ABCG2/BCRP gene expression is related to epithelial–mesenchymal transition inducer genes in a papillary thyroid carcinoma cell line (TPC-1)

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Abstract

Tumor malignancy is associated with the epithelial–mesenchymal transition (EMT) process and resistance to chemotherapy. However, little is known about the relationship between the EMT and the multidrug-resistance gene in thyroid tumor progression. We investigated whether the expression of the *ABCG2/BCRP* gene is associated with *ZEB1* and other EMT inducer genes involved in tumor dedifferentiation. We established a subpopulation of cells that express the *ABCG2/BCRP* gene derived from the thyroid papillary carcinoma cell line (TPC-1), the so-called TPC-1 MITO-resistant subline. The most relevant findings in these TPC-1 selected cells were a statistically significant upregulation of *ZEB1* and *TWIST1* (35- and 15-fold change respectively), no changes in the relative expression of vimentin and *SNAIL1*, and no expression of E-cadherin. The TPC-1 MITO-resistant subline displayed a faster migration and greater invasive ability than parental cells in correlation with a significant upregulation of the survivin (*BIRC5*) gene (twofold change, $P < 0.05$). The knockdown of *ZEB1* promoted nuclear re-expression of E-cadherin, reduced expression of vimentin, N-cadherin, and *BIRC5* genes, and reduced cell migration ($P < 0.05$). Analysis of human thyroid carcinoma showed a slight overexpression of the *ABCG2/BCRP* at stages I and II ($P < 0.01$), and a higher overexpression at stages III and IV ($P < 0.01$). *SNAIL1*, *TWIST1*, and *ZEB1* genes showed higher expression at stages III and IV than at stages I and II. E- and N-cadherin genes were upregulated at stages I and II of the disease (ninefold and tenfold change, respectively, $P < 0.01$) but downregulated at stages III and IV (fourfold lower, $P < 0.01$). These results could be a promising starting point for further study of the role of the *ABCG2/BCRP* gene in the progression of thyroid tumor.

Key Words

- ▶ *ABCG2/BCRP* gene
- ▶ EMT inducer genes
- ▶ papillary thyroid carcinoma cell line (TPC-1)

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Introduction

Most patients with papillary or follicular thyroid tumors present survival rates close to 90% 10 years after diagnosis (Hundahl *et al.* 1998). However, between 2% and 11% of these tumors develop aggressive behavior, with local recurrence or metastases at diagnosis or during follow-up, significantly reducing the survival rate (Cooper *et al.* 2009). The molecular mechanisms and the type of cells involved in the dedifferentiation processes in these tumors are complex and largely unknown. One group of cells that is known to be implicated in the development of these tumors and resistance to chemotherapy is cancer stem cells (CSCs; Mimeault & Batra 2006, Dalerba *et al.* 2007, Akunuru *et al.* 2012). These cells possess stem-cell-like properties and share the expression of several stem cell markers (Li *et al.* 2013). However, their role in thyroid tumors is inconclusive (Lin 2011).

One stem cell marker detected in thyroid glands is the Proliferating Cell Nuclear Antigen (PCNA), also known as CD133. This marker, identified both in human cell lines and in biopsies from patients with thyroid cancer, contributes to radio-resistance and maintenance of the undifferentiated status of the tumors (Friedman *et al.* 2009, Todaro *et al.* 2010, Ke *et al.* 2013). Another stem marker detected in thyroid cancer is nestin. Nestin is also known to be a marker of neuroepithelial stem or progenitor cells in the brain, and to be related to malignancy in some solid cancers (Krupkova *et al.* 2010). In the thyroid, nestin has been identified in differentiated thyroid tumors, but its expression does not relate to malignant characteristics of these tumors (Yamada *et al.* 2009).

In 2007, Hoshi *et al.* identified a cell population which expresses the ABCG2/BCRP gene in the thyroid gland (Hoshi *et al.* 2007). This gene, referred to as the breast cancer resistance protein, is implicated in protection against several xenobiotics, in chemoresistance, and maintenance of the regenerative capacity of adult tissue. However, little is known about its physiological role in the thyroid gland (Krishnamurthy & Schuetz 2006, Robey *et al.* 2007, Thomas *et al.* 2008, Fatima *et al.* 2012).

Knowledge of the molecular mechanisms that control stem cell transformation into stem cell cancer is incomplete. Two of the most important mechanisms identified are the epithelial–mesenchymal transition (EMT) process and the mesenchymal–epithelial transition (MET) induction process. These processes not only maintain normal stem cell phenotypes but also contribute to the development of some types of solid tumors (Klymkowsky & Savagner 2009). Several transcription factors, such as

ZEB1, TWIST, and SNAIL1, have been identified as inducers of the EMT/MET processes, and are known to play a role in cancer progression and metastasis (Montserrat *et al.* 2011). ZEB1 is a master key in these processes. It has been associated with the maintenance of stem cell characteristics through expression of microRNAs and through the inhibition of the sonic hedgehog pathway (Wellner *et al.* 2009, Tang *et al.* 2012).

Another gene that is involved in the MET process is BIRC5 (survivin), a member of the apoptosis inhibitor family. BIRC5 (survivin) is highly expressed in fetal tissue and in most human solid tumors. Its expression is abolished in non-tumor cells and mature cells. Detection of BIRC5 (survivin) in tumors derived from human embryonic stem cells, such as teratoma, indicates that it plays a role in the formation of these tumors and their maintenance by transforming normal embryonic stem cells into embryonic cell-like cells (Brabletz *et al.* 2005, Boidot *et al.* 2008, Blum *et al.* 2009). BIRC5 (survivin) has also been shown to be involved in growth arrest, reduced migration, and resistance to chemotherapy- and radiotherapy-induced apoptosis (Tirrò *et al.* 2006, Nabzdyk *et al.* 2011).

However, the link between the expression of the ABCG2/BCRP transporter gene and genes related to EMT processes in aggressive thyroid tumors is unknown. The aim of the present study was to investigate whether the expression of the ABCG2/BCRP transporter gene is associated with ZEB1 and other EMT-related genes involved in tumor dedifferentiation using a papillary cell line (thyroid papillary carcinoma (TPC-1)) as a cellular model.

Subjects and methods

TPC-1 cell culture and cellular selection

The TPC-1 cell line was provided by Dr Paolo Vigneri (University of Catania, Italy). The short tandem repeat (STR) profiling was carried out using the protocols for the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer/Applied Biosystems, Waltham, MA, USA) and the ABI PRISM 310 Genetic Analyzer (Perkin Elmer/Applied Biosystems) according to the manufacturer's instructions. STR profiles were analyzed using the software package Genemapper 4.1 (Applied Biosystems). The TPC-1 cell line was matched with its STR profiles reported in previous studies (Meireles *et al.* 2007). This cell line was cultured in RPMI 1640 (w L-glutamine) supplemented with 10% fetal bovine serum and 2% streptomycin/penicillin.

To enrich the ABCG2/BCRP-positive cells in the TPC-1 cell cultures and to generate a TPC-1 MITO-resistant subline, we used mitoxantrone, a specific substrate of ABCG2/BCRP. Mitoxantrone was diluted to 12 μ M in the RPMI (low glucose) basal medium and was added to the culture every 2 days, following the protocol described by Mato *et al.* (2009). Cell cultures were maintained in 5% CO₂ at 37 °C in a humidified incubator. The surviving cells were then placed in fresh RPMI medium (low glucose; Gibco-BRL) supplemented with 10% FCS and 5% glutamine. Expression of ABCG2/BCRP transporters was confirmed using the qRT-PCR technique before and after cell selection.

Human thyroid tissue samples

Thyroid tumor tissue samples ($n=34$) were collected from patients undergoing surgery at Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). The study was approved by the Local Ethics Committee and carried out in accordance with the Declaration of Helsinki. Table 1 shows patient data: sex, age at diagnosis, tumor histology, and tumor stage defined by recent international guidelines (Cooper *et al.* 2009). Diagnosis of underlying disease was verified by histological examination 7 days after surgery.

RNA isolation and first strand cDNA synthesis

Total RNAs obtained from the human tissues and cell lines were isolated using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). The RNA yield and the ratio of absorbance at 260–280 nm ($A_{260}:A_{280}$ ratio)

Table 1 Characteristics of patients with malignant thyroid lesions. Tumor stage defined by the recent international guidelines (Cooper *et al.* 2009)

Mean age at diagnosis	59.7 (22–83 years)
Sex (n)	
Female	30
Male	4
Histology	
WDTC (papillary)	31
PDTC	2
ATC	1
Stage (n)	
Stage I	13
Stage II	5
Stage III	6
Stage IV	7 (plus two PDTC and one ATC)

WDTC, well-differentiated thyroid carcinoma, PDTC, poorly differentiated thyroid carcinoma; ATC, anaplastic thyroid cancer.

were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) and the RNA quality was checked by electrophoresis using agarose gels. Total RNA (1 μ g) was reverse transcribed using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science). cDNA samples were stored at –20 °C and diluted with RNase-free water for use as template in real-time PCR analysis.

Quantitative real-time PCR (qRT-PCR) and RT-PCR

Real-time PCR was conducted using an ABI PRISM 7900HF Sequence Detection System, according to the manufacturer's protocol (Applied Biosystems). All reactions were carried out using 100 ng of cDNA in a total volume of 50 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems) and the predesigned and labeled primer/probe set (Assays-on-Demand Gene Expression assay, Applied Biosystems). The samples were analyzed in duplicate, negative controls were included, and PCR products were verified using dissociation curve analysis immediately after RT-PCR. The *GADPH* housekeeping gene was used to normalize gene expression data. The relative changes in gene expression were analyzed by the $2^{-\Delta\Delta C_t}$ method and SDS2.1 and Data Assist V2.1 Software (Applied Biosystems). TaqMan qPCR primers (Applied Biosystems) used in the study were as follows: ABCG2/BCRP (Hs0105379_m1), ZEB1 (Hs01566407_m1), SNAIL1 (Hs00195591_m1), TWIST (Hs003611867_m1), E-cadherin (CDH1) (Hs00170423_m1), N-cadherin (CDH2, Hs00983056_m1), integrin β 1-binding protein 1 (*ITGB1BP1*, Hs00178055_m1), *ITGB1* (Hs00559595_m1), survivin (*BIRC5*, Hs00977611_g1), vimentin (VIM, Hs00185584), and *GAPDH* (4333764F). Commercially available RNA pooled from normal thyroid (636536, Clontech) was used as a control in the real-time PCRs (qRT-PCRs).

siRNA transfection

siRNA transfection in TPC-1 parental cell lines and in the TPC-1 MITO-resistant subline was carried out using Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to the manufacturer's protocol. Approximately 4.5×10^5 cells were plated in six-well plates overnight. The next day, the cells were transiently transfected with either 10 nM predesigned siRNA (Stealth Select RNAi) targeting ZEB1 (sc38643) or control siRNA purchased from Invitrogen. The transfected cells were harvested at 24, 48, and 72 h for further analyses. The efficiency of the siRNA transfection showed a significant

reduction in *ZEB1* mRNA expression level ($P < 0.001$; Supplementary Fig. 1, see section on supplementary data given at the end of this article). Each assay was carried out in triplicate in at least three independent experiments.

Immunocytochemistry

Cytospin slides from cell suspensions of the TPC-1 parental cell line and TPC-1 MITO-resistant subline were immunostained. Samples were then fixed in 4% paraformaldehyde and rinsed with PBS solution. They were then blocked for 10 min at room temperature in a solution containing 1% BSA and 0.2% saponin. The primary antibodies and their dilutions were as follows: *ZEB1* (HPA027524, Sigma–Aldrich), vimentin (MAB3400, Millipore, Darmstadt, Germany), and TWIST (sc-6269, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) at 1/100, E-cadherin (M3612, Dako, Glostrup, Denmark), N-cadherin (M3613, Dako), ITGB1 (610468, Becton Dickinson, East Rutherford, New Jersey, USA), SNAIL1 (sc-10433, Santa Cruz Biotechnology, Inc.) at 1/50, and ABCG2/BCRP (AB3380, Abcam, Cambridge, UK) at 1/1000. All antibodies were diluted with a blocking solution (s3022, Dako) and incubated overnight (O/N) at 4 °C. Biotinylated second antibody and peroxidase-conjugated streptavidin from the Dako Universal LSAB Kit (Dako) were added for 20 min each. Finally, sections were incubated in 3/3-diaminobenzidine for 5 min, followed by hematoxylin counterstaining and mounting. Negative controls were prepared by replacing the primary antibody with non-immune serum. No signals were observed for these samples. The results were visualized with a Leica microscope.

Flow cytometry analysis

Subconfluent cells were harvested using trypsin and 1×10^6 cells were incubated with the primary antibody anti-BCRP, clone 5D3 (MAB4155, Millipore), for 60 min at 4 °C and washed twice with PBS+2% FCS. FITC-conjugated secondary antibody (ab6785, Abcam) was applied to the cells for 30 min at 4 °C. Briefly, cells were washed twice with PBS+2% FCS, and resuspended in 0.5 ml PBS with 10% FCS. Labeled cells were scanned on a FACSCalibur Cytometer (Becton Dickinson) and analyzed using Cellquest Software (Becton Dickinson), acquiring 1×10^4 events. An isotopic control immunoglobulin (ab11-4732, eBioscience, San Diego, CA, USA) was used as a negative control.

In vitro wound healing assay

TPC-1 parental cells, the TPC-1 MITO-resistant subline, and the TPC-1 MITO-resistant subline transfected with *ZEB1* siRNA were plated at high densities and grown to confluence at 90% overnight. The cells were scratched with a pipette tip (10 µl) and washed several times to remove the cellular debris. Using an inverted microscope, the wounds were photographed at 0 h ($t=0$) and again after 16 h of incubation, at the same site, at 37 °C. The cultures were incubated with serum-free medium. Image J Software (National Institutes of Health, Bethesda, MD, USA) was used to analyze photographs. The percentage of wound healing was determined based on three measurements of the wound area. Each result is the mean of three independent experiments.

Invasion assay

TPC-1 (2×10^5) in serum-free DMEM was placed in an insert made of polycarbonate membrane with 8-µm pores and precoated with basement membrane matrix (Cell Biolabs, Inc., San Diego, CA, USA) following the manufacturer's protocol. The outer chamber was filled with 500 µl RPMI containing 10% FCS as the chemoattractant. The chambers were then placed in an incubator at 37 °C with 95% O₂+5% CO₂ for 3 days. The inserts were removed, and the noninvading cells were removed using cotton-tipped swabs. The cells that traversed the membrane pore and spread to the lower surface of the filters were stained with eosin for visualization, and the number of cells that penetrated the membrane was determined by counting the mean cell number of three randomly selected high-power fields (200×; MOTIC AE31). Experiments were carried out in triplicate. The results for the TPC-1 MITO-resistant subline alone and transfected with *ZEB1* siRNA were normalized to the values for TPC-1 parental cells.

Statistical analysis

Results are expressed as mean $\pm$ s.d. The statistical significance was estimated using Student's unpaired *t*-test. Pairwise comparisons were made using Student's *t*-test. A two-tailed $P < 0.05$ was considered statistically significant. The analyses were performed using DATA ASSIST Software, version 3.01 or GraphPad Prism for Windows, version 4.0 (GraphPad Software, San Diego, CA, USA).

Results

Establishment and characterization of the TPC-1 MITO-resistant subline compared with the TPC-1 cell line used as a control: relative expression of the *ABCG2/BCRP* transporter gene and genes related to the EMT process

Stem cell markers analyzed by immunocytochemistry showed a few TPC-1 cells with a weak protein expression for *ABCG2/BCRP* and nestin, and a high protein level for the *CD133* marker. After cell selection, the TPC-1 MITO-resistant cell subline showed an increase in protein expression for *ABCG2/BCRP* but no changes in protein expression for the other markers tested (Fig. 1A). Immunocytochemistry results for the *ABCG2/BCRP* gene correlated with a significant increase in the relative expression of mRNA detected for the *ABCG2/BCRP* gene (eightfold change, $P < 0.05$; Fig. 1B) and a higher mean fluorescence

intensity measured by flow cytometry ($P < 0.05$; Fig. 1C). We also observed a significant increase in expression of the *ZEB1* and *TWIST* genes (35-fold change ($P < 0.05$) and a 15-fold change ($P < 0.05$) respectively) in the same selected cell population. No significant change was observed in *SNAIL1* expression in TPC1 or in the TPC-1 MITO-resistant subline (Fig. 2A), indicating a good correlation with the protein levels detected by immunocytochemistry (Fig. 2B). After cell selection, the TPC-1 MITO-resistant cell subline showed an increase in N-cadherin gene expression (twofold change, $P < 0.01$; Fig. 3A). The results for N-cadherin gene expression were correlated with protein expression (Fig. 3B). No expression of the E-cadherin gene was observed in the TPC-1 or the TPC-1 MITO-resistant subline (data not shown) and the result for gene expression of E-cadherin was confirmed by immunocytochemical analysis (Fig. 3B). The analysis of ITGB1 and

Figure 1

Characterization of TPC-1 parental cells and the TPC-1 MITO-resistant subline. (A) Representatives images of cell stained for *ABCG2/BCRP*, nestin, and *CD133* in both before and after drug selection (20× magnification). (B) Relative expression levels of mRNA for the *ABCG2/BCRP* gene. Data are representative of three independent experiments. Data shown represent

the mean $\pm$ s.d. for three independent experiments for mRNA expression, *** $P < 0.001$. (C) Mean intensity of fluorescence (MFI) of TPC-1 parental cells vs the TPC-1 MITO-resistant subline. Bars represent mean $\pm$ s.d. from three separate experiments (* $P < 0.05$).

Figure 2

Relative expression of protein and mRNA transcription factors: *ZEB1*, *TWIST*, and *SNAIL1* in TPC-1 parental cells and the TPC-1 MITO-resistant subline. (A) qRT-PCR analysis shows a significant higher expression at the mRNA level *ZEB1* and *TWIST* in the TPC-1 MITO-resistant subline. Significant changes were observed in *ZEB1* ($***P < 0.001$) and *TWIST* ($**P < 0.01$) genes. No significant change in *SNAIL1* gene expression was found.

Bars represent mean $\pm$ s.d. from three separate experiments. TPC-1 cells were used as a control. (B) Representative images of cells stained for *ZEB1* (20 $\times$ magnification), *TWIST1*, and *SNAIL1* (40 $\times$ magnification) by immunocytochemistry both before (TPC-1 parental cells) and after mitoxantrone selection (TPC-1 MITO-resistant subline).

integrin cytoplasmic domain-associated protein 1 molecule (ITGB1BP1) showed significant downregulation of the relative expression in selected cells (twofold lower for both genes) compared with parental cells ($P < 0.05$) (Fig. 4A and B). No significant changes were detected in the relative expression of vimentin at both the mRNA and protein levels (Fig. 4C and D).

Effects of silencing of the *ZEB1* gene on EMT-related genes in the TPC-1 MITO-resistant cell subline

The effects of silencing of the *ZEB1* gene at 48 h on EMT-related genes resulted in a significant induction of E-cadherin gene expression (eightfold change, $P < 0.001$) and nuclear localization of E-cadherin protein in 35% of cells (Fig. 5A and B). In contrast, vimentin gene expression showed significant downregulation at 24 h (twofold change, $P < 0.05$) and an even higher downregulation at 48 h (fourfold change, $P < 0.001$), although some expression remained (Fig. 5C and D). After silencing of the *ZEB1* gene for 24 h, analysis of *ABCG2/BCRP* gene

expression showed a significant upregulation (twofold change, $P < 0.01$). However, a significant reduction in *ABCG2/BCRP* gene expression was noted at 48 and 72 h, although some expression remained (fourfold lower, $P < 0.05$; Fig. 6A). A significant downregulation at 24 h was observed in N-cadherin gene expression (fourfold lower, $P < 0.05$) and was maintained at 48 and 72 h (Fig. 6B). Significant upregulation of *ITGB1* gene expression was detected at 48 and 72 h (twofold change and 2.5-fold change, ($P < 0.01$), respectively; Fig. 6C). The ITGB1BP1 (ICAP1) molecule interacted with ITGB1 and had a low level of expression in the TPC-1 MITO-resistant cell subline at 24 and 48 h. This molecule exhibited high upregulation at 72 h (12-fold change, $P < 0.05$; Fig. 6D).

Effect of the *ZEB1* knockdown on survivin (*BIRC5*) gene expression in cell migration and invasion

The survivin gene was expressed in the parental TPC-1 cell line, but it showed a statistically significant upregulation

Figure 3

Relative expression of mRNA and protein for N- and E-cadherin in TPC-1 parental cells and the TPC-1 MITO-resistant subline. (A) qRT-PCR analysis shows a significant increase in expression of N-cadherin in the TPC-1 MITO-resistant subline (twofold change, $**P < 0.01$). Bars represent mean $\pm$ s.d. from three separate experiments. TPC-1 parental cells were used as a control. (B) Representative image of cells stained for N-cadherin (20 $\times$ magnification) and E-cadherin (20 $\times$ magnification) by immunocytochemical staining both before and after drug selection.

in the TPC-1 MITO-resistant subline (twofold change, $P < 0.05$; Fig. 7A). The ability to close a wound, analyzed by wound-healing assays, showed a statistically significantly faster migration in the TPC-1 MITO-resistant subline cells

culture (70% closure of wound area) than in the TPC-1 parental cell line (40% closure of wound area) ($P < 0.05$) (Fig. 7B). The wound healing effects after silencing the *ZEB1* gene showed statistically significant downregulation of *BIRC5* gene expression at 48 and at 72 h of transient transfection (fourfold change, $P < 0.05$; Fig. 7C). The faster cell migration observed in the TPC-1 MITO-resistant subline was reverted after knock-down of the *ZEB1* gene, $P < 0.05$ (Fig. 7D). The TPC-1 MITO-resistant subline showed higher ability to invade a reconstituted ECM than the TPC-1 parental cell line at 92 h of cell culture ($P < 0.05$; Fig. 8).

Relative expression of mRNA ABCG2/BCRP transporter, and EMT-related genes in human thyroid carcinoma tissues

The analysis of gene expression carried out in a cohort of 34 patients with different stages of the disease (see Table 1) showed a statistically significant upregulation in the relative expression of the *ABCG2/BCRP*, *SNAIL1*, *TWIST1*, and *ZEB1* genes compared with control tissue ($P < 0.05$). These results correlated with the stage of the tumors (Fig. 9A, B, C and D). The analysis of E- and N-cadherin genes showed a statistically significant upregulation for both genes (tenfold change and 11-fold change respectively ($P < 0.05$)) in patients with stages I and II of the disease. In contrast, downregulation (fourfold change) was statistically significant for both genes in patients with stages III and IV disease. (Fig. 9E and F).

Discussion

In this study, we identified for the first time, to our knowledge, cells positive for ABCG2/BCRP, nestin, and CD133 in a papillary cell line (TPC-1). However, we did

Figure 4

Relative expression of mRNA and protein for *ITGB1*, *ITGB1BP1*, and vimentin in TPC-1 parental cells and the TPC-1 MITO-resistant subline. (A, B and C) qRT-PCR analysis shows a significant downregulation of the expression of *ITGB1* and *ITGB1BP1* (twofold change, $*P < 0.05$). No significant change in the expression of the vimentin gene was observed in

selected cells. Bars represent mean $\pm$ s.d. from three separate experiments. TPC-1 parental cells were used as a control. (D) Representative image for vimentin (20 $\times$ magnification) stained by immunocytochemistry both before (TPC-1 parental cells) and after mitoxantrone selection (TPC-1 MITO-resistant subline).

Figure 5

Effect of knockdown of *ZEB1* on EMT inducer genes (E-cadherin and vimentin). (A) qRT-PCR analysis showed a significant increase in the expression of the mRNA for the E-cadherin gene (** $P < 0.001$). (B) Representative image of cells positively stained for E-cadherin protein showing nuclear. The arrows indicate staining at 48 h of *ZEB1* silencing (20 $\times$ magnification). (C) qRT-PCR analysis showed a significant reduction

of expression at 24 and 48 h of silencing of *ZEB1* for vimentin mRNA (** $P < 0.01$ and *** $P < 0.001$). (D) Representative image showed a reduction of expression of protein at 48 h of silencing of *ZEB1* (20 $\times$ magnification). Bars represent mean $\pm$ s.d. from three separate experiments. The TPC-1 MITO-resistant subline transfected with siRNA control was used as a control.

not detect changes in gene expression for nestin or *CD133* genes after enriching the cell culture with cells that expressed the *ABCG2/BCRP* gene, indicating that more than one cell population has a pluripotent capacity in

TPC-1. Furthermore, we observed, for the first time, a relationship between the increase in expression of the adult stem marker *ABCG2/BCRP* gene, a member of the ATP-binding cassette transporters and EMT inducer genes,

Figure 6

Effect of knockdown of *ZEB1* on the relative expression of mRNA for *ABCG2/BCRP*, *N-cadherin*, *ITGB1*, and *ITGB1BP1* at 24, 48, and 72 h. (A) Knockdown of *ZEB1* resulted in upregulation of *ABCG2/BCRP* gene expression at 24 h (** $P < 0.01$). This increment was decreased at 48 and at 72 h, a reduction of expression was detected (** $P < 0.001$). (B) Downregulation of *N-cadherin* was detected at 24 h of *ZEB1* silencing (** $P < 0.001$) and was maintained at 48 and 72 h (** $P < 0.01$).

(C) The relative mRNA expression of *ITGB1* showed an upregulation at 24 h (* $P < 0.05$), 48 h (** $P < 0.001$), and 72 h (** $P < 0.001$). (D) The relative mRNA expression of *ITGB1BP1* gene showed an upregulation of gene expression at 72 h (** $P < 0.001$). Bars represent mean $\pm$ s.d. from three separate experiments. The TPC-1 MITO-resistant subline transfected with siRNA was used as a control.

Figure 7

Analysis of survivin (*BIRC5*) gene expression, cellular migration in TPC-1 parental cells. The TPC-1 MITO-resistant subline, and the TPC-1 MITO-resistant subline transfected with siRNA ZEB1. (A) The relative mRNA expression of survivin gene showed upregulation in selected cells (TPC-1 MITO-resistant subline cells; twofold change, * $P < 0.05$). (B) The wound-healing assay showed significantly faster migration in the TPC-1 MITO-resistant subline compared with TPC-1 parental cells (* $P < 0.05$). The images were obtained by phase-contrast microscope (100 $\times$). (C) Transient ZEB1

knockdown resulted in downregulation of *BIRC5* gene expression at 48 h (** $P < 0.01$) and at 72 h (*** $P < 0.001$). (D) The wound-healing assay showed a significant decrease in the closed wound area in the TPC-1 MITO-resistant subline transfected with siRNA ZEB1 compared with the TPC-1 MITO-resistant subline transfected with siRNA control (* $P < 0.05$). Phase-contrast microscope images ($\times 100$). Bars represent mean $\pm$ s.d. from four separate experiments.

in TPC-1 and in papillary thyroid tumors. Although there is evidence that the EMT process generates CSCs, the connection between the specific genes involved in this process and the radioresistance observed in thyroid

tumors has not been clearly established (Visvader & Lindeman 2008, Singh & Settleman 2010, Chu *et al.* 2013, Lan *et al.* 2013). Recent research using anaplastic thyroid cancer as a model has confirmed an association

Figure 8

Transwell invasion ability of TPC-1 parental cells and TPC-1 MITO-resistant subline cells. (A and B) The ability to invade surrounding extracellular matrix was analyzed at 48, 72, and 92 h of cell culture in complete

medium. The TPC-1 MITO-resistant subline cells showed a high capacity for invasion at 92 h (* $P < 0.05$) compared with TPC-1 parental cells used as a control. Bars represent mean $\pm$ s.d. from four separate experiments.

Figure 9

Gene expression of EMT inducer genes (*ZEB1*, *SNAIL1*, and *TWIST1*). E-cadherin, N-cadherin, and *ABCG2/BCRP* from human thyroid carcinoma (papillary tumors and poorly differentiated/anaplastic tumours). (A, B, C and D) The relative mRNA expression of *ABCG2/BCRP*, *SNAIL1*, *TWIST1*, and *ZEB1* showed a higher increase in expression in tumors from patients with aggressive stages (III and IV) in comparison with those with the more benign stages (I and II). (E and F) The relative mRNA expression of E- and

N-cadherin genes showed a significant increase in expression in tumors from patients with benign stages (I and II), in contrast a decrease in expression of both genes was detected in tumors from patients with more aggressive stages (III and IV). Data shown represent the mean $\pm$ s.d. * P <0.05, ** P <0.01, and *** P <0.001. The tumor tissue was compared with normal thyroid tissue.

between the stem-like phenotype and chemotherapy resistance, indicating that the multidrug transporter system is responsible for this chemotherapy effect (Zito *et al.* 2008, Davies *et al.* 2011, Carina *et al.* 2013). Our results indicate that the cell subpopulation identified in TPC-1 expresses the *ABCG2/BCRP* gene, possibly contributing to the malignant progression of these tumors. This cell subpopulation also showed upregulation of expression of the *BIRC5* gene, faster cellular migration, and greater ability for invasion when the selected cells (TPC-1 MITO-resistant subline) were compared with the parental cell line (TPC-1). These results are consistent with those reported for pancreatic cancer (Arumugam *et al.* 2009, Wellner *et al.* 2009, Palena *et al.* 2011). It was of interest to note that this effect could be reverted when the *ZEB1* gene was knocked down in the selected cells. In these selected cells, the transient silencing of the *ZEB1* gene produced a downregulation of *ABCG2/BCRP* and *BIRC5*, and reduced cell migration *in vitro*. These results indicate that *ABCG2/BCRP*, *ZEB1*, and *BIRC5* genes are linked and could play a role in the more aggressive behavior observed in some well-differentiated papillary tumors.

Other markers that we studied in TPC-1 cells were E- and N-cadherin. These genes belong to transmembrane protein families and are involved in cell-adhesion. We found no expression of the E-cadherin gene in the TPC-1 cell line or in the TPC-1 MITO-resistant subline. However,

the N-cadherin gene was increased in expression in TPC-1 MITO-resistant cells. Other authors have described a relationship between the loss of expression of the E-cadherin gene and the metastatic process. The role of expression of N-cadherin gene in carcinomas is controversial (Hazan *et al.* 2004, Li *et al.* 2010, Shih & Yamada 2012). In our study, we observed that transient *ZEB1* silencing in the TPC-1 MITO-resistant subline promoted increased expression of E-cadherin with a nuclear localization, and downregulation of N-cadherin. This result supports the observation that E-cadherin can translocate to the nucleus and participate in the regulation of Wnt-dependent genes (Kuphal & Behrens 2006).

We also analyzed integrin transmembrane receptors, *ITGB1* and integrin β 1-binding protein 1 (*ICAP1*), genes involved in the progression of malignancy, chemoresistance, morphogenesis, terminal cellular differentiation, and maintenance of the progenitor population in adult tissue (De Toni *et al.* 2006, Piwko-Czuchra *et al.* 2009, Wu *et al.* 2009, Ju *et al.* 2010, Shiraki *et al.* 2011). *ICAP1* is considered to be the most specific ligand for the *ITGB1* cytoplasmic domain and is involved in cellular processes such as cellular adhesion and migration (Fournier *et al.* 2002, Brunner *et al.* 2011). In selected cells, we found that both *ITGB1* and *ICAP1* genes were downregulated, but the *ZEB1* silencing gene was able to promote an up-expression in both. Further research is needed, nevertheless, to

clarify the relationship between these genes and the *ABCG2/BCRP* gene.

Another novel finding in our study is that high increase in expression of the *ABCG2/BCRP*, *SNAIL1*, *TWIST1*, and *ZEB1* genes in poorly differentiated thyroid carcinoma, anaplastic carcinoma, and papillary thyroid tumors correlated with more aggressive disease stages. However, in agreement with published results, reduced expression of the E- and N-cadherin genes at stages III and IV also correlated with more aggressive stages. Studies in larger series and with a longer follow-up will be needed, however, to demonstrate whether these two genes can be considered as biomarkers of poor prognosis for papillary thyroid carcinoma.

In summary, we found that the expression of the *ABCG2/BCRP* gene was associated with *ZEB1* and other EMT-inducer genes involved in papillary thyroid tumor dedifferentiation. These results could be a promising starting point for improving understanding of the role of these cells in tumor progression, and a new tool for designing innovative therapeutic strategies.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0051>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

E M and was responsible for supervision the experiment, analysis of the gene expression, statistical analysis of all data, and prepare the manuscript. A M, J I P, C G, and E L were responsible for collection of all human samples and clinical-histological studies. O B for setting up of the experiment and technical supported. A L responsible for the supervision of experiments and prepare the manuscript.

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