flavopiridol for 10 days. Flavopiridol induced a dose-dependent inhibition of spheroid growth and invasion [Fig 1(E)]. To attain a significant inhibition of spheroid growth and invasion, a combination with another chemotherapeutic drug or small molecule inhibitor with Flavopiridol is required (Lucas *et al.* 2012).

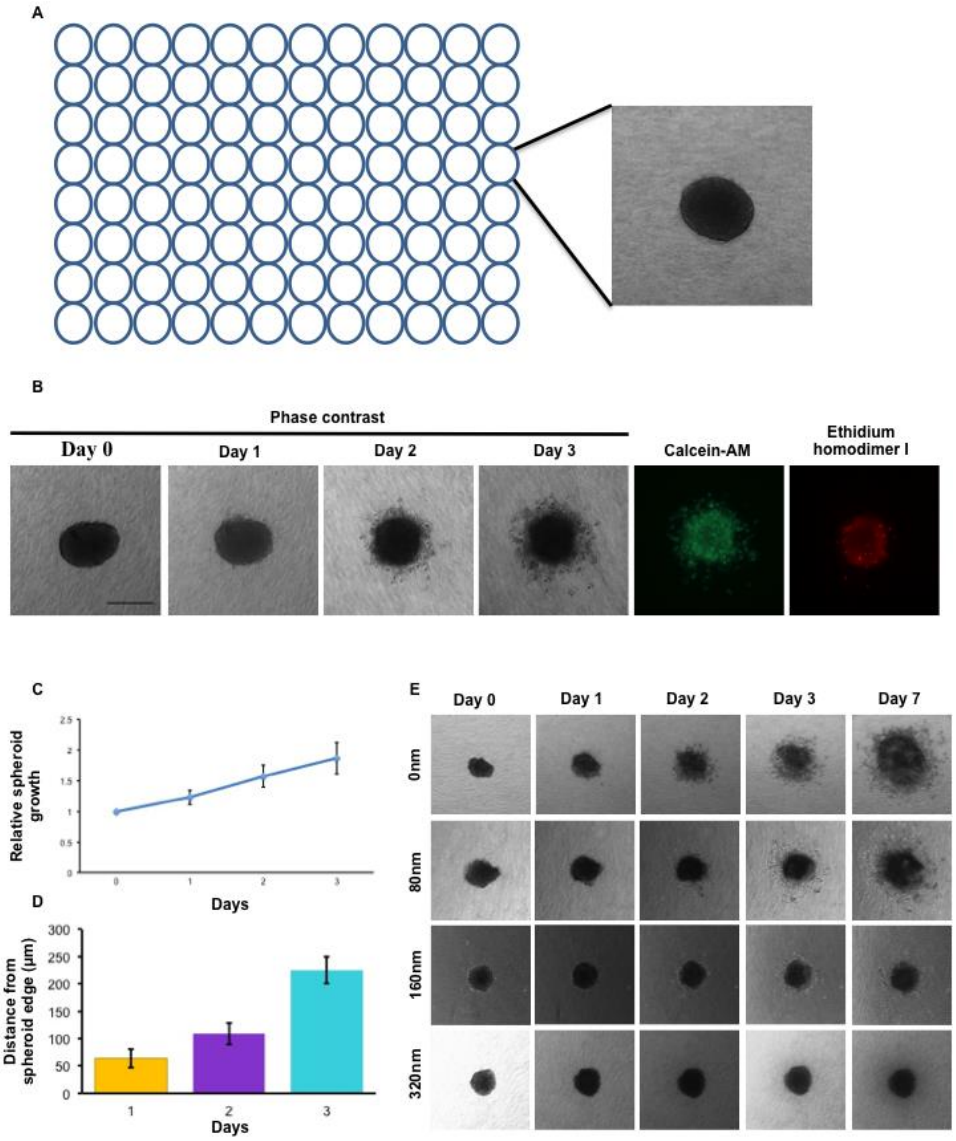

Figure 1: (A) formation of spheroids from the HK1 NPC cells after 72 hours; (B) growth and invasion of the HK1 spheroids over 3 days. Images of spheroids were taken every 24 hours using the Axio Observer Z1 microscope with ApoTome (Carl Zeiss Microscopy, GmbH, Germany) (size bar: 200 µm). Viability of the spheroids were determined by staining the spheroids with Calcein-AM (stains viable cells) and Ethidium homodimer I (stains dead cells); graphs show corresponding quantification of spheroid (C) growth and (D) invasion, errors are SEM, n=3; (E) spheroids were treated with different concentrations of Flavopiridol for 10 days. Medium and drug were replenished every 72 hours. Note the dose-dependent inhibition of growth and invasion of the spheroids.

CONCLUSION

The HK1 cells form compact and uniform spheroids within 72 hours and exhibit gradual growth and progressive invasion into the bovine I collagen matrix over 3 days. Treatment with Flavopiridol exhibited a concentration dependent inhibition of spheroid growth and invasion. Our future endeavour will be to utilise the spheroids to conduct drug sensitivity assays using small molecule inhibitors in combination with chemotherapeutic drugs and to establish spheroids from the Epstein-Barr virus (EBV) positive NPC cell line, C666-1 to observe if the spheroids exhibit the same growth and invasion phenotypes as the HK1 or if there are any changes especially with the presence of the EBV.

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