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DISSERTATION

Doxorubicin fails to eradicate cancer stem cells derived from anaplastic thyroid carcinoma cells: characterization of resistant cells

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To my parents

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Abbreviations

ABCG2	ATP-binding cassette transporter G2
AML	acute myeloid leukemia
ATC	Anaplastic thyroid carcinoma
Bcrp	breast cancer resistance protein
CSC	cancer stem cell
cDNA	complementary deoxyribonucleic acid
^{o}C	degree Celsius
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
ESCs	embryonic stem cells
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FTC	fumitremorgin C
FITC	fluorescein isothiocyanate
×g	G-Force (unit of measurement of rotation speed of a centrifuge)
GBM	glioblastoma multiforme
HBSS	Hank's balanced salt solution
Hela	human cervix carcinoma cell line (initials of Henrietta Laks, the patient from whom the cell line originates)
HNF4	hepatocyte nuclear factor 4
MEM	non-essential amino acids
MgCl2	magnesium chloride
MDR1	multidrug resistance transporter 1
MRP1	multi-drug resistance protein 1

MTT	methyl thiazolyl tetrazolium
μM	micromole per liter solution (unit of concentration)
ml	Milliliter
mRNA	messenger ribonucleic acid
μg	microgram
PBS	phosphate buffered saline
PI	propidium iodide
RT	reverse transcription
PCR	polymerase chain reaction
PH	potentia hydrogenii (negative decimal logarithm of hydrogen-ion concentration)
pNA	p-nitroaniline
SP	side population
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
rpm	revolutions per minute
TBE	Tris-borate EDTA
TBS	Tris buffered saline
TTF1	thyroid transcription factor 1
UV	ultraviolet
VAD	vincristine, doxorubicin, and dexamethasone

1. Introduction

1.1 Epidemiology of thyroid carcinoma

The incidence of thyroid cancer has increased significantly during the past decades (1). Thyroid cancers account for 1% of all neoplasms. It has become one of the ten leading cancer types in females. In 2008, there are estimated to be 37,340 new cases, with 1,590 deaths (2). Anaplastic thyroid carcinoma (ATC) is one of the most aggressive tumours and has a poor prognosis with mean survival of only a few months from the time of diagnosis. ATC accounted for 1-2% of thyroid malignancies (3). From the data of SEER, it is estimated that 37,200 men and women (10,000 men and 27,200 women) will be diagnosed with and 1,630 men and women will die of cancer of the thyroid in 2009 (4).

ATC have a poor prognosis with a mean survival of only a few months (3). Long-term survivors are very rare (5, 6). Cervical lymph nodal involvement is common, and more than 50% of the patients have metastatic disease at presentation. Treatment options for this highly malignant tumour include surgery, radiotherapy and chemotherapy. Unfortunately, surgery alone does not have the potential to alter the course of the disease; sometimes it just palliates the symptoms of local compression. Since ATC is radiation resistant, radiotherapy is only applied to relieve the airway obstruction. In the recent few decades, chemotherapy has been increasingly used. It is used in the clinical treatment with doxorubicin in combination with cisplatin and radiation (7). However, current systemic therapy fails to eradicate this cancer or even to stop tumour progress. For improving the prognosis of anaplastic thyroid carcinomas, it is necessary to investigate the ATC etiopathogenesis.

1.2 Cancer stem cell hypothesis and the evidence of thyroid cancer stem cell

In the cancer research field, both in the laboratory and clinical study, several investigators found that most malignant carcinomas are resistant to different chemotherapy drugs or to radiotherapy

with a high tendency to metastasis and relapse.

There are two conflicting models that attempt to explain tumour formation. The stochastic model suggests that every cell within a tumour has an equal opportunity to initiate a new tumour, but there is a low probability of stochastic events. According to this model, we can not foresee or find the exact cells which would initiate the potential tumour because every cell would have an equal ability to be tumorigenic.

In contrast, another tumour formation model is the hierarchy model, which proposes that only a small subset of cells in a tumour are capable of initiating tumour growth, but the percentage of these cells is low. According to this theory, it should be possible to identify the cells responsible for new tumour formation because not all the cells have the same phenotypic and functional characteristics (8). It's not a new idea. More than a century ago, some researches found that tumours were composed of morphologically heterogeneous cells, and understood that cancer cells also exhibit functional heterogeneity both in vitro (9-12) and in vivo (13, 14). Despite this evidence for tumour cell functional heterogeneity, subsequent researcher emphasized the monoclonal nature of cancers in that they reconciled tumour monoclonality with tumour heterogeneity by hypothesizing that the tumour microenvironment or the presence of clinically inapparent genetic subclones could explain the variable behavior of tumour cells (15, 16). This evidence induced the cancer stem cells (CSCs) hypothesis, which hypothesizes that cancer arises from a subpopulation of tumour-initiating cells, or CSCs (as shown in Fig 1.1). Normal stem cells give rise to multipotent progenitor cells, committed progenitors and mature, differentiated cells. Mutations in stem cells give rise to stem cells with aberrant proliferation and result in a pre-malignant lesion. Additional mutations lead to the acquisition of further increased proliferation and decreased apoptosis. Evasion leads to acquisition of further increased proliferation, decreased apoptosis, evasion of the immune system, and further expansion of the stem-cell compartment which is typical of malignant tumours**.**

The hypothesis of cancer stem cell was first proposed about 150 years ago(17, 18). The progress was slow during the next several decades, because the technology at that time was not advanced

enough to test this hypothesis. In recent years, with the development of new techniques, it became possible to isolate and characterize single cells.

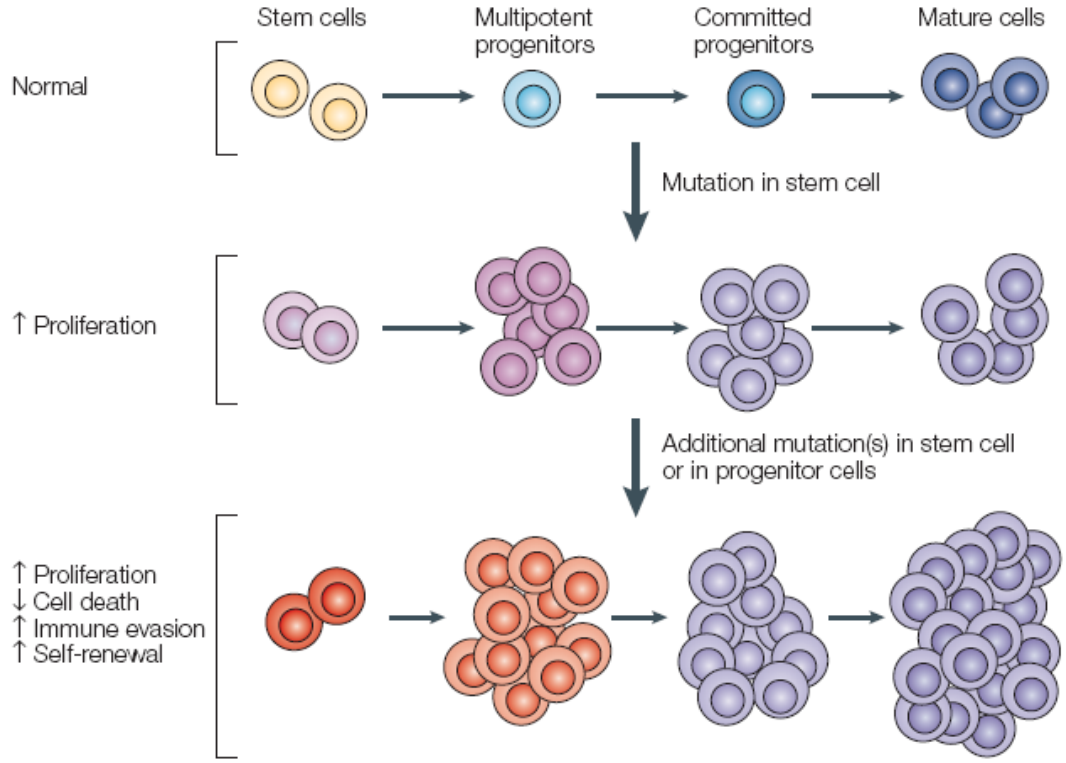

Figure 1.1 Cancer stem cells and tumour progression. Normal stem cells give rise to multipotent progenitor cells, committed progenitors and mature, differentiated cells. Mutation in stem cells give rise to stem cells with aberrant proliferation and result in a pre-malignant lesion, Additional mutations lead to the acquisition of further increased proliferation, decreased apoptosis, evasions lead to acquisition of further increased proliferation, decreased apoptosis, evasion of the immune system, and further expansion of the stem-cell compartment that is typical of malignant tumours. (Taken from: Michale D, Tito F, and Susan B, "Tumour stem cells and drug resistance", 2005, Nature Reviews Cancer, 5: 275-284) (19).

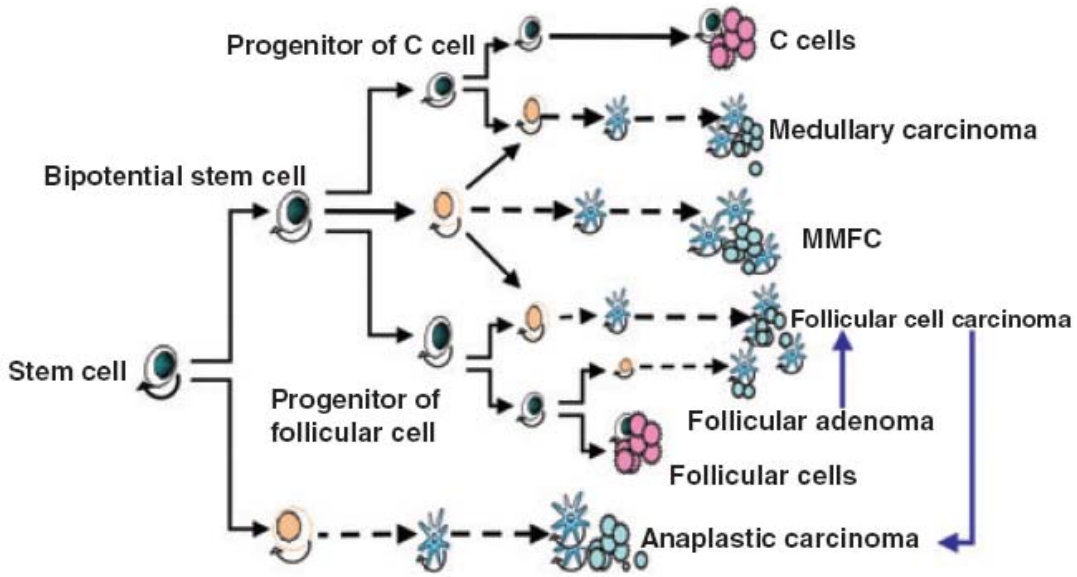

Figure 1.2 Cancer stem cell hypothesis in thyroid carcinogenesis. Stem cells have different capability for self-renewal (indicated by arrows) and exist in both normal tissues (normal stem cells , ; normal cells,) and cancer tissues (cancer stem cells, ; cancer cells,). When specific genetic alterations act on normal stem cells, these cells could be transformed (indicated by) and finally initiate cancer stem cells (follicular cell carcinoma includes both papillary carcinoma and follicular carcinoma). MMFC, mixed medullary and follicular carcinoma. [Taken from: Takano T, Amino N: Fetal cell carcinogenesis: a new hypothesis for better understanding of thyroid carcinoma. 2005, Thyroid, 15: 432-438.] (20).

1.3 Evidence supporting the CSCs hypothesis

Lapidot and colleagues were the first to find solid evidence to support the CSCs hypothesis when they used cell-surface protein markers to identify a relatively rare population of stem-like cells in acute myeloid leukemia (AML) (21). The cells with CD34+CD38– were isolated and displayed stem-like properties. This was confirmed by their ability to engraft mice with compromised immune systems and to produce large numbers of colony-forming progenitors. Later, John Dick and colleagues showed that human AML contains a small percentage of cells (typically 0.1–1%) capable of transferring human AML into immunodeficient mouse hosts (21, 22). Based on a

comparison of the immunophenotypes of leukemia stem cells with normal human haemopoietic stem cells and progenitors, it was predicted that leukemia stem cells arise from the earliest progenitors of the hematopoietic system (21, 22).

After these findings in leukaemia, this model was used in identifying stem-like cells in solid tumours. The first report was from Al-Hajj *et al.* (23). These authors used the markers CD44 and CD24 to isolate tumourigenic cells from human breast carcinoma by using fluorescence-activated cell sorters (FACS). The sub-population of $CD44^{+}CD24^{-}$ was able to form tumours in immunodeficient mice, whereas 100-fold more cells from the $CD44^{+}CD24^{+}$ or $CD44^{-}$ fractions did not form tumours. The tumourigenic subpopulation could be serially passaged: each time a cell within this population generated new tumours containing additional CD44+CD24-/low tumourigenic cells as well as the phenotypically diverse mixed populations of nontumourigenic cells present in the initial tumour. Later, there was strong evidence supporting the existence of stem cell-like populations in brain, colon, prostate, and pancreatic tumours (24-30).

Different markers were used for each kind of solid tumour and cell line to identify stem-like cells. CD133 (also known as prominin-1 or AC133), a transmembrane pentaspan protein, was initially described as a surface antigen specific to human haematopoietic stem cells (31). CD133 has been recognized as a stem cell marker for normal and cancerous tissues. CSCs have been identified in brain, colon, and pancreatic tumours based on CD133+ marker expression (24, 27, 29). The CD133+ cells in colonic cancer have been described as highly tumourigenic after xenotransplantation in immunodeficient mice (29, 32). Other markers were used in different tumours, such as CD138−CD34− surface marker in myeloma (33), CD44+CD24+ESA+ surface marker in pancreas (28), ABCB5+ surface marker in melanoma (34), CD34+CD10− surface marker in B-ALL (35)

Until recently, there have been only very few studies on adult thyroid stem/progenitor cells and thyroid CSCs (36). In our laboratory, my colleagues have recently described and characterized adult stem cells in human thyroid tissues (37-39). These stem or progenitor cells expressed either

the pluripotent marker Oct4 or the endodermal markers GATA4 and HNF4α and the thyroid transcription factors TTF1 and Pax (37). Adult thyroid stem cells have also been characterized in mouse thyroid cells (40).

The existence of cancer stem or progenitor cells as the origin of thyroid cancer has been hypothesized before (Fig. 1.2) (20). Only recently, thyroid CSCs derived from anaplastic thyroid cancer cell lines have been described (41, 42). However, when transplanted onto mice, only some of cell line-derived CSCs displayed a higher tumour-forming capacity than the main population of cancer cells (41, 43).

1.4 Identifying and isolating CSCs

It is very important to identify the exact CSCs that form the tumour cells. Until now, there are two kinds of methods to isolate the CSCs. One method is to use markers to recognize CSCs, as described above, like CD133, CD44, CD34 and others. Unfortunately, not all stem cells or progenitors have unique and specific markers. This task has proven to be challenging because of the lack of universality in morphologic characteristics and marker expression between cancer types.

Another method to identify putative adult stem cells was demonstrated first by Goodell *et al*. (44). They found that when bone marrow-derived cells were incubated with Hoechst dye 33342 and then analyzed by dual-wavelength flow cytometry (Hoechst blue and Hoechst red), a small population of cells did not accumulate an appreciable amount of dye and is thus identified as a low Hoechst side population (SP, Fig 1.3). This SP is defined by Hoechst dye exclusion in flow cytometry and has been commonly used as one of the methods of enriching for cancer stem cells. They have also demonstrated that the exclusion of Hoechst 33343 dye is a dynamic process involving the multidrug resistance transporter 1 (MDR1), a member of the ATP binding cassette (ABC) transporter transmembrane proteins (45). However, later, Zhou *et al*. found that also another ABC transporter transmembrane protein, designated ABCG2, determined the SP

phenotype in mouse hematopoietic stem cells (46).

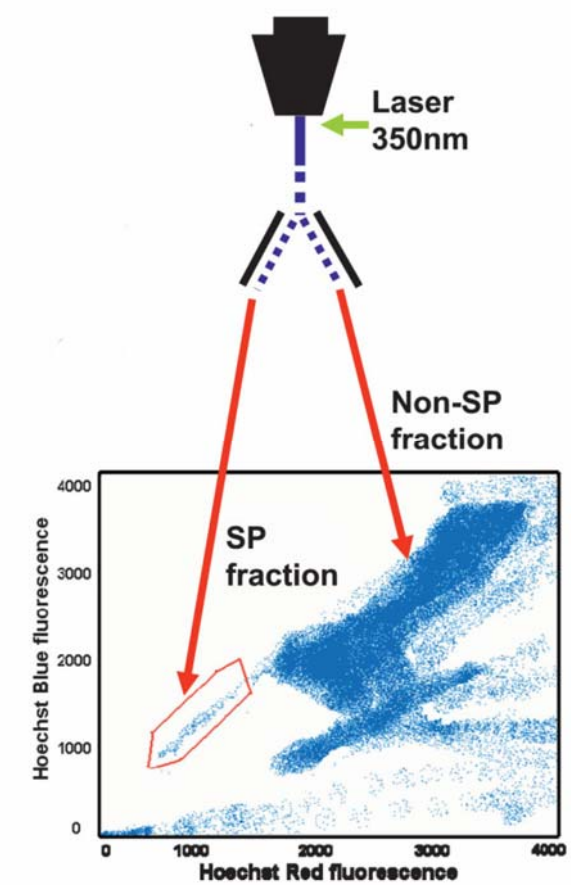

Figure 1.3 The cells are stained with the Hoechst 33342 dye and then separated by FACS. Those small proportion of cells that are able to efflux the dye have a characteristic fluorescent emission profile in dual wavelength analysis, with a low red and low blue fluorescence — the SP fraction. [modified from: Forbes SJ *et al*, Side population (SP) cells: taking center stage in regeneration and liver cancer? 2006, Hepatology, 44: 23-26](47)

ABCG2 (or breast cancer resistance protein: BCRP, ABCP, MXR) is a member of the ABC family of proteins and is a 72 kDa half-transporter of the G subfamily of ATP-binding cassette transporters (48). It was first identified in a breast cancer cell line (49), and later identified in many other cell types, including epithelial cells of the placenta, liver, gut, and stem cells isolated from different organs (49-53). Overexpression of ABCG2 plays a critical role in the development of drug resistance in various malignancies. The importance of ABCG2 activity in

chemoresistance was first supported by studies by Zhou *et al*. who demonstrated that stem cells derived from ABCG2-deficient mice were more sensitive to the cancer drug and ABCG2 substrate mitoxantrone (54). ABCG2 also plays a significant role in doxorubicin-transport and resistance in breast cancer (55), and its role in the transport of drugs in cancer cells has also been suggested (56). Some investigators also found that SP displayed functional ABCG2-mediated efflux. The SP in most of gastrointestinal cancer cell lines were more resistant to doxorubicin, 5-fluorouracil and gemcitabine (57). In the glioma cells, ABCG2 was highly expressed in stem cells, which could reduce the accumulation of chemotherapeutic drugs (58). Therefore, brain cancer stem cells are resistant to drugs may relate the ABCG2 expression.

MDR1 is another ABC transporter transmembrane protein. It was first identified in 1976 as a surface glycoprotein in Chinese hamster ovary cells, which expressed the MDR phenotype(59). MDR1 is a 170-kDa ATP-dependent efflux transporter(60, 61) and exists in various tumours (62-65). MDR1 has been found in CSCs of several types of solid cancer and leukemic cells (66, 67). Tumour cells exposed to a single cytotoxic drug such as one of the anthracyclines, taxanes, doxorubicin or actynomicin D can overexpress MDR1 and therefore acquire a MDR phenotype (68). The mechanism by which MDR1 is able to interact with a large variety of structurally unrelated drugs is currently under investigation.

It has been reported that normal stem cells have a high ability to efflux drugs and dyes due to the high expression of ABC transporters, which actively pump drugs out of the cells (69). The SP technique has been adapted to identify putative stem cells and progenitors in multiple tissues/organs including umbilical cord blood (70), skeletal muscle (71-73), mammary glands (74), lung (75-77), liver (78), epidermis (79, 80), forebrain (81), testis (82, 83), heart (84), kidney (85), limbal epithelium (86), and prostate (87)

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Hirschman-Jax et al. (2004) found that there was a small SP fraction in neuroblastomas as well as in breast cancer, lung cancer and glioblastoma cell lines that could efflux the Hoechst 33342 dye at a rate comparable to normal stem cells (88). To date, side populations of cells with stem cell properties have also been identified in many cancers (84, 89-91), supporting the notion that

certain cancers may arise from a cancer stem/progenitor cell. SP was also identified in normal thyroid tissues and cancer cells. The SP is very low in thyroid normal tissue and thyroid cancer cell lines (less than 1%) (38, 41). Moreover, the SP expressed a high level ABCG2 (38, 41).

1.5 Drug resistance and cancer stem cell

Despite treatment, the mortality of anaplastic thyroid cancer is very high. Although chemotherapy can kill some of the tumour cells, there are cell populations left which are resistant to chemotherapy. Drug resistance seems to be an almost insuperable problem in cancer chemotherapy. It has been hypothesized that this may be explained by the failure of current drugs to effectively target putative cancer stem cells (Fig 1.4**)** (92, 93).

In the CSCs model, a malignant primary tumour arises from cancer stem cells. Furthermore, in the primary tumour, there is a small population of drug-resistant cells that is responsible for relapse after a chemotherapy-induced remission, and/or they can give rise to distant metastases (Fig. 1.4). It is very obvious that if chemotherapy fails to eliminate CSCs, these cells may allow the tumour to relapse. Therefore, it is necessary to first reduce tumour size with a primary compound, and subsequently apply agents that target the remaining CSCs compartment to prevent tumour relapse. It is believed that therapeutic strategies that specifically target cancer stem cells should eradicate tumours more effectively than current treatments and can reduce the risk of relapse and metastasis.

To date, there is some evidence that CSCs may play a role in mediating chemotherapy resistance in tumours. Indeed, studies revealed that the intrinsic or acquired resistance of poorly differentiated leukemic or solid tumour CSCs to current clinical therapy contributed to recurrence (8, 19, 25, 66, 94-102). Recently, it was reported that a subpopulation of pancreatic cancer cells that have a strong resistance to gemcitabine both in vitro and in vivo functionally resemble stem cells (27). One group found that CSCs from gliomas displayed marked resistance to several chemotherapeutic agents (temozolomide, carboplatin, etoposide, and paclitaxel)

relative to the non-CSCs population (95). Leukemic SP cells, which are enriched for CSCs, have an amplified ability to pump chemotherapeutic drugs like daunorubicin and mitoxantrone out of the cell, suggesting that increased drug removal ability may contribute to the chemotherapy resistance of CSCs (89). Hirschmann and colleagues showed that the SP of neuroblastoma cells not only had the characteristics of tumour stem cells, but was also more resistant to drugs such as mitoxantrone and that it may contribute to the overall drug resistance phenotype of relapsed or resistant cancers (88). It has also been shown that SP cells in human malignant glioma cell lines and primary glioblastoma multiforme (GBM) neurosphere cultures increased in the course of temozolomide treatment, and that this SP fraction correlated with stem cell-like activity (103). Taken together, the data demonstrate the link between SP and drug resistance and relapse. These studies highlight the significance of drug-resistant tumour stem cells.

CSCs mediate drug resistance, which may be induced by a resting state, constitutive expression of ABC transporters, detoxifying enzymes, resistance to apoptosis and niche effects (104). One of the most common mechanisms of drug resistance is related to the increased expression of ABC transporters. ABC transporters are a superfamily of ATP-dependent membrane proteins with at least 49 human members classified into seven (A to G) subfamilies (105, 106). Various ABC transporters have also been implicated in multidrug resistance (MDR) of cancer cells to chemotherapy (105, 106). Specifically, the ATP-binding cassette transporters ABCG2 and MDR1 have been implicated in specifically expelling chemotherapeutic agents from cells and thus may mediate chemotherapy resistance when expressed by CSCs (101).

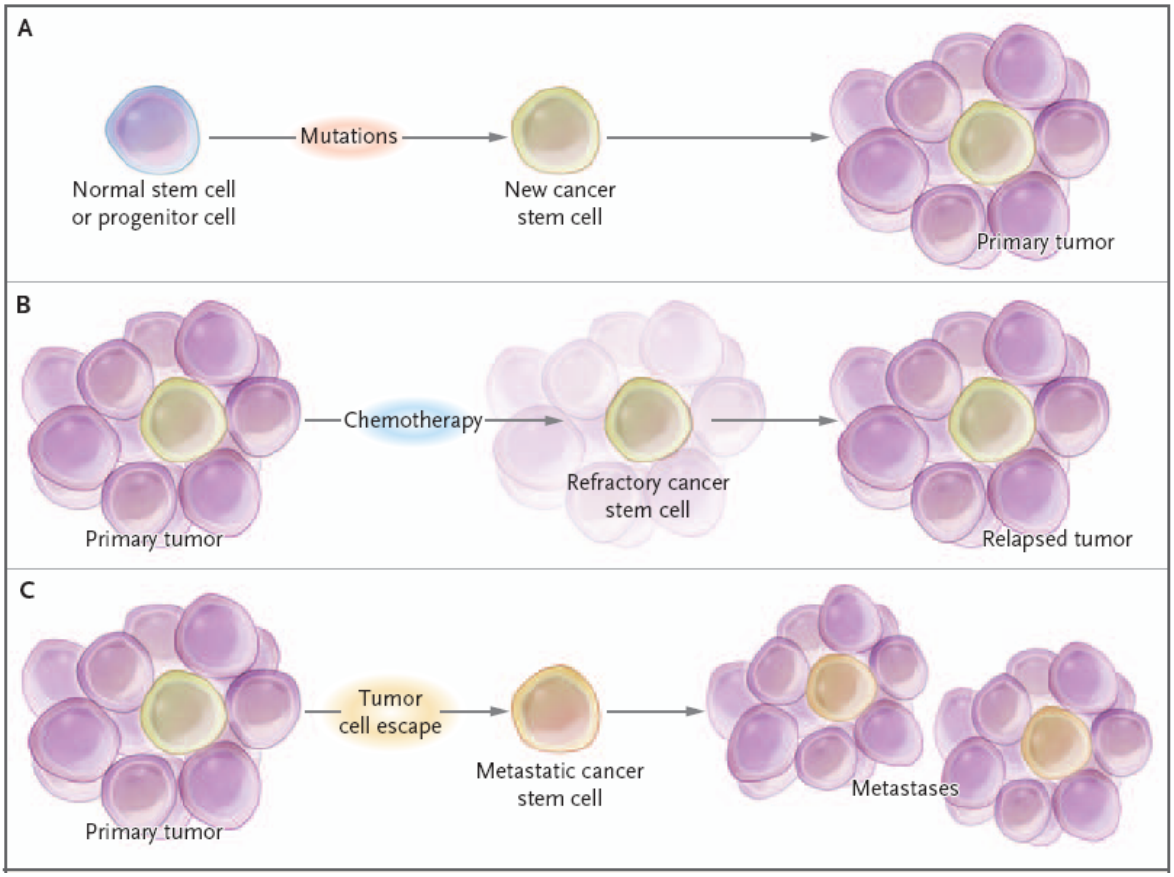

Figure 1.4 Scenarios involving cancer stem cells. For tumours in which cancer stem cells may play a role, at least three scenarios are possible. First, mutation of a normal stem cell or progenitor cell may create a cancer stem cell, which will then generate a primary tumour (Panel A). Second, during treatment with chemotherapy, the majority of cells in a primary tumour may be destroyed, but if the cancer stem cells are not eradicated, the tumour may re-grow and cause a relapse (Panel B). Third, cancer stem cells arising from a primary tumour may emigrate to distal sites and create metastatic lesions (Panel C). [Taken from: Craig T. Jordan, Monica L. Guzman, and Mark Noble. "cancer stem cells", 2006, N Engl J Med**,** 355: 1253 - 1261] (92).

1.6 Aim of the present study

The aim of the present work was (1) to identify whether the putative cancer stem cells exist in anaplastic thyroid carcinoma cells, (2) to generate a stable doxorubicin-resistant anaplastic thyroid carcinoma cell line, (3) to prove that drug resistance is partly due to cancer stem cells

which could expel chemotherapeutic drugs and (4) to detect whether the ABC transporter inhibitors can reverse drug resistance.

2. Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

All thyroid cancer cell lines were stored in liquid nitrogen at -196°C**.** For the experiment, cells were placed in 37°C water bath and shaken gently for 1-2 min and then immediately suspended in pre-warmed culture media.

Anaplastic thyroid carcinoma cell lines used in the experiment were:

The HTh74 human cell line was isolated from thyroid carcinoma tissue of a 73-year-old woman (107). HTh74 cells were shown to express some thyroid-specific genes like functional TSH receptor protein (107), and mRNA for thyroglobulin, albeit both at very low levels (108).

The cell line C643 was established from a fine-needle biopsy of an anaplastic thyroid carcinoma of a 76-year-old man (108). The patient died within 5 months after diagnosis. Demonstration of thyroglobulin mRNA ascertained a thyroid epithelial origin of the cell line (109).

The cell line SW1736 was originally developed by Leibowitz and McCombs III at the Scott and White Memorial Hospital (Temple, TX) in 1977. Their epithelial origin was confirmed by demonstration of cytokeratin expression (110).

All these three cell lines were kindly provided by Prof. Nils-Erik Heldin (Uppsala University, Uppsala, Sweden).

A doxorubicin-resistant HTh74 sub-line (HTh74R) was developed by continuous exposure of HTh74 cell line to doxorubicin (Sigma, USA) starting at 0.01 μg/ml and increasing in a stepwise

manner to 0.5 μg/ml. The resistant cell line was maintained in the medium containing doxorubicin for more than 6 months.

2.1.2 Culture conditions

HTh74 cells were cultured in Ham's F-12 medium (Gibco, Karlsruhe, Germany) with L-glutamine, supplemented with 10 % fetal calf serum (FCS, v/v) (Gibco, Karlsruhe, Germany), 1 % non-essential amino acids (MEM, v/v) (Gibco, Karlsruhe, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (Roche, Mannheim, Germany), and 2.5 μg/ml amphotericin B (Bristol-Meyer Squibb, Germany).

HTh74R cells were grown in Ham's F-12 medium (Gibco, Karlsruhe, Germany) with L-glutamine, supplemented with 10 % fetal calf serum (FCS, v/v) (Gibco, Karlsruhe, Germany), 1 % non-essential amino acids (MEM, v/v) (Gibco, Karlsruhe, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (Roche, Mannheim, Germany), 2.5 μg/ml amphotericin B (Bristol-Meyer Squibb, Germany), and 0.5 μg/ml doxorubicin (Sigma, USA)

C643 and SW1736 were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Karlsruhe, Germany), supplemented with 10 % FCS (v/v), 1 % MEM (v/v), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B.

For the majority of the experiments, all cells were grown as in 100 mm or 60 mm plastic culture dishes and kept in a humidified incubator at 37°C under 5 % CO2, with a media change each 3-4 d. Upon reaching the confluency of 70-80 %, cells were passaged. For passaging, the culture media and trypsin were warmed in 37°C water bath initially. Using the aspirator, the liquid media covering cells was emptied and the cells were briefly washed with HBSS. Then, the cells were incubated with 4ml trypsin (0.05 %, w/v)/EDTA-4Na (0.53 mM) solution in the incubator at 37ºC for 1~5 min, depending on cell type. Trypsin was then inactivated by adding an equal volume of appropriate cell culture medium, after which cells were collected by centrifugation at 310 ×g for 5 min, and reseeded at a splitting ratio of 1:5~6.

2.1.3 Cell counting

A hemacytometer was used for cell counts. After trypsinization and neutralization protocol for cell cultures, a cell suspension was obtained and placed in a centrifuge tube. A 1:2 diluted cell suspension in trypan blue (Sigma, Germany) was loaded on both counting chambers of the hemacytometer and the coverslip was placed over them. The cells were counted under a microscope at 100 × magnification. Cell numbers (total and viable: Trypan blue-unlabeled) overlaying four × 1 mm^2 areas of the counting chamber were determined. And then the total (or viable) cell number of the original cell suspension can be calculated as follows:
Total (or viable) cells recovered = cells/ mm^2 divided by dilution (1/2), and multiplied by 10^4 and total volume of cell suspension

2.2 Fluorescent activated cell sorting of side population after incubation of Hoechst 33342

To isolate the thyroid cancer SP fraction, FACS was performed using the Hoechst staining method as outlined by Goodell *et al*. (45). Before Hoechst staining, the water bath was kept at precisely 37° C (checked with a thermometer), and DMEM+ medium (see below) was pre-warmed. Cancer cells were harvested, suspended at a density of 10^6 cells/ml in DMEM+ medium, and preincubated at 37°C for 10 min. The cells were then labeled in the same medium with 5 µg/ml Hoechst 33342 dye (Sigma, USA) at 37°C for 120 min with periodic agitation, either alone or in combination with 50 µM verapamil (Sigma, USA) or 10 µM fumitremorgin C (FTC) (Sigma, USA), an inhibitor of ABCG2 transporter. During the incubation, tubes were gently inverted every 30 min to prevent cell settling and clumping. Finally, the cells were centrifuged and resuspended in cold HBSS+ medium and counterstained with 2 µg/ml propidium iodide (PI) to exclude dead cells. After 120-min incubation, the cells were spun down at 310 ×g for 5 min at 4°C and re-suspended in 200 µl cold HBSS+.

A 350-nm UV laser was used to excite Hoechst 33342 and PI. Analysis was performed on a FACS Calibur Equipment (Becton-Dickinson Biosciences, Heidelberg, Germany) by using a dual-wavelength analysis (blue, 424-444 nm; red, 675 nm) in the facilities of the German Rheumatology Research Center, Berlin. Dead and dying cells were excluded from the analysis on the basis of PI uptake (fluorescence at 564-606 nm), and only data for viable cells were analyzed for Hoechst labeling using CellQuest software. The SP cells were identified and selected by gating on the characteristic fluorescence emission profile. Equal numbers of SP and non-SP cells were recovered in the culture medium for further experiments or pelleted for RNA isolation.

Reagent solutions:

Hoechst 33342: Obtained from Sigma (called Bis-Benzimide) as a powder. Re-suspended at 1 mg/ml (stock concentration) in water, filter sterilized, and frozen in small aliquots.

HBSS+: Hanks Balanced Salt Solution (Gibco, Germany) with 2 % FCS and 10 mM HEPES buffer.

DMEM+: DMEM with 2 % FCS and 10 mM HEPES buffer.

Propidium Iodide: Obtained from Sigma. Frozen stock is 10 mg/ml in water. Working stock (covered with aluminium foil and kept in the fridge) is at 200 μg/ml in PBS. Final concentration of PI in samples is 2 μg/ml.

2.3 Reverse transcription, polymerase chain reaction (RT-PCR) and quantitative real-time reverse transcription-PCR

2.3.1 Total RNA isolation of FACS sorted cells

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the

manufacturer's specifications. In brief, after aspiration of the culture medium, 350 µl buffer RLT, supplemented with β-mercaptoethanol (β-ME, add 10 µl β-ME per 1 ml buffer RLT), was added to the monolayer cultures. Cell lysates were then collected using a rubber policeman, pipetted in QIA Shredder microcentrifuge tubes (Qiagen, Hilden, Germany) and centrifuged for 2 min at maximum speed for homogenization. Equal volumes of 70% ethanol were added to the homogenized lysate and mixed by pipetting. Samples were then applied to RNeasy mini columns placed in 2 ml collection tubes and centrifuged for 15 sec at 8000 ×g. Flow-through was discarded and 700 µl of RW1 buffer was added to the RNeasy columns and centrifuged for 15 sec at 8000 ×g to wash the columns. Flow-through and collection tubes were discarded and the RNeasy columns were transferred into new 2 ml collection tubes. 500 µl of buffer RPE was pipetted onto the RNeasy columns which were then centrifuged again for 15 sec at 8000 ×g to wash. Flow-through was discarded and another 500 µl buffer RPE was added to the RNeasy columns. Tubes were then centrifuged again for 2 min at 8000 ×g to dry the RNeasy silica-gel membrane. For elution, the RNeasy columns were transferred to new 1.5 ml collection tubes and 30 µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. Tubes were closed gently and centrifuged for 1 min at 8000 ×g. The resulting RNA was either stored at - 20ºC or used directly for spectrophotometry and RT-reaction (see below).

For spectrophotometry, 2 µl of RNA was diluted 1:500 in RNase-free water and absorption was calculated at OD260. For each sample, the mean value of at least two independent readings was adopted as the result.

DNase I working solution:

DNase I stock solution	10 µl
buffer RDD	70 µl

2.3.2 Reverse transcription reaction

For reverse transcription, 1 µl of Oligo$(dT)_{12\text{-}18}$ Primer (0.5 µg/ul) (Invitrogen, Germany) was added to the volume of mRNA solution containing 1 µg of mRNA (as calculated by

spectrophotometry). RNase-free water was added to a final volume of 16.75 µl and the mix was heated to 70°C for 5 min. Probes were placed on ice and the following reagents were added consecutively: 5 µl of M-MLV 5× reaction buffer, 1.25 µl of dNTP nucleotides (10 mM, Roche, Mannheim, Germany), 1 µl (25 Units) of RNAGuard RNase inhibitor (Invitrogen, Germany) and 1 µl (200 Units) of M-MLV Reverse Transcriptase (Promega, Mannheim, Germany). The mixture was warmed to 42°C for 60 min, 95°C for 5 min and the reaction was terminated at 0°C. cDNA samples were stored at -20°C.

2.3.3 Primer preparation

All primers were obtained in powder form from Invitrogen Inc. Upon delivery, primers were diluted in RNAse-free water (concentration 5 pmol/µl), aliquoted and stored at -20°C.

2.3.4 Polymerase chain reaction

For PCR amplification, the Hot Start method was used. In brief, 4 µl of complementary DNA was added to a 45.5 µl master mix containing 5 µl 10× reaction buffer, 1.5 mM MgCl2, 1 µl dNTPs (10 mM) and 30 pmol of sense and antisense primers. Negative controls without template cDNA were included in all cases to exclude carry-over contamination with genomic DNA. 50 µl of mineral oil was added to each reaction tube, the lids were closed and the mixture was preheated to 95 ºC for 10 min before adding Taq polymerase (Invitrogen, Karlsruhe, Germany) to reduce non-specific annealing and primer elongation events. The samples were then cooled to 80 ºC and 0.5 µl of Taq DNA polymerase (5 U/µl) was added to each reaction tube. Using a thermocycler, cycling conditions were carried out at 95°C for 30 sec (initial denaturation), 52-63°C for 30 sec (primer specific, annealing) and 72°C for 1 min (extension), followed by a final extension at 72°C for 10 min and termination at 4°C. The number of cycles used was determined by the log-linear phase of the amplification reaction. In all PCR analyses, β-actin served as an internal control. Primer pair sequences, product lengths and annealing temperatures were as follows:

Table 2.1 Primer sequences, annealing temperatures, cycles and product sizes for RT-PCR

Target gene	Primer sequences[#]	Annealing Temp (^{o}C)	Cycles	Expected size (bp)
β-actin	S: 5'-CCCAGGACCAGGGC GTGAT -3' AS: 5'-TCAAACATGATCTGGGTCAT-3'	59	25	280
ABCG2	S: 5'-AGTTCCATGGCACTGGCCATA-3' AS: 5'-TCAGGTAGGCAATTGTGAGG-3'	53	30	379
Oct4*	S: 5'-GACAACAATGAGAACCTTCAGGAG -3' AS: 5'-CTGGCGCCGGTTACAGAACCA-3'	55	30	216
MDR1*	S: 5'-GCCTGGCAGCTGGAAGACAAATAC-3' AS: 5'-ATGGCCAAAATCACAAGGGTTAGC-3'	59	29	253
18sRNA*	S: 5'-CTCAACACGGGAAACCTCAC-3' AS: 5'-CGCTCCACCAACTAAGAACG-3'	58	-	110
ABCG2*	S: 5'-TGTAGCAACACTTCTCATGACC-3' AS: 5'-TATTCTTCGCCAGTACATGTTG-3'	58	-	234

[#] S: sense primer; AS: antisense primer

* The primers were used for qPCR

2.3.5 Agarose gel electrophoresis

PCR products were separated on 1.5 % or 2 % (w/v) agarose gels (2 % for expected fragment sizes of 250 bp or lower) containing 0.5 μg/ml ethidium bromide. Gels were run at a voltage of 90V in TBE running buffer for 60 min. Bands were visualized on a UV-transilluminator at 312 nm.

TBE Buffer (20X):

1.8 M Tris-base

1.8 M boric acid

25 mM EDTA-Na2.2H2O

PH was adjusted to 8.3

2.3.6 Quantitative real-time reverse transcription-PCR

Real-time quantitative RT-PCR was performed with iCycler iQReal time PCR detector system (Bio-Rad). SYBR Green reactions were performed using ABsolute^{TM} QPCR SYBR Green Fluorescein Mix (Applied Thermo Fisher scientific Inc). The PCR reaction was performed in a 96 well plate. Cycling conditions were as follows: initial enzyme activation at 95 ºC for 15 min, followed by 50 cycles at 95 ºC for 15 s; 58 ºC for 30 s; 72 ºC for 30 s. Relative expression levels of each gene in real time were analyzed using the 2-ΔΔCT method and normalized to the expression of the housekeeping gene 18srRNA. Each sample was replicated twice from 3 independent sets of RNA preparations. Results are tabulated as mean ± SEM of 3 independent experiments. The Ct values of the real-time PCR were calculated by the (2*efficiency) $^{\Delta\Delta Ct}$ method, and normalized by the value of the internal control 18s rRNA. Data were presented as mean of fold change ± SEM vs the control and were derived from at least three independent experiments.

2.4 Immunofluorescence staining

Before doing immunofluorescence staining, coverslips coated with poly-L-lysine (Sigma, USA). Poly-L-lysine was diluted to 0.1 mg/ml with sterile deionized water before coating slides. Poly-L-lysine solution was aspirated 0.5 ml/25 cm^2 to the coverslips. The coverslips were rocked gently to ensure even coating. After 5 minutes, the solution was removed by aspiration and the surface was thoroughly rinsed with sterile deionized water for 3 times. There was at least 2 hours of time allotted for drying before introducing cells and medium.

Cells were plated onto the coverslips and grown in culture medium containing 10% FBS. After 24 h, cells were rinsed in PBS, followed by fixation for 15 min in PBS containing 4% paraformaldehyde. The fixed cells were permeabilized by 0.1%Triton X-100 and blocked by

normal goat serum (10%). Coverslips were incubated overnight at 4°C with the polyclonal anti-ABCG2 antibody or monoclonal antibodies against MDR1 (1:100, Santa Cruz Biotechnology, inc). Unbound antibodies were removed by rinsing in PBS with 0.1% tween-20, followed by incubation for 60 min at room temperature with FITC-conjugated secondary antibody as a secondary antibody in the dark (1:200, Santa Cruz). FITC-labeled cells were analyzed by fluorescence Olympus microscope using standard fluorescent filters (excitation 488 nm).

2.5 Viability assay

Cell viability was measured by the methyl thiazolyl tetrazolium (MTT) test. Cells ($0.5\sim1\times10^4$) were seeded in 96-well plates. After 24 h, they were treated with medium alone or with medium containing different doses of doxorubicin, cisplatin, or doxorubicin plus verapamil for 24h or 48h. The medium containing 0.5 mg/ml MTT (Sigma, USA) was added to each well and incubated at 37°C for 4 h. The formazan product was dissolved in isopropanol and the plates were read at 490 nm using a plate reader. Cell viability was expressed as a percentage of the value of untreated controls. All experiments were repeated at least three times, and each experimental condition was repeated at least in quadruplicate wells in each experiment.

2.6 In vitro clonal analysis

To evaluate the self-renewal potential of HTh74 SP cells, clonal formation assay was performed. SP and non-SP cells sorted by FACS were seeded at clonal density (200 cells per 60-mm dish). The colonies were counted at day 5 and day 10. The percentage of cells that initiated a clone was presented as cloning efficiency. Triplicate samples were run for the experiments.

2.7 Colorimetric assay of caspase-3 activity

The caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate

acetyl-Asp-Glu-Val- Asp 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 (ε^{mM} = 10.5). The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

2×10^6 cells were plated in 10 cm culture dishes. After attaching to the flasks overnight, cells were treated with doxorubicin, verapamil, or doxorubicin plus verapamil, and the control was treated with the culture medium. Caspase 3 was determined using a colorimetric assay kit (CASP3C, Sigma, USA) according to the manufacturer's instructions.

2.8 **Experimental equipment**

2.8.1 Apparatus

FACS Calibur equipment	Becton-Dickinson Biosciences
Laminar flow cabinet	Heraeus, Laminair HB 2448
Cell culture incubator	Heraeus
Phase contrast microscope	Nikon, TMS
Fluorescence microscope	Olympus Soft Inaging Solutions
Digital camera	Sony DSC-W7
Water bath	Kotterman Labortechnik
UV Illuminator	Bachofer Laboratoriumsgeräte
UV camera	Polaroid MP4 Land Camera with Polaroid 545 4X5 film holder
Scanner	CanoScan 5000
Thermocycler	Biometra, Trio-Thermoblock
Spectrophotometer	Pharmacia, Ultrospec II
Autoclave	H+P Varioklav
Shake incubator	Infors HT

Precision scale	Advenurer OHAUS
Voltage generators	Consort, Electrophoresis power supply, E455
Homogenisator	Eppendorf Thermomixer 5436
Centrifuges	Hettich Mikro 200R
	Beckmann Microfuge E
	Hettich Rotina 46R
Heating oven	Memmert
Pipets and tips	Eppendorf
Plastic centrifuge tubes (1.5, 15 and 50 ml)	Sarstedt
Falcon tubes	Becton Dickinson
Plastic culture dishes and plates	Sarstedt
Polystyrene slide flasks	Nunc
Elisa machine	Biotek

2.8.2 Software

Biology Workbench version 3.2	DNA and mRNA sequence analysis
Cellquest	Flow cytometry analysis
Adobe photoshop version 7.0	Figure preparation
Image-Pro Plus 6.0	Figure preparation
GraphPad Prism 4.0	Figure preparation

3. Results

3.1 Characterization of anaplastic thyroid carcinoma cell lines and their stem cells

3.1.1 ABC transporters(ABCG2/MDR1) and stem cell marker Oct4 expression in human anaplastic thyroid cancer cell lines

Expression of ABC transporters (ABCG2/MDR1) and stem cell marker Oct4 mRNA was detected in the human anaplastic thyroid cancer cell lines C643, HTh74 and SW1736 by RT-PCR with human specific primers (Fig. 3.1). The amplified sequences displayed the expected size of 379bp (ABCG2), 253bp (MDR1) and 216bp (Oct4) in all cases.

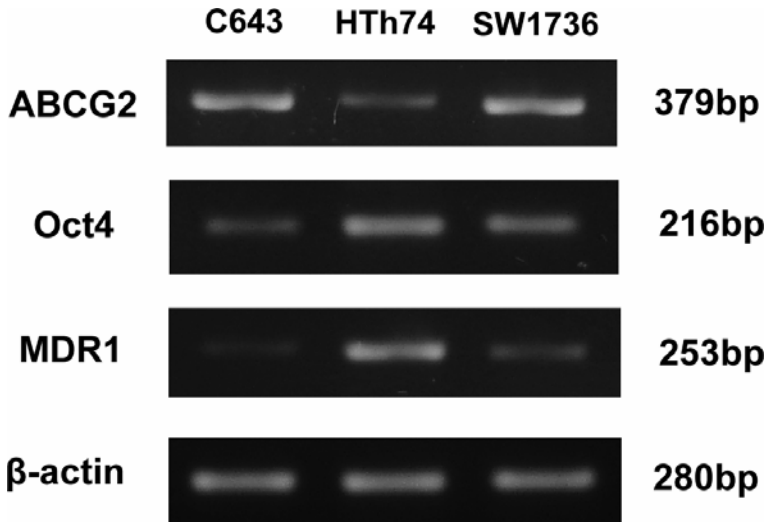

Figure 3.1 Expression of ABC transporter genes and stem cell marker Oct4 in different human anaplastic thyroid cancer cell lines. β-actin was also detected as a control in all samples.

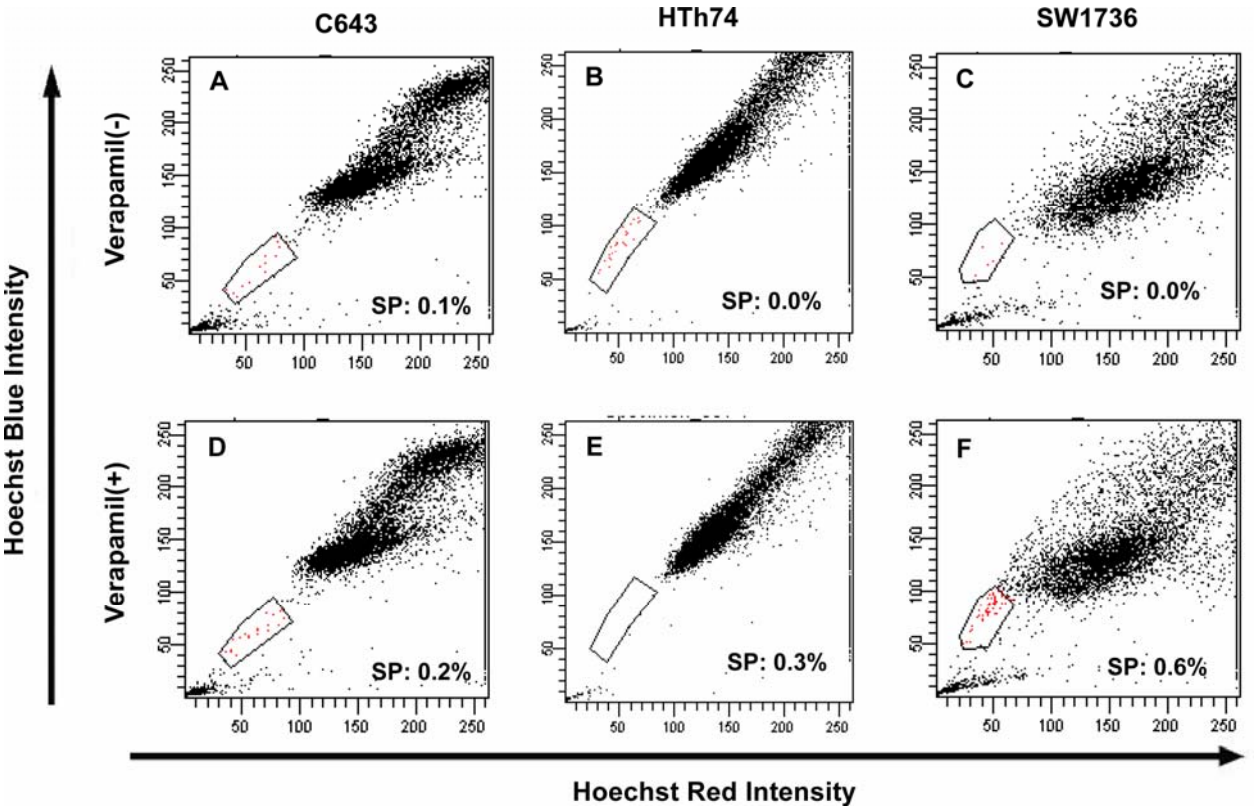

Figure 3.2 Identification of SP cells in human anaplastic thyroid cancer cell lines. C643 (A, D), HTh74 (B, E), and SW1736 (C, F) cells were labelled either with Hoechst 33342 alone (A-C) or in combination with verapamil (D-F) and then analysed by FACS. The SP cells (A-C), which disappeared in the presence of verapamil (D-F), were outlined and shown as a percentage of the total cell population.

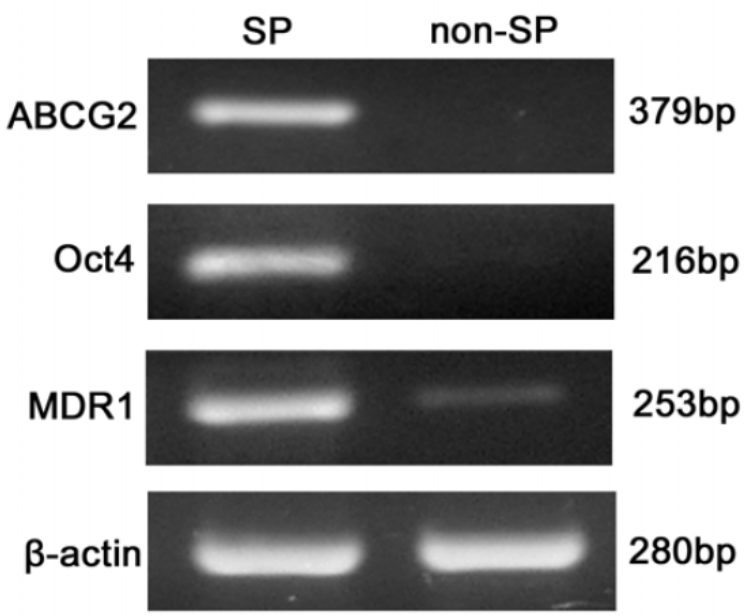

Figure 3.3 Identification of stem cell marker and ABC transporter genes in HTh74 SP cells. SP cells showed expression of Oct4, ABCG2 and significantly higher levels of MDR1, in comparison to non-SP cells.

3.1.2 Existence of side population cells in human anaplastic thyroid cancer cell lines

C643, Hth74 and SW1736 anaplastic thyroid carcinoma cell lines were trypsinized and incubated with Hoechst 33342. As revealed by flow cytometry analysis, 0.2% of C643 cell line, 0.3% of HTh74 cell line and 0.6% of SW1736 cell line presented as an SP of cells that excluded the Hoechst dye due to the ability to express transporters of the ABC transporter family (Fig. 3.2)., Verapamil, an inhibitor of ABC transporters, as the control was used to abolish the SP profile.

Then mRNA levels of Oct4, ABC transporters (ABCG2 and MDR1) were analysed in the SP and non-SP fraction. SP cells expressed Oct4 mRNA, a marker of embryonic and adult stem cells (38, 111), and 2 genes of the ABC transporter family (46), whereas non-SP cells expressed only low amounts of MDR1 mRNA (Fig. 3.3).

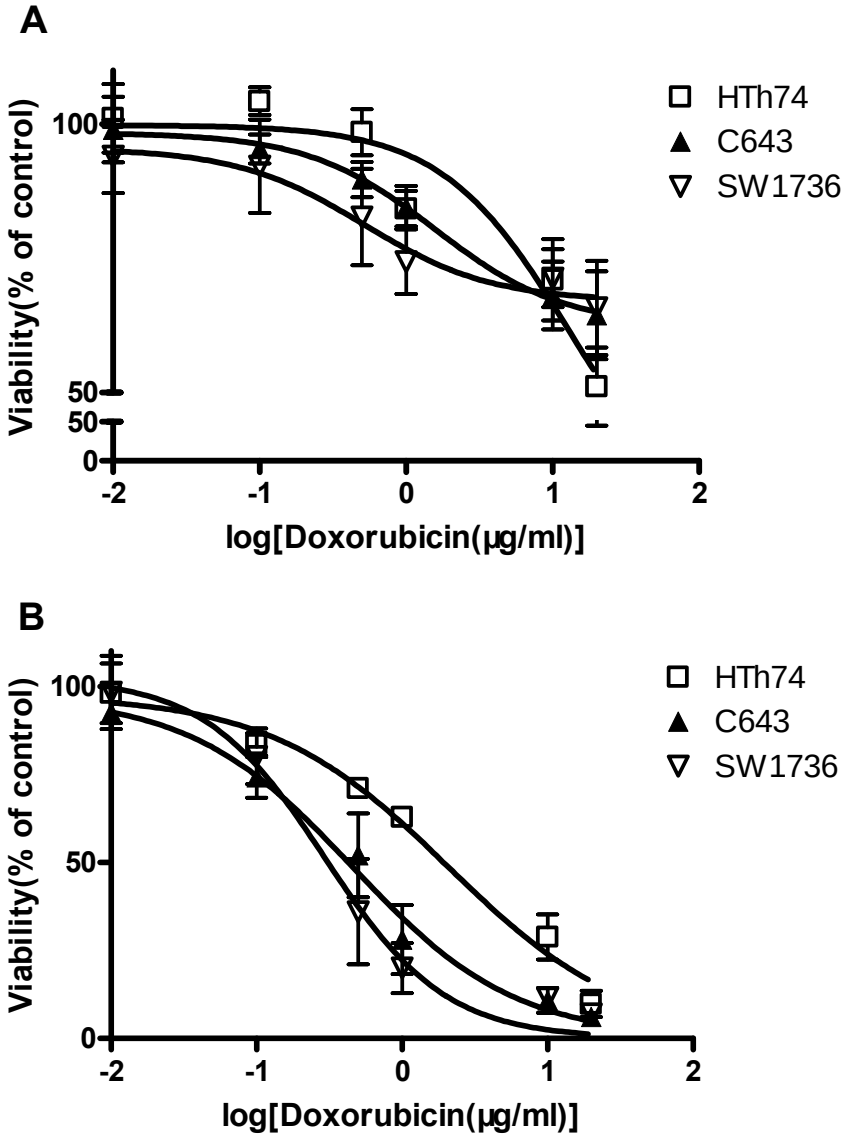

Figure 3.4 Effects of doxorubicin on anaplastic thyroid carcinoma cells viability. (A) Dose response curves for 24h doxorubicin treatment. (B) Dose response curves for 48h doxorubicin treatment. Data are represented by mean S.E.M of at least three independent experiments.

3.2 Effect of doxorubicin on survival of anaplastic thyroid carcinoma cells and establishment of a stable resistant cell line

3.2.1 Effect of doxorubicin on survival of anaplastic thyroid carcinoma cells

When anaplastic thyroid cells were treated with doxorubicin, only high doses killed the large majority of tumour cells (Fig. 3.4). When incubated for 48 hrs, the IC_{50}（the half inhibitory concentration）of doxorubicin for HTh74 cells was 1.80±0.14 μg/ml, for C643 cells 0.89±0.40 μg/ml and for SW1736 cells 0.61±0.26 μg/ml (Table 2).

3.2.2 Establishment of a HTh74 doxorubicin-resistant cell line

A doxorubicin-resistant HTh74 sub-line (HTh74R) was developed by continuous exposure of HTh74 cell line to doxorubicin starting at 0.01μg/ml and increasing the concentration gradually to 0.5μg/ml. By treatment of HTh74 cells with 0.5μg/ml doxorubicin for 6 months a stable cell line, designated HTh74R, was established. There was no morphologic difference between the HTh74 and HTh74R cells (Fig.3.5 A). The growth curves of the cell lines also did not significantly differ (Fig. 3.5 B). After 6 months of culturing in the presence of the drug, HTh74R was more resistant to the doxorubicin than the wide type HTh74. The IC50 value of HTh74R was 153.53±16.43 μg/ml, which was an 85-fold increase over that of the parent HTh74 cell line (IC50 was 1.80±0.14 μg/ml) (Fig. 3.6 and Table 2).

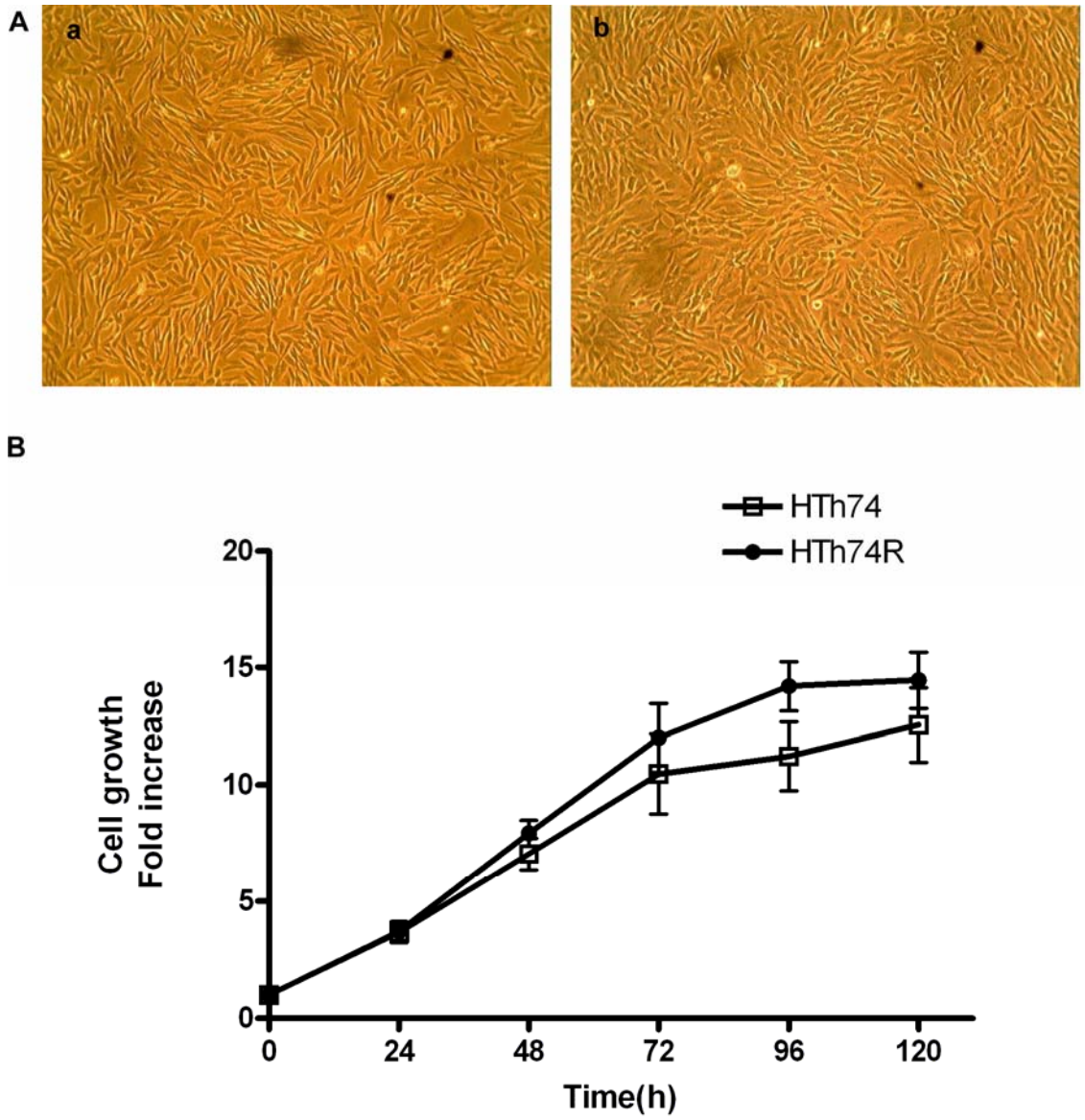

Figure 3.5 (A) a, Parental anaplastic thyroid carcinoma cell line, HTh74. b, Doxorubicin-resistant cell line, HTh74R. There was no morphologic difference between the HTh74 and HTh74R cells. (B) Growth curve of HTh74 and HTh74R cell lines. The growth curves of the cell lines also did not differ. Data were represented by mean ±SEM of at least three independent experiments.

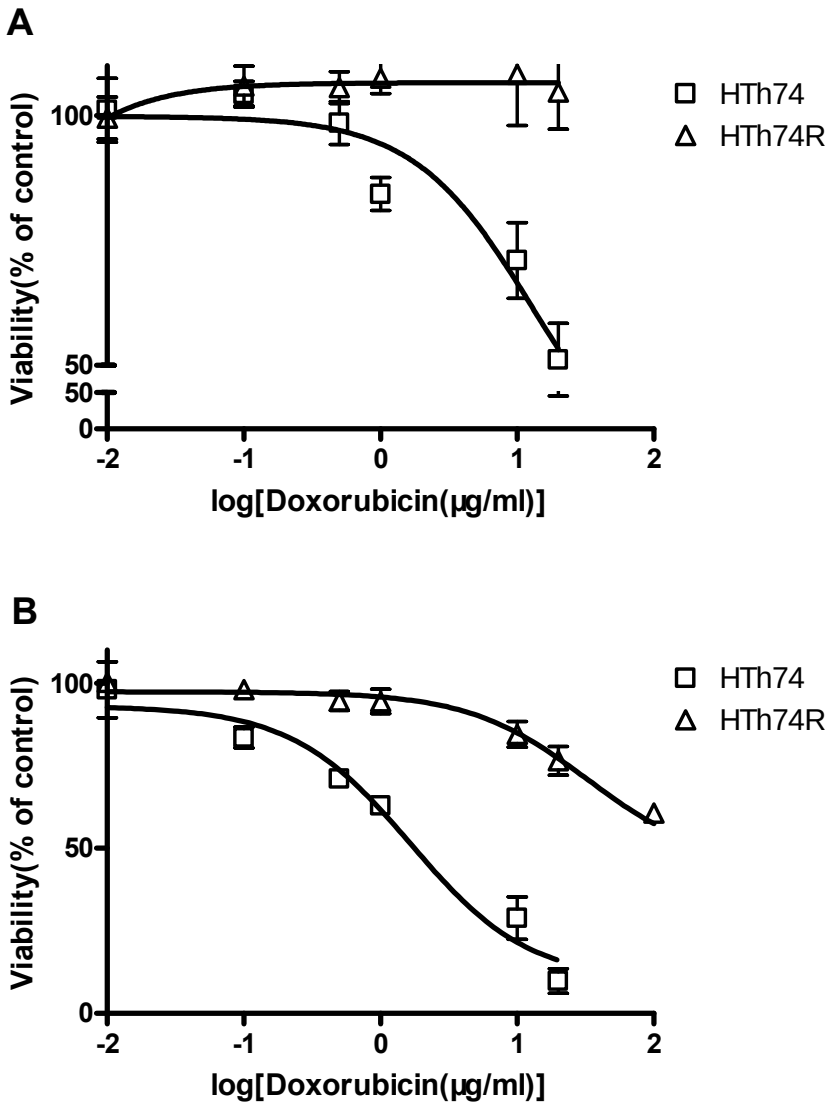

Figure 3.6 The effects of doxorubicin on HTh74 and HTh74R cells viability. (A) Dose-response curves for doxorubicin 24h treatment. (B) Dose-response curves for 48h doxorubicin treatment. Data were represented by mean ±SEM of at least three independent experiments.

3.3 Comparison of the hallmarks of HTh74 and HTh74R cells

3.3.1 HTh74R cells were more clonogenic than HTh74 cells

To further investigate whether the HTh74R cells are associated with some of the intrinsic stem cell properties, HTh74R cells and HTh74 cells were used in a clonal formation assay, which measures the self-renewal capacity of the cells. As shown in Fig. 3.7, about 7 % of total HTh74 cells grew as colonies. Whereas about 50 % of the HTh74R cells sustained a clonal growth and formed characteristic compact circular colonies with a cobblestone appearance (Fig. 7B), HTh74R cells were more clonogenic than HTh74 cells

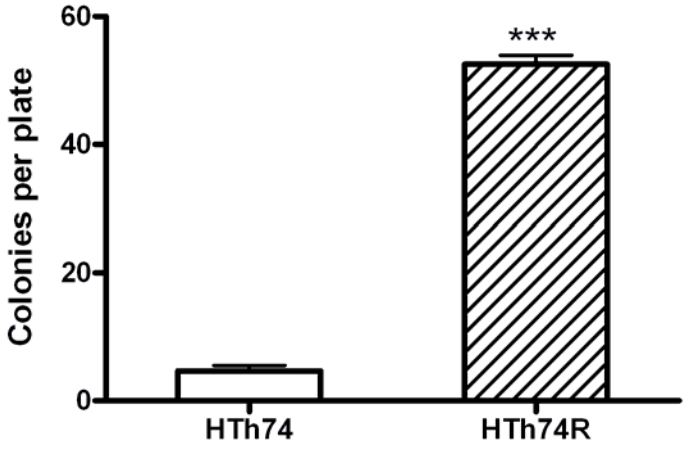

Figure 3.7 Clonal assay of HTh74 and HTh74R. Data were represented by mean ± SEM of three independent experiments (***, $P<0.001$).

3.3.2 HTh74R cells expressed high levels of ABCG2, Oct4 and MDR1

Gene expression profiles of HTh74R cells and HTh74 cells were further analyzed by RT-PCR and real-time PCR (Fig. 3.8). Real-time PCR analysis indicated that HTh74R cells expressed about 3 times higher levels of Oct4 than HTh74 cells. HTh74R also expressed approximately 90-fold higher levels of MDR1 and 2-fold higher levels of ABCG2 than the HTh74. However, the expression of MRP1 was not increased in HTh74R compared with HTh74.

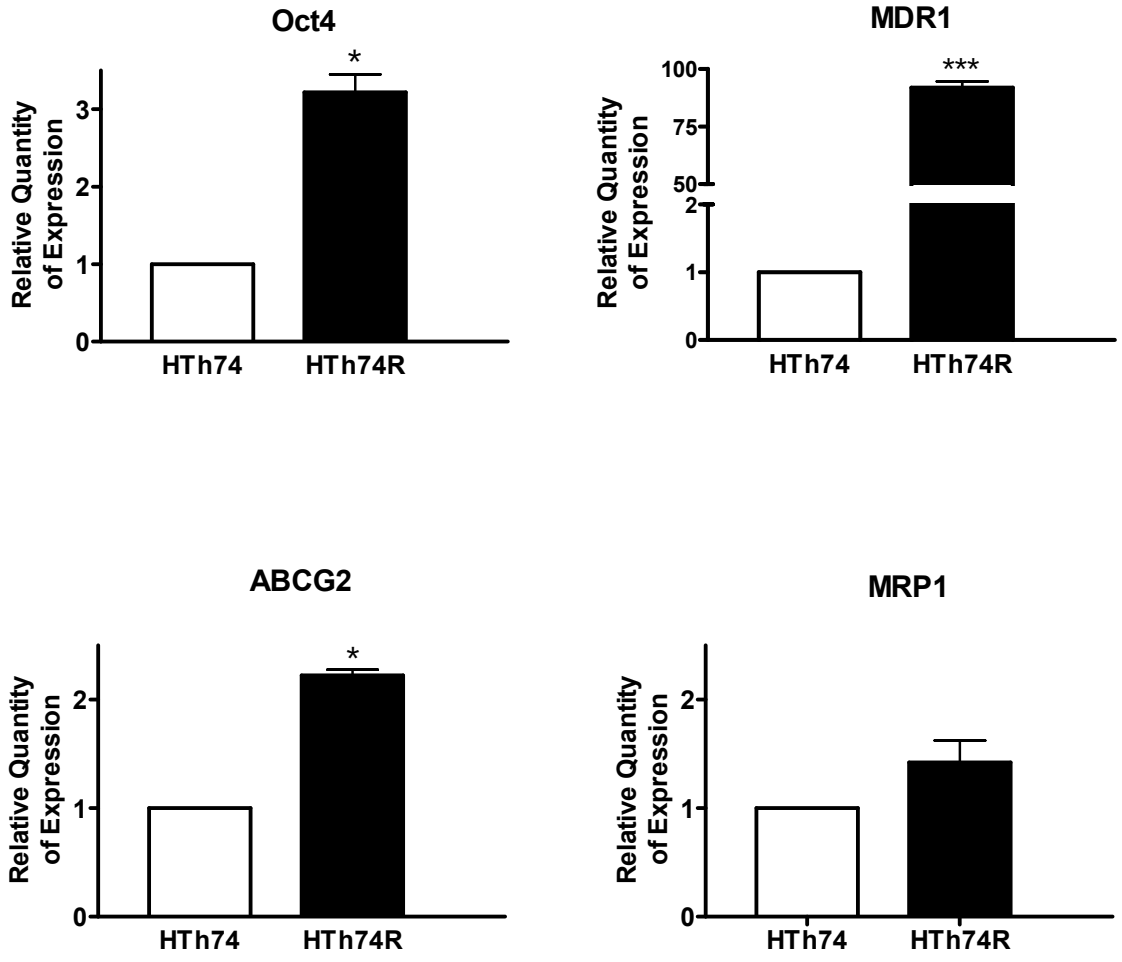

Figure 3.8 Real-time RT-PCR analysis of mRNA expression of ABC transporter genes and stem cell marker Oct4 in HTh74 and HTh74R. The Ct values of real-time PCR were calculated by the (2*efficiency) $^{\Delta\Delta Ct}$ method, and normalized by the value of the internal control 18s rRNA. Data were presented as mean of fold change ±SEM *vs.* control and were derived from at least three independent experiments (*, $P<0.05$; *** $P<0.001$)

3.3.3 HTh74R cells contained more SP cells than HTh74

To determine the effect of doxorubicin in HTh74 cells, HTh74 cells were treated with doxorubicin 0.5 µg/ml over a short-term (3 days) and over a long-term (more than 6 months, to establish a resistant cell line HTh74R). Then cells were dissociated from culture dishes by trypsin, incubated with Hoechst 33342 and analyzed by FACS. Representative density dot-plots of FACS profile are shown in Figs. 3.9- 3.11. By treatment of HTh74 cells with 0.5µg/ml doxorubicin for 3 days, the percentages of SP cells in C643, HTh74 and SW1736 were increased to 0.4 %, 7.3 % and 3.1 %, respectively (Figs. 3.9-3.11). For more than 6 months, about 70% of cells were detected as SP cells (Fig. 3.12). In each case, addition of verapamil completely abolished the SP profile, illustrating the specificity of the staining.

These results demonstrate that by long-term treatment of anaplastic thyroid cells with doxorubicin, the majority of the main population non-SP cells died whereas the SP survived.

3.3.4 Immunofluorescence staining of ABCG2 and MDR1 in HTh74 and Hth74R cells

To evaluate the expression of ABC transporters (ABCG2 and MDR1) in HTh74R cells, which contained about 70% SP cells, HTh74 and HTh74R cells were stained with the polyclonal anti-ABCG2 antibody or monoclonal antibodies against MDR1. As shown in Fig 3.13, the numbers of ABCG2-positive cells and MDR1-positive cells are much higher in HTh74R than in HTh74 cells.

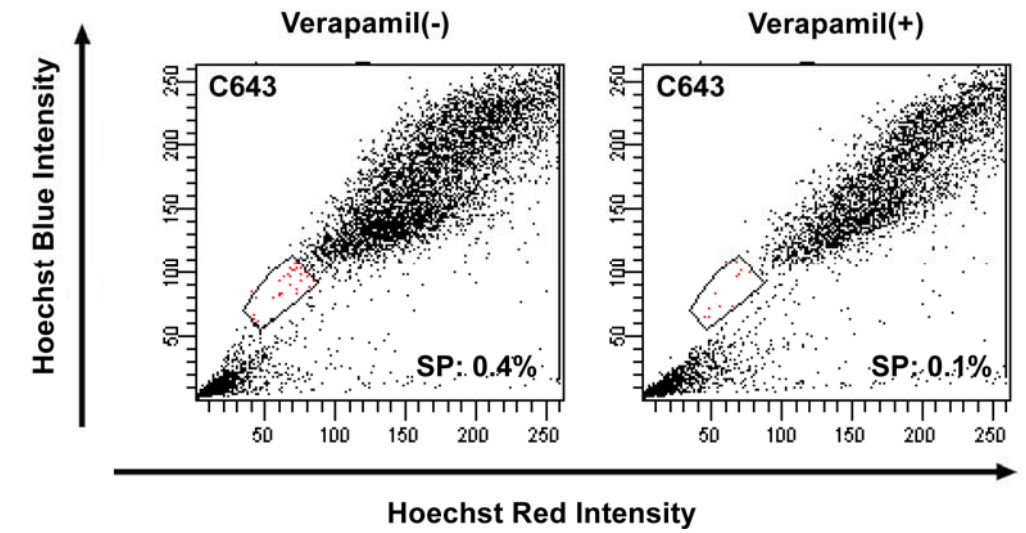

Figure 3.9 Analysis of SP in C643 cells treated with doxorubicin 0.5µg/ml for 3 days. Cells were labelled either with Hoechst 33342 alone or in combination with verapamil and then analysed by FACS.

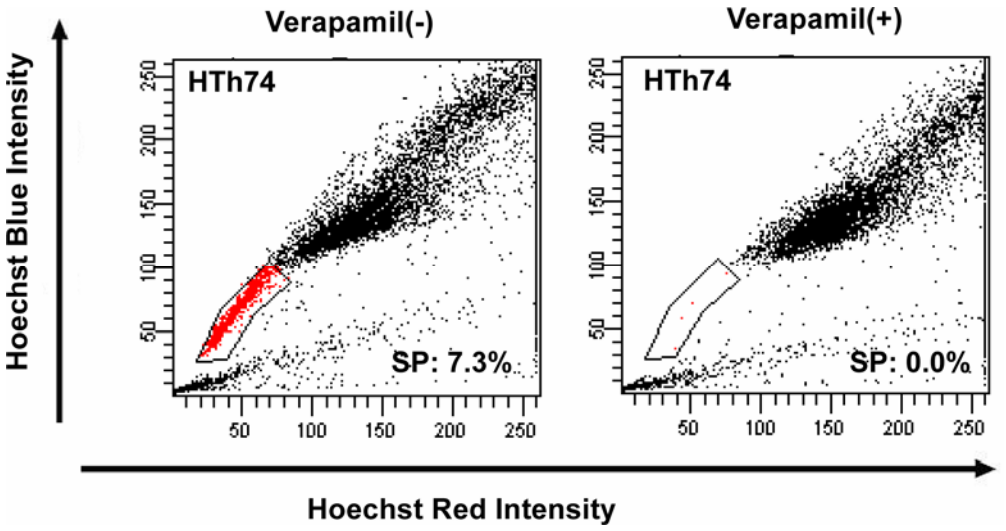

Figure 3.10 Analysis of SP in HTh74 cells treated with doxorubicin 0.5µg/ml for 3 days. Cells were labelled either with Hoechst 33342 alone or in combination with verapamil and then analysed by FACS.

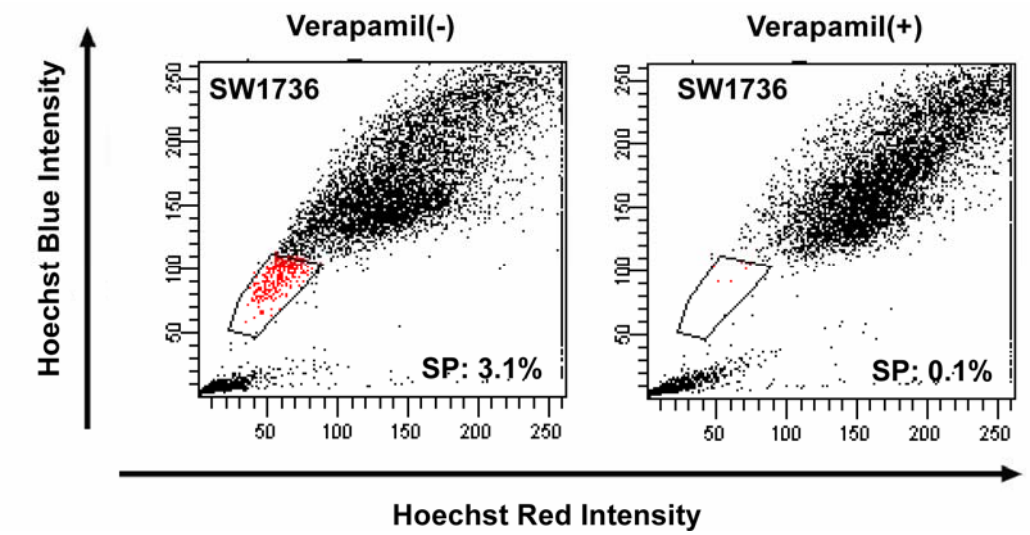

Figure 3.11 Analysis of SP in SW1736 cells treated with doxorubicin 0.5μg/ml for 3 days. Cells were labelled either with Hoechst 33342 alone or in combination with verapamil and then analysed by FACS.

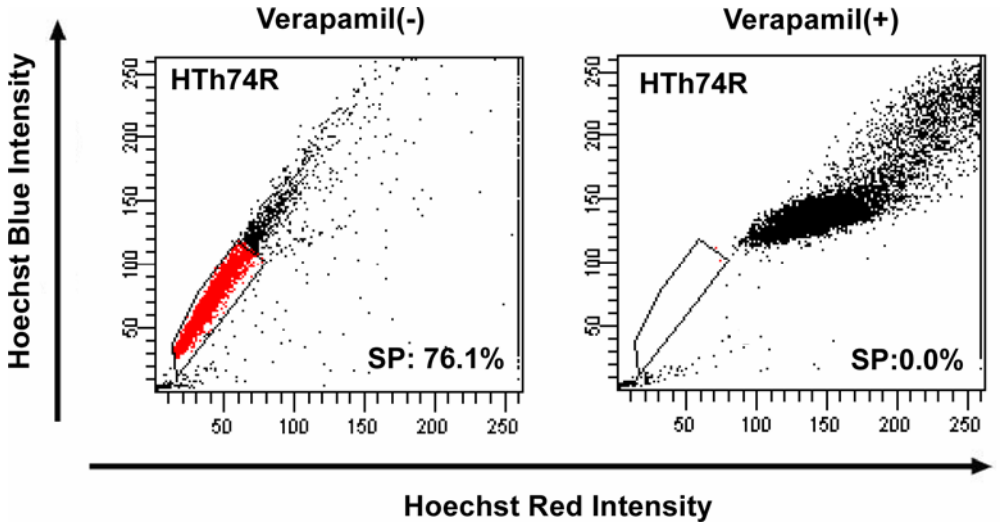

Figure 3.12 Analysis of SP in HTh74R treated with doxorubicin 0.5μg/ml more than 6 months. Cells were labelled either with Hoechst 33342 alone or in combination with verapamil, and then analysed by FACS.

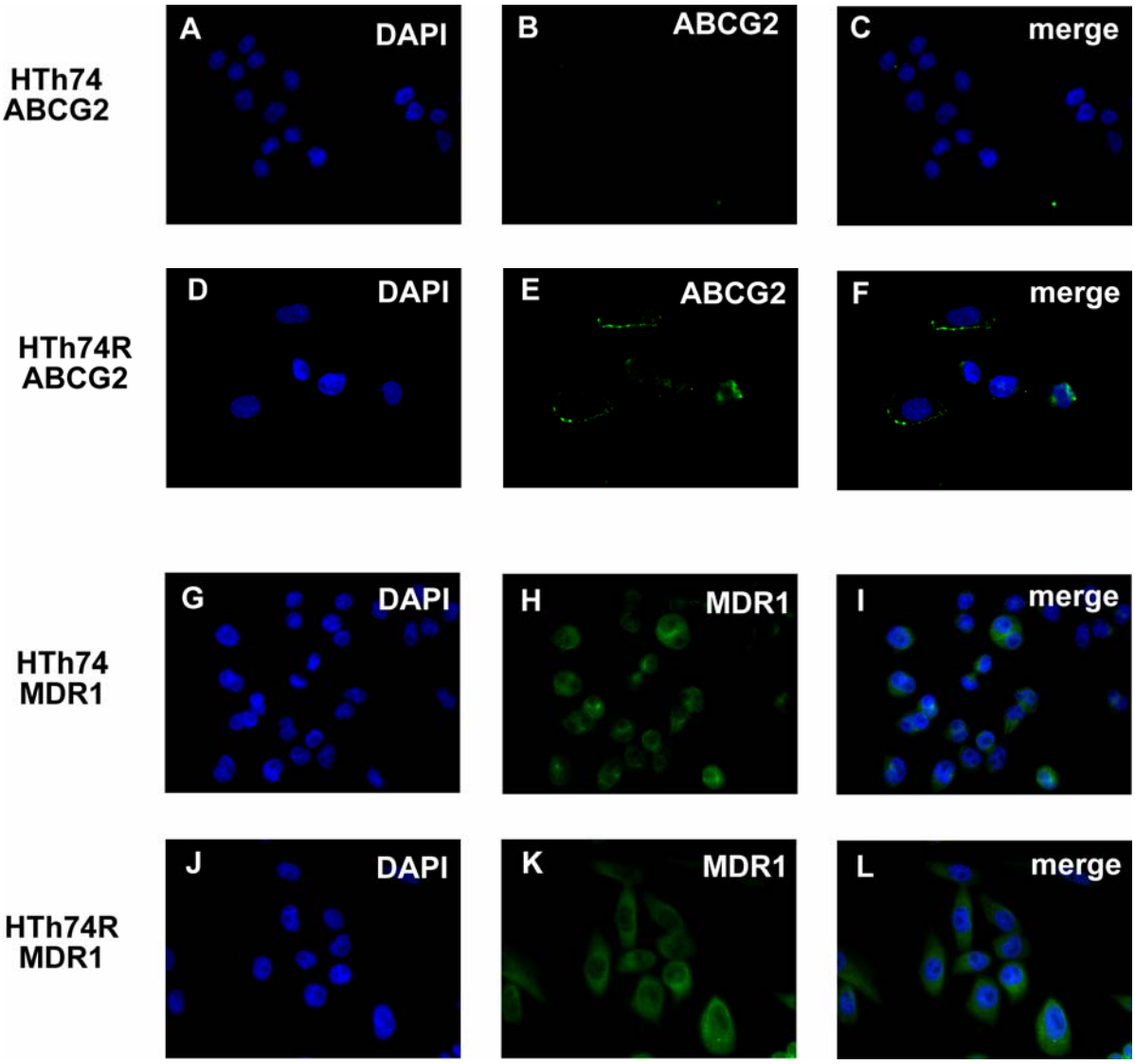

Figure 3.13 Immunofluorescence staining of ABCG2 and MDR1 in HTh74 cells and HTh74R cells. HTh74 cells and HTh74R cells were plated onto coverslips and subjected to immunofluorescence labeling with polyclonal anti-ABCG2 antibody or monoclonal anti-MDR1 antibody. (A-C) ABCG2 of HTh74. (D-F) ABCG2 of HTh74R. (G-I) MDR1 of HTh74. (J-L) MDR1 of HTh74R.

3.4 ABC transporter inhibitors reverse the resistance of doxorubicin treatment

To investigate if verapamil can increase the sensitivity of anaplastic thyroid carcinoma to doxorubicin and reverse the resistance, HThR cells were treated with doxorubicin combined with verapamil. Fig. 3.14 showed the effects of doxorubicin alone or with verapamil on HTh74R cells' viability. After the treatment of 48 hrs, the IC_{50} of doxorubicin for HTh74R cells was 157.60±13.98μg/ml, the IC_{50} of doxorubicin with verapamil for HTh74R cells 2.04 ± 0.60 μg/ml. Verapamil enhanced the sensitivity of HTh74R to doxorubicin.

To determine if the ABCG2 transporter is responsible for chemoresistance to doxorubicin, fumitremorgin C (FTC), a specific inhibitor of ABCG2, was used to treat HTh74R cells. Fig. 3.14 showed the effects of doxorubicin alone or with FTC on HTh74R cells' viability. After the treatment of 48 hrs, the IC_{50} of doxorubicin with verapamil for HTh74R cells was 14.14±1.73 μg/ml. FTC increased the sensitivity of HTh74R to doxorubicin.

The ability of FTC to inhibit the ABCG2 transporter was confirmed by Hoechst 33342 staining and FACS. HTh74R cells were labelled either with Hoechst 33342 alone or in combination with 10 μM FTC and analysed by FACS. In contrast to verapamil, which completely inhibits the exclusion of Hoechst dye and thus the SP phenotype, FTC specifically blocks only the ABCG2 transporter (112, 113). As depicted in Fig 3.15, FTC partly inhibited the SP phenotype in HTh74R cells.

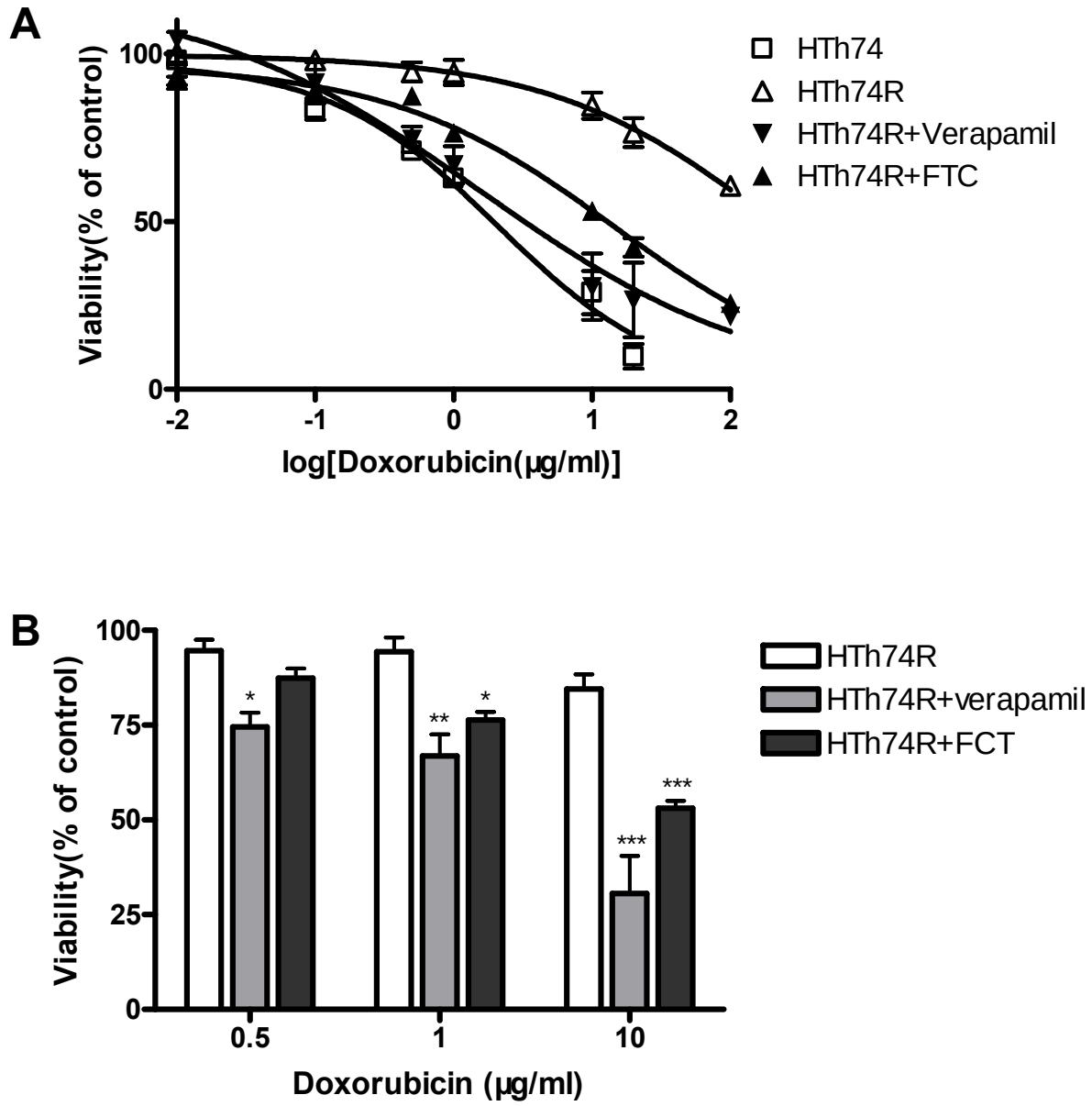

Figure 3.14 Effects of doxorubicin alone or with verapamil or FCT on HTh74R cells' viability. (A) Dose-response curves for doxorubicin or doxorubicin plus verapamil or FTC 48h treatment. (B) Dose-response column for doxorubicin or doxorubicin plus verapamil or FTC 48h treatment. Each data point represents the mean of at least three independent experiments. (*: $P<0.05$, **: $P<0.01$, ***, $P<0.001$)

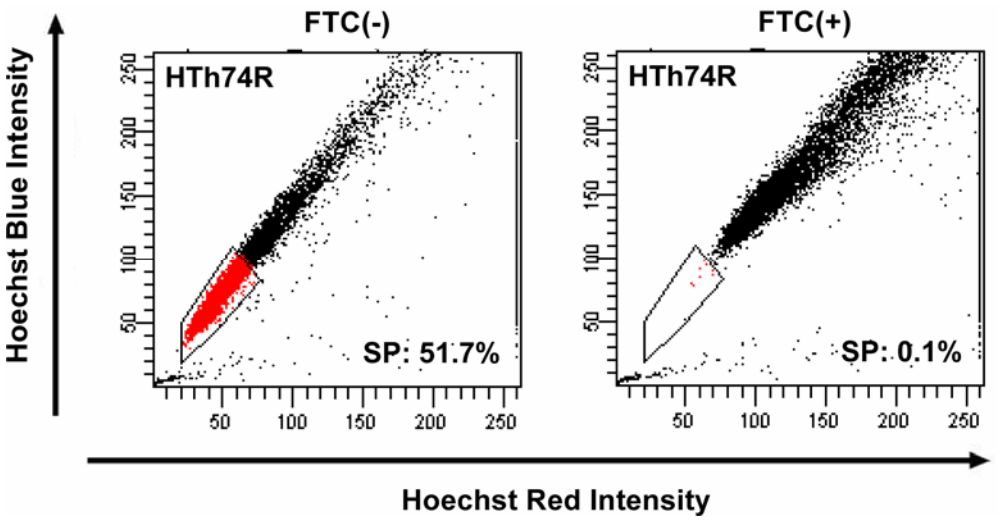

Figure 3.15 Analysis of SP in doxorubicin-resistant cell line HTh74R. HTh74R cells were labelled either with Hoechst 33342 alone or in combination with FTC for 120min, and then analysed by FACS.

Table 2 Sensitivity of anaplastic thyroid carcinoma cell lines to doxorubicin. IC50 values were determined by the MTT assay. Each data represented the mean of at least three independent experiments.

Cell lines	Drug	IC50 µg/ml	SEM
SW1736	DOX	0.61	0.26
C643	DOX	0.89	0.40
HTh74	DOX	1.80	0.14
HTh74R	DOX	153.53	16.43
HTh74R	DOX+Verapamil	2.04	0.60
HTh74R	DOX+FTC	14.14	1.73

3.5 Caspase-dependent apoptosis

Caspase 3 is a key enzyme of the apoptotic pathways (114). Apoptosis execution in many carcinomas including anaplastic thyroid carcinomas depends on caspase 3 and other caspases(115). When HTh74R cells were incubated with doxorubicin alone, the caspase-3

activity was similar to HTh74R cells without doxorubicin (Fig. 3.16). However, when HTh74R cells were incubated with verapamil which inhibits expulsion of doxorubicin from putative cancer stem cells, caspase-3 activity was increased (Fig. 3.16). Under these conditions there was an 87.8% increase in caspase 3 activity (Fig. 16).

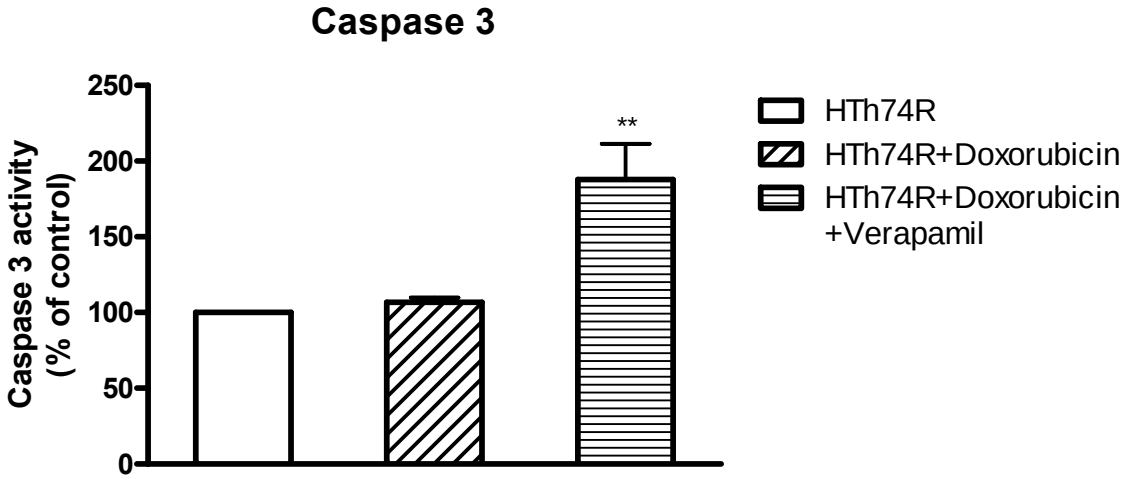

Figure 3.16 Effect of verapamil on doxorubicin-induced apoptosis. HTh74R cells were treated with doxorubicin (0.5μg/ml) alone or plus verapamil (10μM) for 48h. After treatment, caspase 3 activity was measured as described in the text. Activity of caspase 3 in untreated cells was taken as 100%. Results of at least 3 independent experiments are expressed as mean ± S.E.M. (**, $P<0.01$).

4. Discussion

The purpose of cancer research is to find the exact mechanisms of tumourigenesis, to find specific drugs or methods which can increase the survival rate of patients, and to cure cancer at the end. However, so far, even if there was early detection and diagnosis of cancer, treatment can often only shrink the size of the cancer but not completely eradicate the tumour. Distant metastasis often occurs and drug resistance frequently develops.

During recent decades, normal stem cells have been identified and isolated in many organs (38). The progress has been utilized in several biological and medical fields. For example, haemopoietic stem cells can be isolated and used to treat leukemia, and it is hoped that neuron regeneration through neural stem cells maybe a means of treating Alzheimer's disease. It is now widely believed that tumour initiation, progression and metastasis are driven by a small population of CSCs, also termed tumour-initiating cells (116). Indeed, the existence of CSCs, in hematopoietic malignancies and even in solid tumours such as brain, breast or lung cancer (117-119) were well confirmed (21, 22).

Until recently, there were two methods to isolate the stem cell. Some researchers have demonstrated the existence of CSCs with molecular markers such as CD133, CD44 in isolated cells possessing tumour initiator-like properties. However, uncertainty still exists regarding the benefits of using these markers alone or in various combinations when identifying and isolating cells for stem cell research, since in some tissue or cell lines these markers are negative.

Another method to identify CSCs is based on the activity of membrane ATP-binding cassette (ABC) drug transporters, which mediate the efflux of Hoechst 33342 from the cytoplasm (120). These cells with low levels of Hoechst 33342 dye are referred to as the SP when separated by FACS. SP cells have also been identified in many tumour cell lines and shown, in a few cases, to be highly tumourigenic (88).There is evidence indicating that due to the enhanced drug efflux, which is also mediated by ABC transporters (41, 88, 91, 121), tumour SP cells are more resistant

to the chemotherapeutic drugs than non-SP cells

Anaplastic thyroid cell lines have been frequently used as a model to study molecular aberrations, cellular dysregulation and therapeutic strategies (122). Indeed, gene profiling analysis revealed that most thyroid carcinoma cell lines present a common undifferentiated phenotype that resembles that of undifferentiated thyroid tumours (123). These data suggest that anaplastic thyroid cell lines are also an appropriate model to study CSCs.

Our group has recently detected adult stem cells that are derived from human goiters (38). These cells account for only 0.1% of the cell population. In the present work we demonstrated that CSCs derived from anaplastic thyroid carcinoma cell lines expressed ABCG2, MDR1 transporters of the ABC gene family (Fig. 3.3), which enabled the exclusion of the Hoechst dye from the CSCs that otherwise binds to the DNA in non-stem cells (124). The expression of these transporters on the one hand is a prerequisite to isolate stem cells by FACS as an SP (Fig.3.2) and on the other hand the reason for the export of drugs and thus for resistance to chemotherapy. The percentage of stem cells in different anaplastic thyroid carcinoma cell lines was very low (less than 1%, Fig. 3.2) , which is in accordance with previous reports of CSCs in anaplastic thyroid carcinoma cell lines (41, 125). The fraction of CSCs in anaplastic thyroid carcinoma cell lines is also comparably low which suggests that niches, microenvironmental cells that regulate growth stem cells, are operative (116)

CSCs also expressed Oct4 (Fig 3.2), a characteristic marker for adult and embryonic stem cells (ESCs) (37, 38, 126) that is also expressed in some human tumours and some cancer cell lines but not in normal differentiated cells(127). Oct4 is a member of the POU (pit, oct, unc) family of transcription factors (128), which was discovered in 1990 (128-130). It was found in ovulated oocytes, mouse pre-implantation embryos, ectoderm of the gastrula (but not in other germ layers) and primordial germ cells, as well as in ESCs but not in their differentiated daughters (131). Oct4 has been consistently associated with pluripotent or stem-like cells, and it is hypothesized that Oct4 is necessary for the maintenance of pluripotency. Recently, Oct-4 transcripts have consistently been detected in human embryonal carcinomas, testicular germ cell tumours,

seminomas and bladder carcinomas (127, 132-134). These Oct4-expressing cancer stem cells possess self-renewal capabilities and are derived from dysregulated self-renewal pathways in normal stem cells or early progenitor cells (135, 136). Other studies suggest that over-expression of Oct4 in epithelial tissues may lead to dysplasia by inhibiting progenitor-cell differentiation, an effect similar to that of Oct4 in ESCs (137, 138). Specific knockdown of Oct4 in ESCs and embryonic cancer cells results in the onset of differentiation and loss of pluripotency (111, 139). The expression of Oct-4 has further been shown in human breast cancer stem-like cells (140), suggesting that its expression may be implicated in self-renewal and tumourigenesis via activating its downstream target genes. Additionally, Oct4 increases the malignant potential of ESCs in a dose-dependent manner (127). All of these studies suggest that Oct4 may play a role in oncogenesis and that Oct4 may have potential as a biomarker for human cancers.

The present results indicate that Oct4 was highly expressed in SP cells of the anaplastic thyroid cell line but not in non-SP cells which points to an undifferented state of SP cells. To analyze the relationship between CSCs and chemotherapy drug resistance, HTh74 cells were cultured with doxorubicin for a short and long period of time. After short-term stimulation with doxorubicin, only the high concentration of doxorubicin killed most of the cancer cells. After long-term culture with increasing amounts of doxorubicin, stepwise for more than 6 months, the stable doxorubicin resistance sub-line, HTh74R, was established. The IC50 of HTh74R cells was 85-fold higher than that of HTh74 cells. HTh74R showed strong resistance to doxorubicin. Accordingly, when the HTh74 cells were cultured with 0.5μg/ml doxorubicin for 3 days, the percentage of SP was only slightly increased (Fig 3.9-3.11), whereas HTh74R cells consisted of about 70% SP that expelled the chemotherapeutic drug from the cells (Fig. 12). The qPCR results revealed that Oct4, MDR1 and ABCG2 expression was up-regulated in HTh74R cells, compared to the HTh74 cells. In addition, fluorescence immunostaining results also demonstrated that ABCG2 and MDR1 were up-regulated on the protein level. However, there was no up-regulation of MRP1 in doxorubicin resistant cell line. From these data, it is evident that (1) the HTh74R SP fraction that is enriched with CSCs is responsible for doxorubicin resistance and (2) the ABC transporters ABCG2 and MDR1 but not MRP1 are carrier of this resistance. Oct4 was also up-regulated in the resistant cells, which again suggests the nature of

CSCs. High Oct4 expression may help to maintain cancer cells in an undifferentiated state, thus increasing their capacity for self-renewal or proliferation, which also contributes to drug resistance. In the HTh74R cells, the remaining 30% non-SP cells may have gained other molecular mechanisms that conferred drug resistance to these cells.

The CSCs model supports the morphologic heterogeneity in cancer. Morphologic heterogeneity is a typical feature of malignant cell lines and has been attributed to genetic instability and clonal evolution. In the hierarchical model, primary tumours and metastatic cancer are initiated by rare cancer stem cells (8, 141). With this new insight into the cellular mechanism leading to cancer, it has been suggested that the metastatic potential of tumour cells may be a reflection of the ability of the cancer stem cells to clonally initiate tumourigenicity at distant sites (142, 143). Self-renewal is a very important characteristic of CSCs (140, 144, 145). Clonogenic formation assay, the potency to form single cell-derived clones in culture, is a useful method to estimate self-renewal ability. As in vivo assays are particularly difficult to apply to stem cells (146), more reliance has been placed on in vitro clonal assays (147)**.**

Clonal assays were used in many normal and malignant cell lines (8, 148-151), since they provide a robust and reliable method for the identification and isolation of cells with stem cell properties from tumour or cancer cell lines and can provide systems for the characterization of CSCs responses to various factors and therapeutic agents. In our laboratory, my colleague found that HTh74 SP cells are more clonogenic than non-SP cells (unpublished data not shown). In this study, to determine whether the higher tumourigenicity in the resistant cells might be associated with some of the intrinsic stem cell properties, clonalities of HTh74 and HTh74R cells were compared. HTh74R cells displayed a higher clonality than the wide type of HTh74. (Fig. 3.7)

Experimental evidences and clinical studies found that currently available drugs can shrink tumour size but usually only transiently, since existing therapies may kill the bulk of cancer cells but fail to eradicate CSCs that are more resistant to chemotherapeutics (8) (Fig. 4.1) . Reversing drug resistance is one of the major issues in the treatment of malignant tumours. In 1981, Tsuruo *et al.* found that verapamil reversed drug-resistance effectively in the rat P388/VCR cell line

(152). Since that time, drug resistance reversal agents have been widely studied and many compounds and drugs have been found, such as verapamil, cyclosporine A, and trifluoperazine (153). However, most have only been used in the laboratory, and rarely used in clinic conditions due to detrimental side effects.

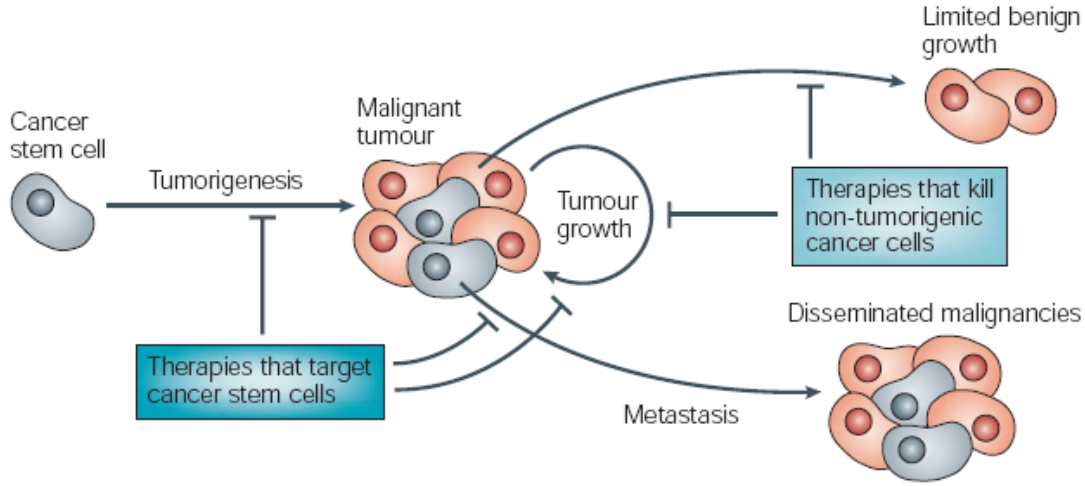

Figure 4.1 Therapeutic implications of cancer stem cells. Cancer stem cells (grey) self-renew and differentiate within tumours to form additional cancer stem cells as well as non-tumourigenic cancer cells (orange), which have limited proliferative potential. As the tumour grows, these cells can either undergo limited benign growth or form disseminated malignancies. Therapies that kill, induce differentiation or prevent the metastasis of cancer stem cells represent potential cures. Therapies that kill primarily non-tumourigenic cancer cells can shrink tumours, but will not cure the patient because the cancer stem cells will regenerate the tumour. By prospectively identifying and characterizing cancer stem cells it might be possible to identify more effective therapies. The intrinsic differences in tumourigenic potential among cancer cells might also explain why it is possible to detect disseminated solid cancer cells in patients that never develop metastatic disease. The identification and characterization of cancer stem cells should therefore also lead to diagnostic methods that can distinguish between disseminated tumourigenic and non-tumourigenic cells, as well as provide a better understanding of the mechanisms that regulate migration of cancer stem cells. [Taken from: Ricardo Pardal, *et al*, "Applying the principles of stem-cell biology to cancer", 2003, Nature Reviews Cancer, **3**: 895-902] (154).

It has been demonstrated that CSCs that expressed ABCG2 and MDR1 transporters were relatively resistant to doxorubicin in anaplastic thyroid carcinoma cells. It may be possible to sensitize CSCs to chemotherapy by blocking the function of one or more of these ABC transporters, so that CSCs would be unable to efficiently efflux the cytotoxic agents as, resulting in enhanced cell death. The importance of these transporters was emphasized by inhibition experiments with verapamil and FTC.

Verapamil is a clinically approved drug, used for the treatment of arrhythmia, hypertension, angina, cardiomyopathy, and migraine headaches. It is an L-type calcium ion influx inhibitor and a potent vasodilator of coronary vessels. As long ago as 1981, it was discovered that drug resistance could be reversed by verapamil (152). Many researches found verapamil interferes with ABC transporter activity, because of the effect of calcium channel. Later it was found that verapamil was a potent inhibitor of a wide range of the multidrug efflux pumps, a competitive inhibitor of MDR1 (155) and also effective for MRP1 and ABCG2 and a calcium blocker (156, 157). Thus, verapamil and functionally similar drugs have been considered for anticancer therapies against multidrug-resistant tumours. However, effects of the clinical application in different tumours were dissimilar. A phase Ⅲ trial combining VAD (vincristine, doxorubicin, and dexamethasone) chemotherapy with verapamil in myeloma did not improve the outcome (158). Another two trials in breast cancer and lung cancer demonstrated verapamil's benefit for survival (159, 160). Until now, there is no report about verapamil applied to the treatment of the thyroid anaplastic thyroid carcinoma.

FTC is a tremorgenic mycotoxin isolated from aspergillus fumigatus (161). In mammalian cells, it is tremorgenic and causes cell cycle arrest (161). FTC was shown to reverse resistance to doxorubicin, mitoxantrone, and topotecan in non-MDR1, non-MRP (multidrug resistance protein) multidrug-resistance cells (162). FTC is a specific and potent inhibitor at micromolar concentrations of ABCG2 (112) and also inhibits ATPase activity (163). It had little effect on MDR1- or MRP1- mediated drug resistance, making it very useful for cell pharmacological studies of ABCG2 (112, 162). This reversal of resistance is associated with an increase in drug accumulation (162).

In this study, to determine if either MDR1 and/or ABCG2 are really responsible for chemoresistance to doxorubicin, verapamil and FTC were used to block ABC transporter. The reversal activity of verapamil and FTC in HTh74R cells was determined by using verapamil (10μM) and FTC (10μM) in combination with increasing doses of doxorubicin drugs (Fig. 3.14 and table 2). Verapamil potentiated the toxicity of doxorubicin (75.3-fold) and FTC potentiated the toxicity of doxorubicin (10.9-fold). These findings closely resembled those on the activity of FTC in three drug-selected cell lines that overexpress ABCG2 (162). Thus both ABCG2 and MDR1 transporters are responsible for chemoresistance to doxorubicin in HTh74R cells.

Apoptosis or "programmed cell death" represents the regulated activation of a pre-existing death program encoded in the genome. It plays a central role in the control of tissue cell numbers during development and homeostasis together with other important functions, such as cell proliferation and differentiation (164-166). Dysregulation of apoptosis, on the other hand, may be involved in the pathogenesis of human disease (167).

The onset of apoptosis is associated with the proteolytic activation of caspases. Caspases, a family of cysteine proteases, play a critical role in the execution of apoptosis. It is not only an initiator but also a marker for apoptosis (168). More than 10 caspases have been identified. Some of them (e.g., caspase 8 and 10) are involved in the initiation of apoptosis, others (caspase 3, 6, and 7) execute the death order by destroying essential proteins in the cell (169).

Caspase-3 is an effector caspase, and is activated by extrinsic and intrinsic cell death pathways. It plays a central role in the execution phase of cell apoptosis (170-173). Moreover, depletion of caspase-3 in a cell-free apoptotic system causes inhibition of various downstream events (174). Caspase-3 can cleave and destroy (or modifies the functions of) hundreds of cellular protein substrates (175). Two major caspase-3-activating pathways have been identified, the extrinsic (receptor-initiated) and intrinsic (mitochondrial) apoptosis pathways (176-179) (Fig. 4.2).

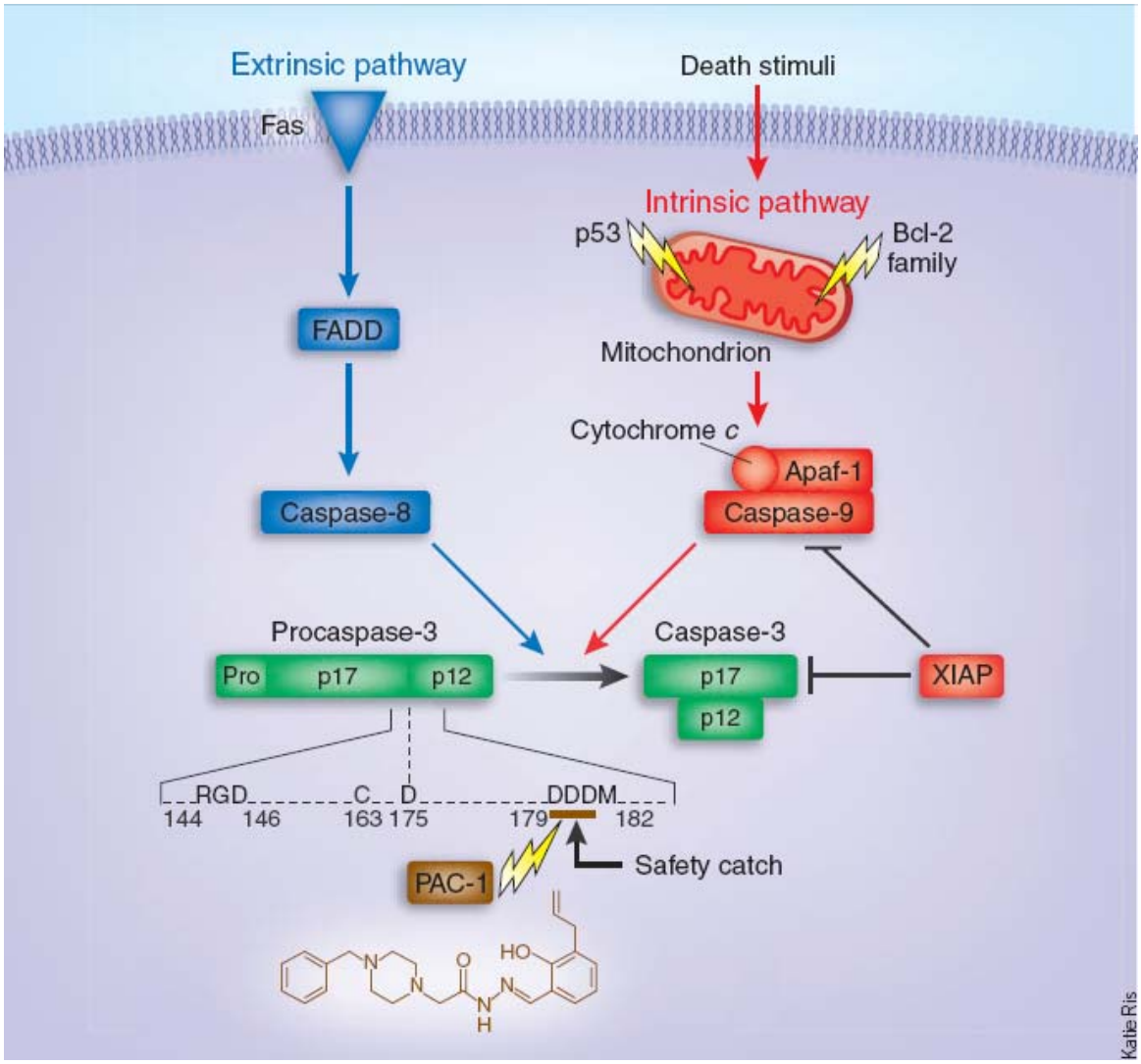

Figure 4.2 Summary of cell death pathways that converge on the proteolytic activation of caspase-3. Caspase-3 activation via tumour necrosis factor (TNF) family receptors (for example, Fas), FADD (Fas-activated death domain protein) and caspase-8 represents the extrinsic pathway (blue), whereas caspase-3 activation via the mitochondrial release of cytochrome *c* and Apaf-1–mediated processing of caspase-9 represents the intrinsic pathway (red) (176). For clarity, not all of the players are shown. [Taken from: Alan G Porter, "Flipping the safety catch of procaspase-3", 2006, Nature Chemical Biology, 3: 509-510] (180).

It has been reported that chemotherapeutic drugs induce cell death by activating the caspase apoptotic pathway (181, 182). Doxorubicin is a key adjuvant drug for the treatment of anaplastic thyroid cancer. It triggers apoptosis through several mechanisms. As with many chemotherapeutic agents, it induces DNA damage by interacting with topoisomerase II, leading to DNA breakage (183). It has been reported that up-regulation of the Fas/Fas ligand system, which involves the extrinsic caspase-3 pathway, may also be involved in doxorubicin-mediated killing (184). It has been shown that doxorubicin involves apoptosis in a wide variety of cell lines, including neuroblastoma cells (185), thyrocytes (186) and others (187).

It was demonstrated that ABC transporter overexpression confers resistance to a wide range of caspase-dependent apoptotic agents not only by removing drugs from the cell but also by inhibiting the activation of proteases involved in apoptotic signaling (188). In the multidrug resistant human CEM and K562 tumour cell lines, it was also shown that resistant cells were less sensitive to multiple forms of caspase-dependent cell death. Inhibition of ABC transporters enhanced drug- or Fas-mediated activation of caspase-3 (189). Doxorubicin selection led to changes in gene expression that reduced the apoptotic response to death-inducing stimuli and thus contributed to the multidrug resistance phenotype (190).

In this study, caspase-3 activity was measured. When HTh74R cells were incubated with doxorubicin alone, the caspase-3 activity was similar to that of HTh74R cells without doxorubicin. However, when HTh74R cells were incubated with verapamil that inhibited the efflux of doxorubicin from putative cancer stem cells, caspase-3 activity was increased by about 87.8% (Fig. 3.16). This revealed that verapamil potentiated the doxorubicin-induced apoptosis by activating the caspase-dependent pathway in resistant anaplastic thyroid carcinoma cells. Recently, a similar result was reported in the resistant human hepatic cancer cells (191).

In conclusion, the present work suggests that the failure of doxorubicin to eradicate all anaplastic thyroid carcinoma cells is mainly due to resistance of CSCs to the chemotherapeutic drug although there is a smaller fraction of resistant cells that do not express drug-exporting ABC transporters. ABC transporter inhibitors can reconstitute the effect of doxorubicin in resistant

cells, which is mediated by caspase-dependent pathways. Further therapeutic strategies have to be developed that target not only the main population of cancer cells but also to eradicate CSCs that are responsible for tumour progression and recurrence.

5. Summary

Current chemotherapy with doxorubicin fails to eradicate anaplastic thyroid cancer or even to stop tumour progress. It is hypothesized that cancer initiation, progression and metastasis are driven by a small population of cancer stem cells (CSCs), which are also responsible for drug resistance. The aim of the present work was (1) to identify whether the putative cancer stem cells exist in anaplastic thyroid carcinoma cells, (2) to generate stable doxorubicin resistant anaplastic thyroid carcinoma cell line, (3) to prove whether or not drug resistance is partly due to cancer stem cells which could expel chemotherapeutic drugs and (4) to detect whether the ABC transporter inhibitors can reverse drug resistance.

To test these hypotheses, anaplastic thyroid cell lines were characterized by FACS for their content of cancer stem cells, their in vitro sphere-forming capacity and their expression of multidrug resistance transporters of the ABC gene family which may confer drug resistance to the cells. Cells were treated with doxorubicin in short-term and long-term culture up to 6 months to establish a resistant cell line. The survival of cancer and cancer stem cells and the differential expression of transporters were analyzed.

This work demonstrated that anaplastic thyroid cancer cell lines (C643/ HTh74/ SW1736) consisted of 0.2 – 0.6 % side population (SP) cells, which enabled the exclusion of the Hoechst dye that otherwise binds to the DNA in the major fraction of non-SP cells. SP cells highly expressed stem cell marker Oct4 and ABCG2 and multi-drug-resistant 1 (MDR1) transporters of the ABC gene family which were characterized as cancer stem cells.

HTh74 anaplastic thyroid cancer cells were treated with doxorubicin in short-term and long-term culture up to 6 months to establish a resistant cell line, designated HTh74R. The survival of cancer and cancer stem cells and the differential expression of transporters were analyzed. Treatment with doxorubicin killed the large majority of cancer cells derived from anaplastic thyroid carcinoma cell lines. This conferred a growth advantage to cancer stem cells which in turn overgrew the culture. HTh74R cells consisted of about 70% SP cells, expressed high levels of ABCG2, MDR1 and Oct4 and were more clonogenic than HTh74 wild type cells. Inhibitors of ABCG2 and/or MDR1 (verapamil and fumitremorgin C) sensitized HTh74R to doxorubicin.

They potentiated the doxorubicin-induced apoptosis by activating the caspase-dependent pathway in resistant anaplastic thyroid carcinoma cells and thus partly resolved drug resistance.

In conclusion, the present work suggests that the failure of doxorubicin to eradicate all anaplastic thyroid carcinoma cells is mainly due to resistance of CSCs to the chemotherapeutic drug although there is a smaller fraction of resistant cells that do not express drug-exporting ABC transporters. ABC transporter inhibitors can sensitize the effect of doxorubicin to resistant cells, which is mediated by a caspase-dependent pathway. Further therapeutic strategies have to be developed that target not only the main population of cancer cells but also to eradicate CSCs that are responsible for tumour progression and recurrence.

ZUSAMMENFASSUNG IN DEUTSCHER SPRACHE

Die gegenwärtig in der Behandlung des anaplastischen Schilddrüsenkarzinoms empfohlene Chemotherapie mit Doxorubicin führt weder zu einer ausreichenden Zerstörung der Tumormasse noch kann sie die Tumorprogression aufhalten. Nach einer mittlerweile weitverbreiteten Hypothese ist die Tumorentstehung, die Wachstumsprogression und die Metastasierung auf eine kleine Population von sogenannten Karzinomstammzellen (CSCs, cancer stem cells) zurückzuführen, die auch für die Chemotherapeutika – Resistenz verantwortlich sind. Das Ziel der gegenwärtigen Arbeit war (1) mögliche Karzinomstammzellen in anaplastischen Schilddrüsenkarzinomen zu identifizieren und zu charakterisieren, (2) eine stabile Doxorubicin-resistente anaplastische Schilddrüsencarcinom-Zelllinie herzustellen, (3) zu analysieren, ob Karzinomstammzellen Chemotherapeutika aus der Zelle ausschleusen können und daher für Chemotherapieresistenz in diesen Karzinomzellen verantwortlich sind und (4) zu untersuchen, ob Hemmer der ABC-Transporter die Chemotherapieresistenz rückgängig machen können.

Diese Arbeit wies nach, dass anaplastische Schilddrüsenkarzinomzelllinien (C643 / HTh74/ SW1736) 0,2 – 0,6 % sogenannter side population - Zellen aufweisen, die die Ausschleusung des Hoechst Farbstoffes aus CSC ermöglichen, während bei nicht-CSC- Zellen der Farbstoff an die DNA bindet. HTh74 side population-Zellen exprimierten den Stammzellmarker Oct4 und ABCG2 und multiple drug resistance 1 (MDR1)-Transporter der ABC Gene-Familie.

HTh74 - Zellen wurden mit Doxorubicin in einer Kurzzeit- und einer Langzeitkultur bis zu 6 Monaten behandelt mit dem Ziel einer Etablierung einer resistenten Zelllinie HTh74R. Das Überleben der Karzinom – und Karzinomstammzellen und die differentielle Expression der Transporter wurden analysiert. Die Behandlung mit Doxorubicin zerstörte die große Mehrheit von Karzinomzellen der anaplastischen Schilddrüsenkarzinomzelllinien. In der Folge kam es zu einem Wachstumsvorteil der Karzinomstammzellen, die die Kultur überwuchsen. HTh74R – Zellen bestanden aus etwa 70% side population - Zellen, die eine höhere Expression von ABCG2,

MDR1 und Oct4 und ein ausgeprägteres klonales Wachstum im Vergleich zu HTh74 – Zellen aufwiesen. Hemmstoffe der ABCG2- und/oder MDR1 – Transporter (Verapamil und Fumitremorgin C) machten die resistenten HTh74R – Zellen wieder empfindlich gegenüber Doxorubicin und verminderten so deren Resistenz. Verapamil potenzierte die durch Doxorubicin induzierte Apoptose über eine Aktivierung caspase - abhängiger Stoffwechselwege in den resistenten anaplastischen Schilddrüsenkarzinomzellen.

Zusammenfassend lässt sich sagen, dass die unzureichende chemotherapeutische Wirkung von Doxorubicin bei anaplastischen Schilddrüsenkarzinomzellen im Wesentlichen auf eine Resistenz von Karzinomstammzellen zurückzuführen ist, wobei eingeschränkt gesagt werden muss, dass eine kleinere Anzahl resistenter Zellen die Chemotherapeutika – exportierenden ABC - Transporter nicht exprimierten. Hemmstoffe der ABC - Transporter führen folglich dazu, dass Doxorubicin in den resistenten Zellen wieder wirksam wurde. Dieser Effekt wird im Wesentlichen über den caspase - abhängigen Stoffwechselweg vermittelt.

Künftige therapeutische Strategien müssen darauf zielen, nicht nur die Hauptpopulation der Karzinomzelllinien sondern auch die Karzinomstammzellen zu vernichten, die für die Tumorprogression und für die Rezidive verantwortlich sind.

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Erklärung

„Ich, Xuqin Zheng, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Doxorubicin fails to eradicate cancer stem cells derived from anaplastic thyroid carcinoma cells: characterization of resistant cells“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe, die Arbeitohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum 14-May-2010 Unterschrift Xuqin, Zheng

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.