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

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Abstract. Current chemotherapy with doxorubicin fails to eradicate anaplastic thyroid cancer or even to stop tumor progress which may be due to the failure of these drugs to effectively target putative cancer stem cells. To test this hypothesis, 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. Anaplastic thyroid cancer cell lines that consisted of 0.4-0.8% side population cells, expressed ABCG2 and multi-drug-resistant 1 (MDR1) transporters. Treatment with doxorubicin gradually killed the non-side population of cancer cells derived from anaplastic thyroid carcinoma cells. This conferred a growth advantage to cancer stem cells which in turn overgrew the culture. Resistant cell line consisted of a 70% side population fraction enriched with Oct4-positive cancer stem cells. Inhibition of ABCG2 and/or MDR1 revealed that resistance of cancer stem cells to doxorubicin may be mainly due to the expression of these ABC transporters that were highly up-regulated in the resistant subline. The poor outcome of chemotherapy with doxorubicin in anaplastic thyroid carcinoma may be partly explained by up-regulation of ABCG2 and MDR1 transporters that confers resistance to cancer stem cells. Thus an effective treatment of anaplastic thyroid cancer has not only to destroy cancer cells that represent the bulk of tumor cell population but also cancer stem cells that may drive tumor progression.

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Introduction

Anaplastic thyroid carcinomas have a poor prognosis with a mean survival of only few months (1). Standard treatment options for this highly malignant tumor include chemotherapy with doxorubicin in combination with radiation (2). However, current systemic therapy fails to eradicate this cancer or even to stop tumor progress. It has been hypothesized that this may be explained by the failure of current drugs to effectively target cancer stem-like cells (CSC) (3,4).

To date, CSC have been reported in several solid tumors and in cancer cell lines (5-10). It is widely believed that only the small cell population of CSC or tumor-initiating cells within a tumor has the capacity to sustain tumor growth. This subpopulation of cells can self-renew, differentiate into other tumor cells and regenerate a phenocopy of the cancer when injected into an *in vivo* microenvironment (4).

Until recently, there are only very few studies on adult thyroid stem/progenitor cells and thyroid CSC (11-13). We and others have recently described and characterized adult stem cells in human thyroid tissues (14-16). These stem or progenitor cells express either the pluripotent marker Oct4 or the endodermal markers Gata4 and HNF4α and the thyroid transcription factors TTF1 and Pax (14).

The existence of cancer stem or progenitor cells as the origin of thyroid cancer has been hypothesized before (17). Only recently thyroid CSC derived from anaplastic thyroid cancer cell lines have been described (11,12,18). However, when transplanted onto mice, only some of the cell line-derived CSC displayed a higher tumor-forming capacity than the main population of cancer cells (11,18).

Based on these results, we hypothesized that some cancer cells and CSC derived from anaplastic thyroid carcinoma cell lines may differ in their responsiveness to chemotherapy rather than their proliferative and tumor-forming potency. Infrequently proliferating CSC that are quite resistant to chemotherapeutic drugs may only overgrow the tumor when the main population of cancer cells are destroyed by drugs.

To test this hypothesis anaplastic thyroid cell lines that were characterized for their content of CSC, their sphere-forming capacity and expression of transporter of the ABC gene family, were treated with doxorubicin in short-term and long-term culture up to 6 months to establish a resistant cell line. Here we report for the first time that in long-term culture doxorubicin killed the large majority of rapidly proliferating cancer cells derived from anaplastic thyroid carcinoma cell line. This conferred a growth advantage to cancer stem cells which in turn overgrew the culture. Resistant cell line consisted of a 70% side population (SP) fraction enriched with Oct4-positive cancer stem cells. Resistance to doxorubicin was due to high expression of multidrug-resistant genes of the ABC transporter. These data suggest that an effective treatment of anaplastic thyroid cancer has not only to destroy cancer cells that represent the bulk of tumor cell population but also CSC that may drive tumor progression.

Materials and methods

Cell cultures. Human anaplastic thyroid cancer cell line HTh74 was grown in Ham's F-12 medium. Two other human anaplastic cancer cell lines, C643 and SW1736, were cultured in Dulbecco's modified Eagle's medium (Invitrogen). Each medium was supplemented with 10% fetal calf serum (FCS, v/v) (Invitrogen), 1% MEM (v/v) (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (Roche). All cells were grown as monolayers and kept in a humidified incubator at 37˚C under 5% CO_2, with media change every 3-4 days.

Establishment of a stable doxorubicin-resistant sublines from HTh74 cell line. A doxorubicin-resistant subline (HTh74R) was developed by continuous exposure of the HTh74 cell line to doxorubicin (Sigma) starting at 10 ng/ml and increasing in a stepwise manner to 0.5 μg/ml. The resistant cell line was maintained in the medium containing doxorubicin for >6 months.

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 days 5 and 10. The percentage of cells that initiated a clone was presented as cloning efficiency. Triplicate samples were run for the experiments.

Cell invasion assay on a Matrigel-coated membrane in a transwell. Cell invasion potential was measured using a transwell migration apparatus (Becton-Dickinson) according to the manufacturer's instructions. Before invasion assay, the BD Falcon cell culture inserts with 8 μm-pores in their PET (Polyethylene terephthalate) track-etched membranes were coated by Matrigel (Sigma, 50 μg/well) and placed in 24-well culture plates. Cells were harvested, washed once, and resuspended in DMEM medium. To start the assay, $1x10^4$ of SP or non-SP cells were individually suspended in 300 μl F12 medium containing 10% FCS and seeded into the upper chamber of the inserts. Additional 700 μl of the same medium was loaded into the lower chamber. The transwell apparatus was incubated at 37˚C for 24, 48 and 72 h, respectively. At the end of the incubation, the cells on the top surface of the filters were wiped off with cotton swabs and those on the lower surface of the insert were fixed and stained with haematoxylin (Dako). The number of infiltrating cells was determined by counting in six random visual fields under a light microscope for each membrane. The results were expressed as cells/field, and the values represented the mean ± SEM of three independent experiments performed in triplicate.

Sphere formation assay. Tumor spheres were generated by placing HTh74 cells ($1x10^4$ cells/ml) into serum-free DMEM/F-12 (1:1, v/v) medium containing B27 (1:50 dilution, Invitrogen), bFGF (20 ng/ml, Invitrogen) and EGF (20 ng/ml, Invitrogen). Every 2-3 days, B27, bFGF and EGF were added. After 14 days, some cells had formed intact floating spheres. Sphere cells were then collected and dissociated enzymatically (15 min in 0.05% trypsin, 0.53 mm EDTA-4Na at 37˚C). Then the cells were stained with Hoechst 33342 and re-sorted by FACS to detect the SP percentage.

Fluorescence cytometry and fluorescence-activated cell sorting. To isolate the thyroid cancer SP fraction, FACS was performed using the Hoechst staining method as outlined by Goodell *et al* (19). In brief, cancer cells were harvested, suspended at a density of 10^6 cells/ml in DMEM medium containing 2% FCS and 10 mM HEPES, 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) at 37˚C for 120 min with periodic agitation, either alone or in combination with 50 μM verapamil (Sigma) or 10 μM fumitremorgin C (FTC) (Sigma), inhibitors of ABCG2 transporter. Finally, the cells were centrifuged and resuspended in cold HBSS containing 2% FCS and 10 mM HEPES, counterstained with 1 μg/ml propidium iodide (PI) to exclude dead cells.

A 350-nm UV laser was used to excite Hoechst 33342 and PI. Analysis was performed on fluorescence-activated cell sorter (Becton-Dickinson Biosciences, Heidelberg, Germany) by using a dual-wavelength analysis (blue, 424-444 nm; red, 675 nm). PI-positive dead cells were excluded from the analysis. 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 culture medium for further experiments or pelleted for RNA isolation.

Immunofluorescence staining. HTh74 cells were double stained in suspension with Hoechst 33342 and ABCG2 antibody. After being dissociated from culture dishes, cells ($2x10^6$) were labeled with Hoechst as described above. Then they were washed, resuspended in 200 μl PBS and incubated with 1:100 diluted ABCG2 antibody (Santa Cruz) on ice for 30 min. This step was followed by incubation with TRITC conjugated secondary antibody (1:100 dilution, Santa Cruz) for 30 min in the dark on ice. After incubation, cells were washed twice, pipetted onto a slide and then cover-slipped for fluorescent microscopic viewing.

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

Table I. Primer sequences, annealing temperatures, cycles and product sizes for semi-quantitative RT-PCR and qPCR.

Target gene	Primer sequences[a]	Annealing temp. (˚C)	Cycles	Expected size (bp)
ß-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[b]	S: 5'-GACAACAATGAGAACCTTCAGGAG-3' AS: 5'-CTGGCGCCGGTTACAGAACCA-3'	55	30	216
MDR1[b]	S: 5'-GCCTGGCAGCTGGAAGACAAATAC-3' AS: 5'-ATGGCCAAAATCACAAGGGTTAGC-3'	59	29	253
MRP1[b]	S: 5'-ACCCCTCTCTGTTTAAGGTGTT-3' AS: 5'-AAGCAGATGTGGAAGTACTGGT-3'	58	-	238
18S RNA[b]	S: 5'-CTC AAC ACG GGA AAC CTC AC-3' AS: 5'-CGC TCC ACC AAC TAA GAA CG-3'	58	-	110
ABCG2[b]	S: 5'-TGTAGCAACACTTCTCATGACC-3' AS: 5'-TATTCTTCGCCAGTACATGTTG-3'	58	-	234

[a]S, sense primer; AS, antisense primer. [b]The primers were used for qPCR.

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). 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 dark (1:200, Santa Cruz). FITC-labeled cells were analyzed by fluorescence Olympus microscope using standard fluorescent filters (excitation 488 nm).

RNA isolation and real-time RT-PCR. Total RNA was extracted using the RNeasy Micro kit (Qiagen) according to the manufacturer's specifications. RT-PCR was performed as described previously (20). For PCR amplification, reactions were carried out at 95˚C for 10 min, 25-30 cycles of 95˚C for 30 sec, 52-63˚C (primer specific) for 30 sec (primer specific) and 72˚C for 1 min, followed by a final extension at 72˚C for 10 min and termination at 4˚C. The number of cycles used was determined to be in the log-linear phase of the amplification reaction.

In all PCR analyses, ß-actin was used as an internal control. Primer sequences, product sizes, cycles and annealing temperatures are listed in Table I. The PCR products were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Signals corresponding to target gene expression were normalized relative to ß-actin for each sample.

Real-time quantitative RT-PCR was performed with iCycler iQ_Real-time PCR detector system (Bio-Rad). SYBR Green reactions were performed using ABsolute™ 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˚C for 15 min, followed by 50 cycles at 95˚C for 15 sec; 58˚C for 30 sec; 72˚C for 30 sec. 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 18s rRNA. Each sample was replicated twice from 3 independent sets of RNA preparations. Results are tabulated as mean ± SEM of 3 independent experiments.

BrdU incorporation. To test the proliferative potential of HTh74 SP cells which were grown as spheres in serum-free medium with EGF and bFGF, BrdU was added to the medium at a final concentration of 10 μM. After incubation with BrdU for 48 h, the spheres were fixed with ethanol fixative and further analyzed for BrdU incorporation using BrdU Labeling and Detection Kit I (Roche). Samples were then mounted in Vectashield (Vector Laboratories) and observed under the fluorescence microscope.

Viability assay. Cell viability was measured by the methyl-thiazolyl-tetrazolium (MTT) test. Cells (0.5-1x10^4) were seeded in 96-well plates. After 24 h, they were treated with medium alone or with medium containing different doses of doxorubicin, or doxorubicin plus verapamil or FTC 48 h. The medium containing 0.5 mg/ml MTT (Sigma) 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

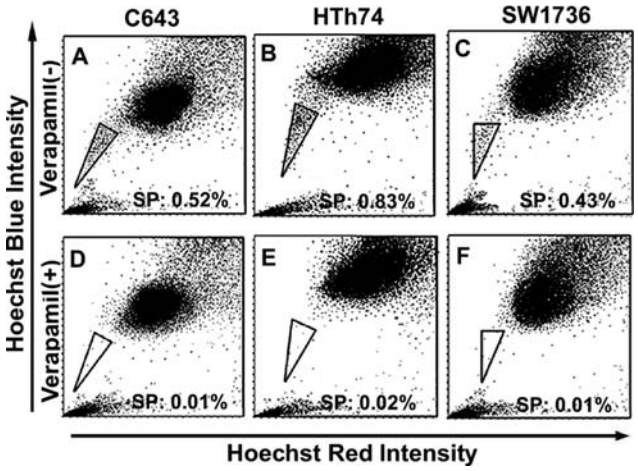

Figure 1. Identification of SP cells in human anaplastic thyroid cancer cell lines. C643 (A and D), HTh74 (B and E), and SW1736 (C and F) cells were labelled either with Hoechst 33342 alone (A-C) or in combination with verapamil (D-F) and then analysed by FACS. The SP cells (A-C), which disappeared in the presence of verapamil (D-F), were outlined and shown as a percentage of the total cell population.

were repeated at least three times, and each experimental condition was repeated at least in quadruplicate wells in each experiment.

Statistical analysis. Statistical analysis was performed with Prism software. Numerical data are expressed as mean ± SEM Statistical differences are considered significant at $P<0.05$.

Results

Characterization of anaplastic thyroid carcinoma cell lines and their stem cells. C643, Hth74 and SW1736 anaplastic thyroid carcinoma cell lines were analyzed for the content of CSC. As revealed by FACS, 0.52% of C643 cell line, 0.83% of HTh74 cell line and 0.41% of SW1736 cell line presented as SP cells that excluded the Hoechst dye due to the ability to express transporters of the ABC transporter family (Fig. 1).

FACS-isolated SP cells and the major fraction of non-SP cells were then analyzed for clonal and invasive growth *in vitro*. In comparison to non-SP cells, SP cells showed an almost 10 times higher clonality (Fig. 2A). When grown in Matrigel invasive growth of SP cells was also much higher than that of non-SP cells (Fig. 2B).

We have recently reported that adult stem cells may grow out from human thyroid cell culture to form spheres, designated thyro-spheres, when maintained in serum-free medium and stimulated by EGF and bFGF (15). Under the same conditions stem cells derived from HTh74 cells also grew out to form spheres (Fig. 3D). FACS analysis revealed that spheres contained almost 5% SP and >95% non-SP (Fig. 3E).

Expression of Oct4 stem cell marker, ABCG2, MDR1 mRNAs and immunostaining. SP cells expressed Oct4 mRNA, a marker of embryonic and adult stem cells (15,21), and ABCG2 and MDR1 genes of the ABC transporter family (22) whereas non-SP cells expressed only low amounts of MDR1 mRNA (Fig. 3A). Immunostaining demonstrated single cells of HTh74 cell culture that expressed Oct4 (Fig. 3B), ABCG2 (Fig. 4A and B) or MDR1 (Fig. 4E and F). When cells were incubated with Hoechst dye that is taken up by all cells but only excluded by transporter-expressing SP cells, ABCG2 immunofluorescence staining was co-localized with low Hoechst staining, while, as expected, ABCG2 negative cells showed strong Hoechst fluorescence (Fig. 3C).

Effect of doxorubicin on survival of HTh74, C643 and SW1736 anaplastic thyroid carcinoma cells and establisment of a stable

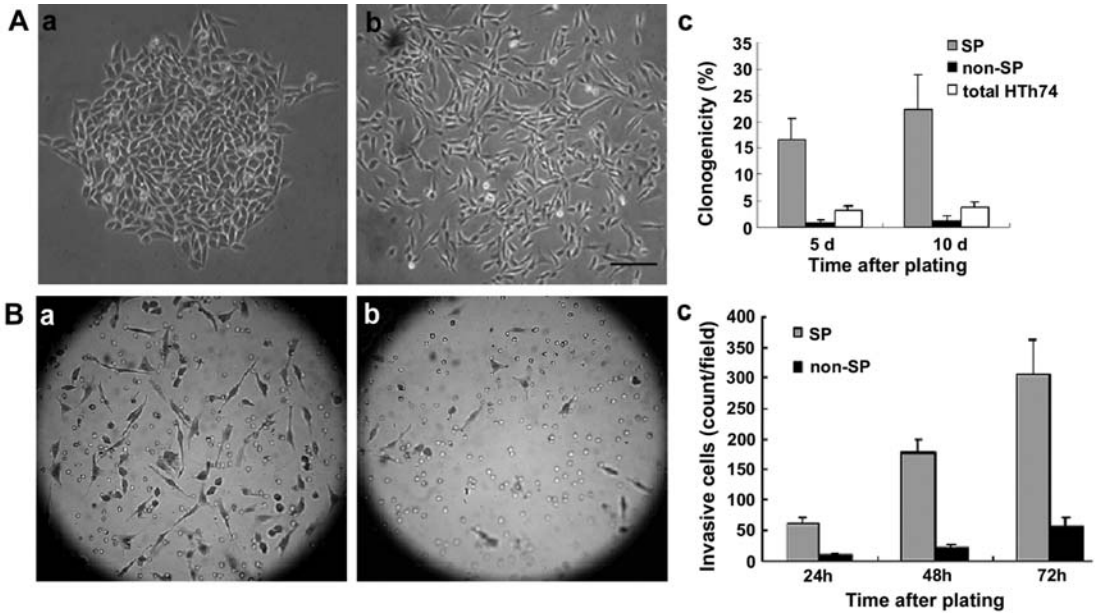

Figure 2. (A) SPs of HTh74 cells are more clonogenic than non-SP cells. a, HTh74 SP cells sustained a clonal growth and formed characteristic compact circular colonies. b, The non-SP cells scattered and failed to proliferate as clones. Scale bar, 20 μm. c, The cloning efficiency of SP, non-SP and unselected HTh74 cells at days 5 and 10. (B) SP of HTh74 cells show high invasive potential *in vitro*. a, Representative field of SP cells that migrated under the membrane was photographed at 24 h (x200). b, The migrated non-SP cells at 24 h. c, The migrated cells of both SP and non-SP increased in a time-dependent manner.

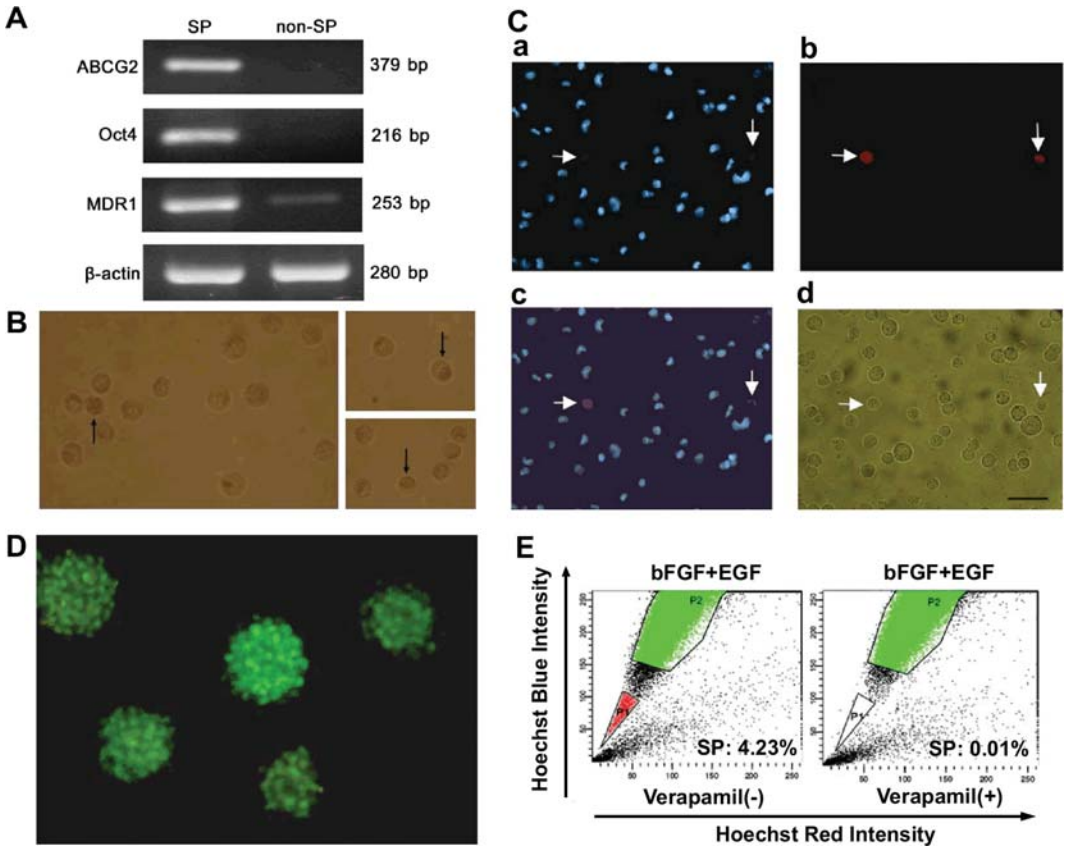

Figure 3. Expression of Oct4 stem cell marker, ABCG2, MDR1 mRNAs in HTh74 SP and non-SP cells. HTh74 SP can be enriched in serum-free medium containing bFGF plus EGF. (A) Identification of stem cell marker and ABC transporter genes in HTh74 SP cells. SP cells showed expression of Oct4 and significantly higher levels of stem cell marker ABC transporter genes, ABCG2 and MDR1, in contrast to non-SP cells. (B) Oct-4 positive cells were detected in HTh74 cells by immunocytochemistry with a polyclonal antibody. Only nuclear staining was considered specific. (C) Co-localisation of ABCG2 immunoreactivity with Hoechst low-staining cells in HTh74 cell cultures. All four panels illustrate the same field. Hoechst low-staining cells (a, arrows) showed ABCG2 immunoreactivity (b, arrows). Hoechst low-staining cells co-localised with ABCG2 immunoreactive cells in a merged image (c, arrows). (d) Brightfield images of these cells. Scale bar, 20 μm. (D) BrdU incorporation of 10 d spheres derived from sorted SP cells cultured in serum-free medium containing bFGF and EGF showed that most cells in 10 d spheres were labeled with BrdU, indicating that spheres developed and grew in size by cell division. (E) HTh74 cells were cultured in serum-free medium containing bFGF plus EGF for 2 weeks, and then analyzed for SP fraction by FACS; the SP percentage of HTh74 cells was substantially increased.

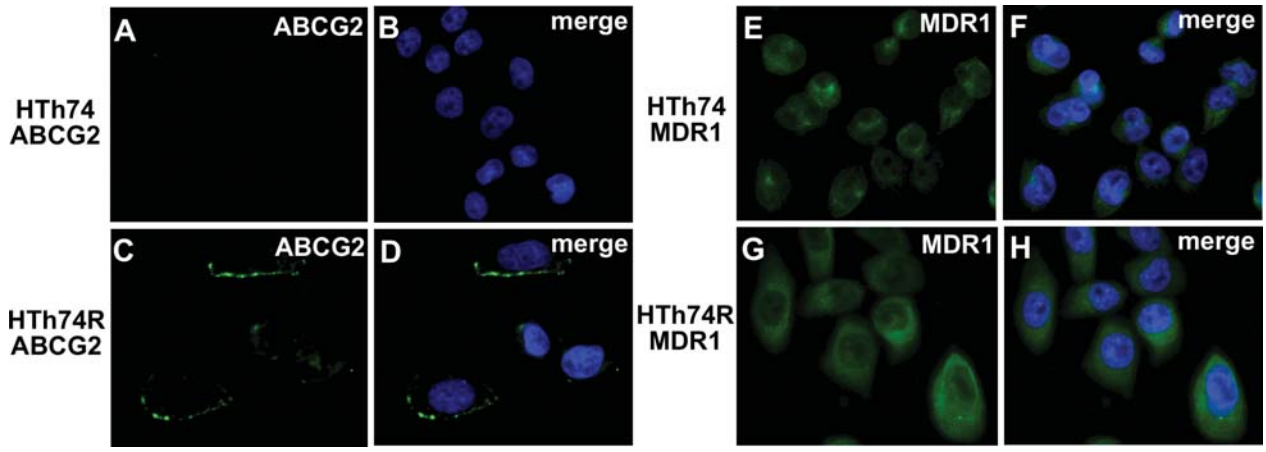

Figure 4. Immunofluorescence staining of ABCG2 and MDR1 in HTh74 cells and HTh74R cells. HTh74 cells and HTh74R cells were plated onto coverslips. After 24 h, cells were washed with PBS and fixed with 4% PFA, and subjected to immunofluorescence labeling of polyclonal anti-ABCG2 antibody or monoclonal anti-MDR1 antibody, DAPI for nuclear staining. (A and B) ABCG2 of HTh74. (C and D) ABCG2 of HTh74R. (E and F) MDR1 of HTh74. (G and H) MDR1 of HTh74R.

resistant cell line. When anaplastic thyroid cells were treated with doxorubicin, only high doses killed the large majority of tumor cells (Fig. 5). When incubated for 48 h, 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, respectively.

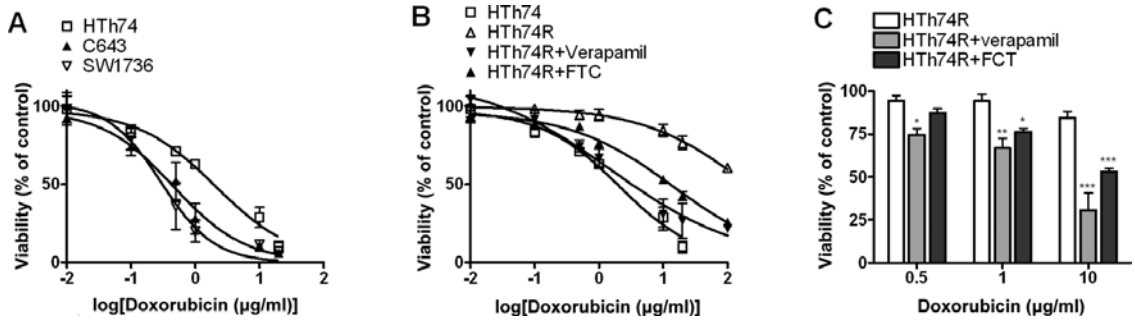

Figure 5. The effects of doxorubicin alone or combined with verapamil or FTC on anaplastic thyroid carcinoma cell viability. (A) Dose-response curves for doxorubicin 48-h treatment in HTh74, C643 and SW1736 cell lines. (B) Dose-response curves for doxorubicin or doxorubicin plus verapamil or FTC 48-h treatment in HTh74 and HTh74R cell lines. (C) Comparison of dose-response for doxorubicin or doxorubicin plus verapamil or FTC 48-h treatment in HTh74 and HTh74R cell lines. Each data-point represents the mean of at least three independent experiments. *P<0.05; **P<0.01; ***P<0.001.

Table II. Sensitivity of anaplastic thyroid carcinoma cell lines to doxorubicin.[a]

Cell lines	Drug	IC_{50} µg/ml	SEM
SW1736	Doxorubicin	0.61	0.26
C643	Doxorubicin	0.89	0.40
HTh74	Doxorubicin	1.80	0.14
HTh74R	Doxorubicin	153.53	16.43
HTh74R	Doxorubicin + Verapamil	2.04	0.60
HTh74R	Doxorubicin + FTC	14.14	1.73

[a]IC_{50} values were determined by the MTT assay. Each data-point represents the mean of at least three independent experiments.

By treatment of HTh74 cells with 0.5 µg/ml doxorubicin for 6 months a stable cell line, designated HTh74R, was established. After 6 months of culture in the presence of the drug, the IC_{50} value was 153.53±16.43 µg/ml, which corresponds to a 85-fold increase of resistance compared to parental HTh74 cells (Table II).

Flow cytometry of Hoechst staining demonstrated that about 70% of doxorubicin-resistant cells were detected as SP cell fraction, CSC that expressed transporters of the ABC gene family (Fig. 6). In fact, immunofluorescence staining of HTh74R cells confirmed that the majority of resistant cells expressed ABCG2 or MDR1 transporters (Fig. 4C and D and G and H). Although doxorubicin killed the majority of cancer cells that lack ABC transporters, there was still a fraction of almost 30% of cells that were resistant to doxorubicin due to other molecular mechanisms.

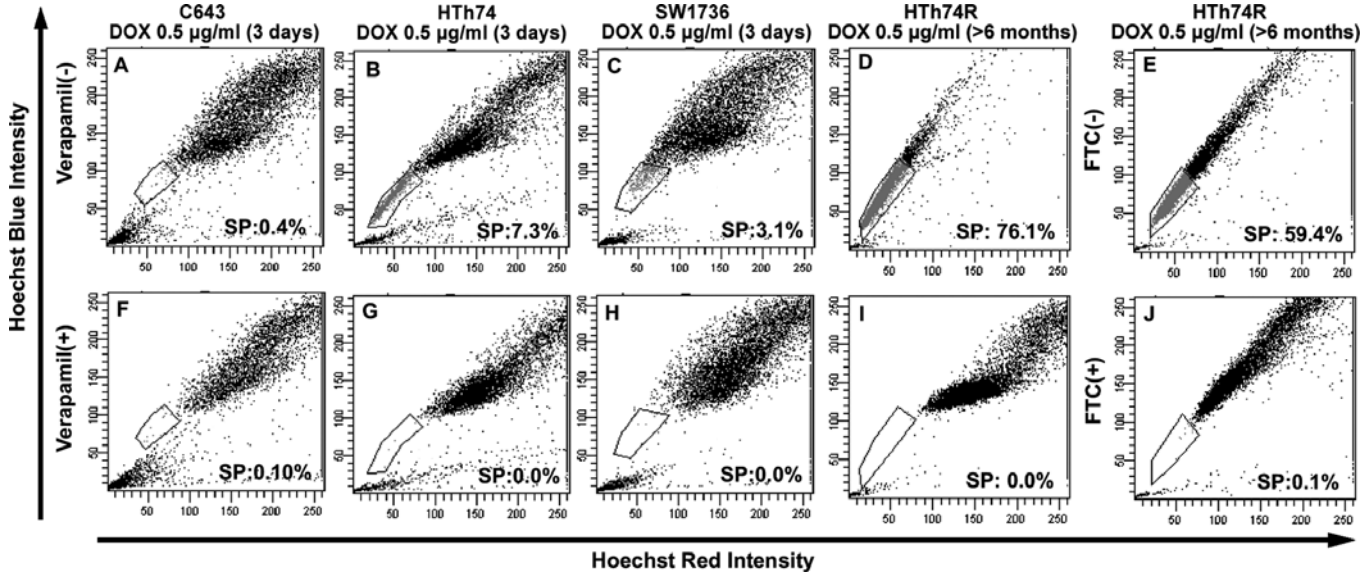

Figure 6. Analysis of SP in anaplastic thyroid carcinoma cell lines treated with doxorubicin 0.5 µg/ml for a short and long time. C643, HTh74 and SW1736 cells treated with doxorubicin 0.5 µg/ml for 3 days. C643 (A and F), HTh74 (B and G), and SW1736 (C and H) cells were labelled either with Hoechst 33342 alone (A-C) or in combination with verapamil (F-H); HTh74R treated with doxorubicin 0.5 µg/ml >6 months. Cells were labelled either with Hoechst 33342 alone (D) or in combination with verapamil (I); HTh74R cells were labelled either with Hoechst 33342 alone (E) or in combination with 10 µM FTC (J) for 120 min, and then analysed by FACS.

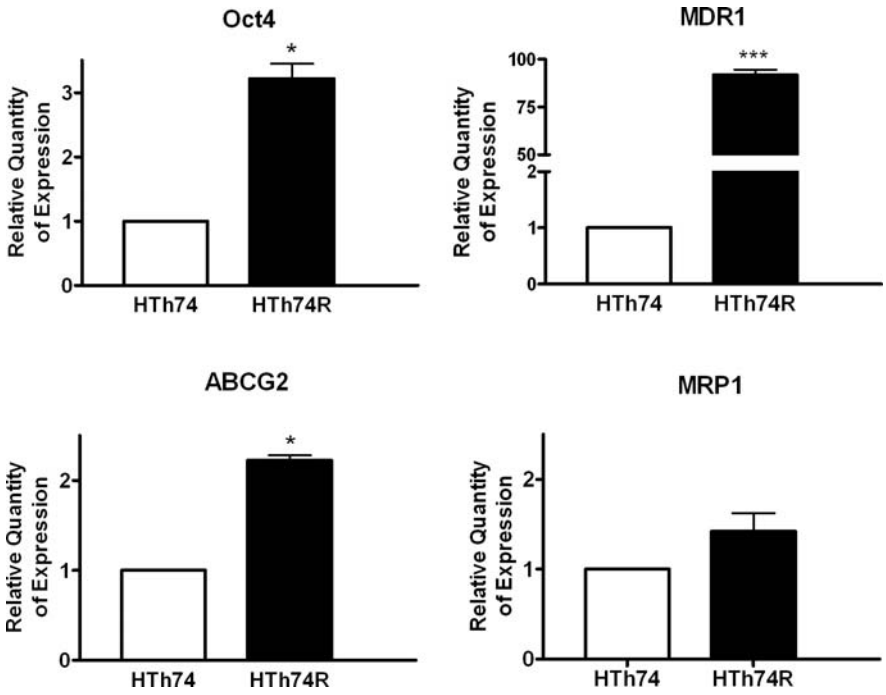

Figure 7. Real-time RT-PCR analysis of mRNA expression of ABC transporter genes and stem cell marker Oct4 in HTh74 and HTh74R. The Ct values of the real-time PCR were calculated by the $(2^{*}\text{efficiency})^{\Delta\Delta Ct}$ method, and normalized by the value of the internal control 18s rRNA. Data are presented as mean of fold change ± SEM vs control and were derived from at least three independent experiments. $^{*}P<0.05$; $^{***}P<0.001$.

Inhibition of transporters by FTC or verapamil partly or completely abolished the resistant state of HTh74R cells. Accordingly, FTC decreased the IC_{50} of HTh74R to 14.14±1.73 μg/ml whereas verapamil decreased the IC_{50} to 2.04±0.60 μg/ml and, which is in the order of magnitude of wild-type cells (Table II).

Both ABCG2 and MDR1 transporters are responsible for chemoresistance to doxorubicin. Whereas verapamil completely inhibited the SP phenotype, FTC only partly suppressed it (Fig. 6). FTC is a specific blocker of ABCG2 transporter but does not affect MDR1 (23). This underlines the importance of MDR1 for the drug-resistance of these cells.

As a hallmark of the resistant state ABCG2 and MDR1 were up-regulated in HTh74R cells (Fig. 7). In contrast, MRP1 transporter was equally expressed in HTh74 and HTh74R cells, which indicates that this ABC transporter is not involved in doxorubicin-mediated resistance (Fig. 7). It is also remarkable that stem cell marker Oct4 displayed higher expression in HTh74R than wild-type cells which points to CSC as carrier of resistance.

Discussion

It is now widely believed that tumor initiation, progression and metastasis are driven by a small population of CSC, also designated tumor-initiating cells (23). This concept is suggested by experimental evidence and clinical studies including the finding that in many tumors currently available drugs can shrink tumor size but usually only transiently, since existing therapies may kill the bulk of cancer cells but fail to eradicate CSC that are more resistant to chemotherapeutics (24).

In the present work we demonstrated that CSC derived from anaplastic thyroid carcinoma cell lines expressed ABCG2 and MDR1 transporters of the ABC gene family (Fig. 3), which enabled the exclusion of the Hoechst dye from CSC that otherwise binds to the DNA in non-stem cells (25). The expression of these transporters is on the one hand a prerequisite to isolate stem cells by FACS as SP fraction (Fig. 1) 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 at only 0.41-0.83% (Fig. 1) which is in accordance with previous reports of CSC in anaplastic thyroid carcinoma cell lines (11,18). CSC also expressed Oct4 (Fig. 3), a characteristic marker for adult and embryonic stem cells (14,15,26) that is also expressed in some human tumors and some cancer cell lines but not in normal differentiated cells (27).

SP cells isolated from anaplastic thyroid cell lines displayed a higher clonality, the potency to form single cell-derived clones in culture (Fig. 2A) and a more invasive *in vitro* growth in comparison to non-SP cells (Fig. 2B), a hallmark of these cells (18).

Adult stem cells and CSC share the common features of self-renewal and slow-cycling (23). Both types of stem cells may give birth to rapidly proliferating progenitor cells although it is still debated whether CSC are derived from mutated (quiescent) stem cells or proliferating progenitor cells (23). We have recently detected adult stem cells that are derived from human goiters (15). These cells account for only 0.1% of the cell population. The fraction of CSC in anaplastic thyroid carcinoma cell lines is also comparably low which suggests that niches, microenvironmental cells that regulate growth stem cells (23), are operative. However, under the condition of serum deprivation and intense growth

stimulation with EGF and bFGF, CSC grow out to form spheroids (Fig. 3).

When anaplastic cell lines were treated with doxorubicin, the large majority of cancer cells were killed (Fig. 5). This conferred a growth advantage to cancer stem cells which in turn overgrew the culture. Resistant cell line HTh74R consisted of a 70% SP fraction enriched with Oct4-positive cancer stem cells. The resistance of CSC to doxorubicin can be attributed, at least in part, to the expression of ABC transporters. The importance of these transporters is emphasized by inhibition experiments with verapamil that completely inhibits the SP phenotype and make CSC sensitive to cell death (Fig. 5). However, specific inhibition of ABCG2 by FTC demonstrated that this transporter is only partly responsible for drug resistance in HTh74R cells and that MDR1 also contributed to doxorubicin-resistant state in HTh74R cells (Fig. 6). Furthermore, the results confirmed that CSC are highly heterogeneous and are a subject to selection, dependent on the chemotherapeutic drug that was applied (28).

Anaplastic thyroid cell lines have been frequently used as a model to study molecular aberrations, cellular dysregulation and therapeutic strategies (29). Indeed, gene profiling analysis revealed that most thyroid carcinoma cell lines present a common undifferentiated phenotype that resembles that of undifferentiated thyroid tumors (30). These data suggest anaplastic thyroid cell lines also as an appropriate model to study CSC.

We have demonstrated here that CSC that expressed ABCG2 and MDR1 transporters are relatively resistant to doxorubicin. However, even stable HTh74R cells that were raised by long-term treatment with doxorubicin consisted of only 70% CSC that excluded the chemotherapeutic drug from the cell (Fig. 6). From these data it is evident that the remaining cells may have gained other molecular mechanisms that conferred drug resistance to these cells.

In conclusion, the present work suggests that the failure of doxorubicin to eradicate all anaplastic thyroid carcinoma cells is mainly due to resistance of CSC to the chemotherapeutic drug although there is a smaller fraction of resistant cells that do not express drug-exporting ABC transporters. Further therapeutic strategies have to be developed to target not only the main population of cancer cells but also to eradicate CSC that are responsible for tumor progression and recurrence. The isolation of CSC and their progeny as spheres may be a useful tool to study the efficacy of new drugs that also kill ABC transporter expressing cancer cells.

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