Thyroid

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TITLE: Reprogramming of energy metabolism: increased expression and roles of pyruvate carboxylase in papillary thyroid cancer

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ABSTRACT

Background: Energy metabolism is described to be deregulated in cancer and the Warburg effect is considered to be a major hallmark. Recently, cellular heterogeneity in tumors and tumor microenvironment have been recognized to play an important role in several metabolic pathways in cancer. However, their contribution to papillary thyroid cancer (PTC) development and metabolism is still poorly understood.

Methods: We performed a proteomic analysis of 5 PTC and investigated the cellular distribution of several upregulated metabolic proteins in the cancerous and in the stromal cells of these tumors.

Results: MS/MS analysis revealed the upregulation of many metabolism-related proteins, among them pyruvate carboxylase (PC). PC knockdown in thyroid cell lines alters their proliferative and motility capacities, and measurements of oxygen consumption rates show that this enzyme is involved in the replenishment of the TCA cycle. Immunostainings of several upregulated metabolic proteins show that thyroid cancer cells have an increased mitochondrial oxidative metabolism compared to stromal cells.

Conclusion: PTC have a very active TCA cycle, continuously replenished by a pyruvate carboxylase mediated anaplerosis. This is specifically observed in the tumor cells.

INTRODUCTION

Deregulated energy metabolism is one of the hallmarks of cancer, playing a crucial role in tumor development (1–3). Tumor cells have high energy requirements to support many biosynthesis pathways and promote cell growth. The Warburg effect has been considered for a long time to be the major metabolic reprogramming in cancer. In this scheme, glucose uptake is increased, metabolized to pyruvate, then transformed into lactate, instead of entering the TCA cycle, even when the level of oxygen is normal in the tumor microenvironment (4). This allows the oxidation of the NADH produced by glycolysis into a NAD^+ sustaining glycolytic flux (4, 5).

Since a few years, another concept is emerging: the TCA cycle can maintain its functions and participate in the synthesis of energy and biosynthetic intermediates as metabolic precursors out of the mitochondria (6). In addition, the metabolism of cancer cells is influenced by the metabolic microenvironment of the tumor. Indeed, several studies have described for example the important role of cancer-associated fibroblasts (CAFs) in the majority of tumors (7). They contribute to tumor proliferation by exchanging cytokines, growth factors or pro-angiogenic factors (8, 9), but it is still unclear how they are involved in tumor metabolism. Recent studies showed that CAFs carry out aerobic glycolysis and release lactate in the tumor microenvironment (10), which then become available for oxidative mitochondrial metabolism sustaining the cancer cells (11). This model, named “The Reverse Warburg Effect”, allows exchanging resources between cells within tumors and defines CAFs as feeders of lactate, which is then transformed into pyruvate by oxidative tumor cells in order to replenish the TCA cycle.

The TCA cycle intermediate replenishment, or anaplerosis, is known to be carried out by two major pathways: 1) glutaminolysis, which consists in using glutamine, metabolized to glutamate by glutaminase (GLS), itself converted into α-ketoglutarate by glutamate dehydrogenase (GDH); and 2) the carboxylation of pyruvate to oxaloacetate via ATP-dependent pyruvate carboxylase (PC). In cancers, a compensatory relationship between GLS and PC has been demonstrated when cells deprived of glutamine switched to a glucose-dependent anaplerosis via pyruvate (12). At the opposite, intracellular lactate signaling promotes glutamine uptake, that becomes the major substrate (13).

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Thyroid cancer is the most frequent endocrine cancer in human, and papillary thyroid carcinoma (PTC) represents up to 85% of all malignant thyroid tumors. PTC is usually biologically indolent and has an overall 5- to 10-years survival rate of 80–95%. The typical therapy consists of thyroidectomy followed by radioiodine treatment. Dedifferentiated PTC can display resistance to therapy. Several cancers have been metabolically characterized to define new therapeutic approaches using inhibitors against metabolic enzymes. For instance, Doherty et al. developed inhibitors against the lactate dehydrogenase (LDH) proteins, such as gossypol or galloflavin (14). Accordingly, characterizing the energy metabolism of PTC could offer an opportunity to treat more aggressive forms. For now, thyroid tumor metabolism has been poorly described but appears to vary according to the histological subtype (15). An increased level of labeled glucose consumption by poorly differentiated thyroid tumors compared to differentiated tumors has been reported, with a strong correlation with the presence of BRAF mutations (16). The enhanced glucose metabolism was validated by the demonstration of the overexpression of glucose transporters 1 and 3 (GLUT1, GLUT3), and of hexokinase 2 (HK2) in BRAF-mutated PTC (17, 18). The expression levels of GLS and of GDH were also increased in BRAF-mutated PTC, suggesting an overall increase in glutamine metabolism (15).

On the other hand, the role of the tumor microenvironment is now recognized to play a major metabolic role in numerous cancers (19, 20). However, its contribution to thyroid tumor development is still poorly described. The cellular heterogeneity of PTC resulting in part from the presence of CAFs has been characterized and revealed a positive correlation with tumor aggressiveness (21). Another study showed respective overexpression of monocarboxylate transporters 1 and 4 (MCT1, MCT4) in cancer and stromal cells, each one being responsible of lactate transport across the plasma membrane (22).

In this study, we performed a proteomic analysis of PTC, which revealed an upregulation of a high number of metabolism-related proteins. We first focused on PC whose expression and functional role were studied in thyroid tumors and in thyroid cell lines. We then extended our study by investigating the expression and cellular localization of other metabolic enzymes. Our data show that PTC have a very active TCA cycle, continuously replenished by a PC mediated anaplerosis. This is specifically observed in the tumor cells.

MATERIAL AND METHODS

Cell culture

The human thyroid cancer cell lines TPC1 (RET/PTC1 rearranged) and 8505C ($BRAF^{V600E}$ and TP53 mutated) (23), and HTori-3 (SV40-immortalized human thyrocytes) (24) were cultured in RPMI 1640 (+ L-glutamine + 25 mM Hepes) (Life Technologies) supplemented with 10% FBS, 2% streptomycin/penicillin and 1% amphotericin B (Life Technologies). The cells were grown at 37°C in a humidified atmosphere with 5% CO_2 and 20% O_2. STR analyses performed previously for TPC1 and 8505C cells (25) showed that they were identical to those published by Schweppe et al. (26). STR analysis of HTori-3 cells is provided in Supplementary Figure. Their genotype is identical to the STR profile of Nthy-ori 3-1, a subclone of HTori-3 (European Collection of Cell Culture-ECACC 90011609), except a heterozygosity detected for *THO1*. Moreover these cells express TTF1 and PAX8, suggesting that they are of thyroid origin.

Thyroid tissue samples

Paired samples of normal and tumor thyroid tissues were obtained from patients undergoing surgery for papillary thyroid cancer (n=28). All the tissues were provided by the Anatomical Pathology Department of the J. Bordet Institute (Brussels, Belgium) where the diagnoses were made by pathologists. 5 paired samples, containing at least 70% of tumor cells, were selected for MS/MS and transcriptomic analyses, and the remaining samples were used for validation. Tissue samples were embedded in OCT and directly stored at −80°C until processing. Paraffin sections were used for immunostaining (n=6). Protocols have been approved by the Ethics Committee of the J. Bordet Institute.

MS/MS analysis

Five PTC (classical variant) and their normal adjacent tissues were analyzed by mass spectrometry. All of them carried the BRAF V600E mutation, the most common mutation in PTC (relevant clinical data are provided in Supplementary Table 1). Protein extraction and analysis were performed at the proteomic platform of Professor Rudy Wattiez (University of Mons), with the TripleTOF mass spectrometer (ESI sources). Proteins were identified with the UniProt database.

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RNA purification

Total RNA was extracted from thyroid samples or from thyroid cell lines using a TRIzol Reagent kit (Invitrogen Carlsbad, CA, USA) followed by purification on RNeasy columns (Qiagen Hilden, Germany). RNA concentrations were spectrophotometrically quantified, and their integrity was verified using an automated electrophoresis system (Experion, Bio-Rad).

Affymetrix microarray hybridization

The 5 samples analyzed by mass spectrometry and 3 additional samples for which no proteins were available were analyzed by Affymetrix microarrays. RNA amplification, cDNA synthesis and labeling were performed according to manufacturer's instructions (Affymetrix Santa Clara, CA, USA). RNA (100 ng for each sample) from 8 PTC and their normal, non-neoplastic adjacent thyroid tissues were hybridized on Affymetrix Human Genome U133 Plus 2.0 Arrays. CEL file data were normalized by GCRMA (GenePattern - http://www.broad.mit.edu/cancer/software/genepattern/). For each spot, data were expressed as the log2 ratio of fluorescence intensities from tumor and normal tissues.

Quantitative RT-PCR for pyruvate carboxylase mRNA expression

RNA extraction from tissue samples and cell lines was carried out according to manufacturer's instructions (Invitrogen Carlsbad, CA, USA). RNA samples were treated by "DNase I amplification Grade" and subjected to reverse transcription using "Superscript II RNase H Reverse Transcriptase". PC mRNA expression was quantified on an Applied Biosystems 7500 Fast Real Time PCR with SyberGreen (Eurogentec, Liège, Belgium), using oligonucleotides designed with Primer Blast (forward sequence: 5'-GGCGACGGCGAGGAGATAG-3', reverse sequence: 5'- GAGTAGATGGCTACGGTGCG-3'). NEDD8 and TTC1 mRNA expressions were used for normalization (27).

Protein extraction

Thyroid tissues or cells were lysed in Laemmli lysis buffer, supplemented by phosphatase and protease inhibitors (NaF, Vanadate, Pefabloc, Leupeptin and Tablet Roche Inhibitor

25x (Roche Applied Science)). Protein extracts were denatured at 100 C° for 3 min and then quantified by the PAR/IDCR method (Thermo Fisher Scientific Waltham, MA, USA).

Western blotting

Proteins samples (30 µg/well) were loaded on a 7.5% acrylamide gel and run for 90 minutes at 20 mA/gel. The proteins were transferred to a nitrocellulose membrane at 80 V during 90 minutes. The membrane was first blocked 1 hour at room temperature in Odyssey/PBS (v/v), and then incubated overnight at 4°C with the primary antibody [1:200 anti-PCB rabbit polyclonal antibody, Santa-Cruz (sc-67021); 1:100 anti-PDHE1α mouse monoclonal antibody, Santa-Cruz (sc-377092); 1:5000 anti-GLS rabbit monoclonal antibody, Abcam (ab156876)] in the solution (Odyssey/PBS (v/v) + Tween 0.1%). After incubation for 1 hour in the dark at room temperature with the secondary antibody solution (Odyssey/PBS (v/v) + Tween 0.1% + SDS 0.001%), images were acquired using the Azure Biosystems C500.

Immunostainings

Immunohistochemical analyses were performed on 10 µm thick sections prepared from paraffin-embedded tissues. Expression of smooth-muscle actin was analyzed by pathologists from the J. Bordet Institute to distinguish CAFs from cancer cells. The sections were deparaffinized, rehydrated, treated with citrate buffer (10 mM, pH 6, 95°C, 1h) for antigen retrieval, and their endogenous peroxidase activity was blocked. The sections were then incubated with the primary antibody [anti-PCB (Santa Cruz; sc-67021), anti-PDK (Santa Cruz; sc-28783), anti-PCCB (Abcam; ab96729), anti-ECH1 (Abcam; ab153720), anti-GLS (Abcam; ab156876), anti-LDHA (Abcam; ab47010), anti-LDHB (Santa Cruz; sc100775), anti-MCT1 (Santa Cruz; sc365501), anti-MCT4 (Santa Cruz; sc50329), anti-PCK2 (Abcam; ab137580)] and the detection was performed with the peroxidase AEC substrate (K3464, DAKO). The primary antibody [anti-PDHα1 (Santa Cruz; sc-377092)] was revealed by immunofluorescence using Alexa Fluor (594nm). As negative controls, immunostainings were carried out in the absence of primary antibodies.

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siRNA transfection

For transient gene knock-down, a PC-specific siRNA (siPC) sequence (s10089, Ambion) was transfected into different thyroid cell lines (TPC1, HTori-3 and 8505C) using lipofectamine RNAiMAX reagent according to manufacturer's instructions (LifeTechnologies Carlsbad, CA, USA). An unrelated, non-targeting, siRNA (siCTRL) sequence (4390843, Ambion) was used as a negative control. Cells (2 x 10^5/well) were seeded in 6-well plates and transfected 24 h later with 25 pmol of siRNA. RNA and protein samples were prepared 48 h and 72 h post-transfection, respectively.

Proliferation assays

Cell proliferation rate was assessed using the Click-It® Plus EdU Flow Cytometry Assay kit (Thermo Fisher Scientific Waltham, MA, USA) according to manufacturer's instructions. Briefly, cells (75,000 cells/well) were seeded in 12-well plates 24h post-transfection. After 48 h, they were incubated for 6 h with 10 µM 5, 5-ethynyl-2'-deoxyuridine (EDU). After a final incubation with Alexa Fluor 488-containing buffer, EDU incorporation was analyzed by flow cytometry on the BD LSRFortessa™ cell analyzer.

Migration / Invasion tests

Forty-eight hours post-transfection, cells were incubated in a serum-free medium for 24 h. They were plated in migration or invasion chambers (20,000 cells/chamber – 8 µm pore size) (Corning Biocoat Matrigel Invasion Chamber Kit, Corning) with 10% FBS in the lower chamber, as a chemoattractant, and then incubated for 22 h. Cells were removed from the upper part with a cotton swab, and cells that have crossed the membrane were stained with the Diff-Quick Stain Kit (Polysciences Hirschberg, Germany) and 5 fields were counted on the ZOE cell imager.

Metabolic profiling

Oxygen-consumption rate (OCR) was measured using the Seahorse XF96 plate reader (Agilent Technologies). As mentioned above, the cells were routinely cultured in RPMI 1640. This medium contains 11.11 mM D-glucose. For respirometry experiments, cells (5000 cells/well in 96-well plates) were transfected as described above and then incubated

(24h post-transfection) in a substrate-free DMEM medium (DMEM D5030, Sigma) for 24 h before OCR measurements. Metabolic substrates were given to the cells at 10 mM per well 1 h before measurements: glucose, lactate, pyruvate, or glucose + glutamine. Where indicated, cells were treated with 10 µM BPTES (Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide) to inhibit glutaminase activity. Substrate-induced OCR was evaluated by calculating the difference of OCR values before and after substrate addition in each experimental condition.

Statistical analyses

Results are expressed as mean ± SD with statistical parametric t-tests for *in vitro* experiments, and as median ± quartiles with statistical non-parametric Mann-Whitney tests for *in vivo* experiments, of at least three independent experiments, unless otherwise noted. All statistical analyses were performed with GraphPad Prism version 6.01. Sample sizes (n) are reported in the corresponding figure legends.

RESULTS

MS/MS analysis reveals numerous metabolism-related proteins with deregulated expression.

Five BRAF mutated PTC and their normal adjacent tissue were analyzed by mass spectrometry. The clinical data, mutational status and patient information are given in Supplementary Table 1. The analysis identified about 1100 proteins in paired tumor and normal tissues. Each protein was quantified by calculating the expression ratio in log2 (tumor/normal) and was selected if this ratio was ≥ |1|. A total of 85 proteins showed deregulated expression in the 5 PTCs (Supplementary Table 2), SH3BP4 being the only one downregulated in all of them. 84 proteins were upregulated, among which several proteins involved in cellular energy metabolism. We identified proteins involved in the TCA cycle: SUGLG2, PDHA1, CS, PC; in fatty acid β-oxidation: ACADVL, HADH2, DECR1, DCI, PCCA, ECH1; in amino acids degradation: AK2, LONP1, IVD, HIBADH, HIBCH; in ATP production and oxidative stress protection: ATP5B, ATP5L, PRDX3, SOD2; and mitochondrial membrane proteins: CH3CH3, VDAC1, VDAC2.

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The discovery of the upregulation of PC in papillary thyroid carcinomas, implicated in the oxidative mitochondrial metabolism through its anaplerotic role in replenishing the TCA cycle, led us to explore the expression of other metabolic proteins in PTC (Figure 1). Most of them showed increased protein levels. This was particularly striking for the TCA cycle enzymes. PTC-c appeared to be the most active in terms of metabolism. Indeed, all the investigated proteins were overexpressed at least two times in this sample. PGK1 was downregulated but this was not verified in the 4 other PTC. PTC-a appeared as the less metabolically altered tumor with the smaller number of deregulated proteins, especially for the glycolysis pathway. PTC-e was mainly altered in TCA cycle enzymes and in the "others" metabolic protein category, while glycolysis enzymes showed only a few deregulations. Although the low number of samples does not allow to have sufficient statistical power, our data suggest there might be a correlation between the metabolic profiles and the TNM staging for each one of the PTC examined. For example, PTC-c, which is the most metabolically deregulated tumor, presented a TNM stage of pT4aN1a, while PTC-a was classified as pT1bN0 (Supplementary Table 1). Of course, this trend should be confirmed with a higher number of samples.

As a complementary approach to evaluate the expression pattern of metabolic proteins in PTC, we performed Affymetrix gene expression analyses on the same 5 PTC analyzed by mass spectrometry and on 3 additional PTC. A heatmap of the mRNA levels for different metabolic proteins following Affymetrix microarray analysis is presented in Figure 2. There was no correlation between proteomic and transcriptomic expression levels: an overall increase in mRNA expression of genes encoding proteins involved in glycolysis and inversely an overall decrease in mRNA expression of genes encoding proteins of the TCA cycle were noticed, with some exceptions. For instance, PC mRNA was strongly overexpressed in the tumors and was even the most overexpressed mRNA among all the investigated genes. The mRNA of the enzymes from the "others" metabolic pathway category showed variable deregulated expression patterns depending on the gene considered. This lack of correlation has already been reported and reflects different levels of regulation during protein synthesis, e.g. post-transcriptional, translational, or post-translational regulation (28).

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Pyruvate carboxylase expression is increased in PTC.

Among the proteins identified by proteomic analysis, PC was consistently upregulated in all the PTC investigated (Figure 1). Similarly, an increase at the mRNA level was found in the same samples and in three additional PTC (Figure 2), as well as in 49 independent PTC analyzed previously, with a mean ratio in log2 of 1.7 (data not shown) (29). The upregulation of PC mRNA expression has been validated by qRT-PCR in 12 independent PTC: 9 were compared to their normal adjacent tissues (Figure 3a) and 3, for which no adjacent tissue was available, were compared to a pool of 22 normal thyroid tissues (Figure 3b). PTC9 has been compared both to its normal adjacent tissue and to the pool of normal thyroids, which explains the difference between PC mRNA relative expression. The mean of expression (log2 of expression ratios between tumor and normal tissues) was 1.75, and 9/12 PTC showed a more than 2 fold upregulation. Increased protein expression of PC has been validated by Western blotting (Figure 3c) on 4 samples already tested for mRNA expression (PTC1-4) and on 11 additional samples. Altogether, these data provided good evidence that PC is upregulated in PTC and this led us to further explore its function in thyroid cancer cells.

Pyruvate carboxylase knockdown in thyroid cell lines alters their proliferative and motility capacities.

Three thyroid cell lines (TPC1, 8505C, HTori-3) were used to investigate the functional role of PC, following siRNA transfection. The PC mRNA expression level was significantly decreased 48 hours following siPC transfection in TPC1 cells (Figure 4a), and the downregulation of the protein was validated by Western blotting (Figure 4b) in the three cell lines, 72 hours after transfection. PC protein levels were measured in cells transfected with siPC, with siCTRL, and in non-transfected cells. No difference was observed between non-transfected cells and cells transfected with siCTRL, whereas PC expression was clearly downregulated following siPC transfection.

PC knockdown was accompanied by changes in the proliferative capacity of TPC1 and 8505C cells (Figure 4c). After 72 hours of transfection, there was no change in the proliferation rate when the cells were transfected with the siCTRL compared with non-transfected cells, while siPC transfection markedly reduced the percentage of EdU positive

cells for TPC1 and less importantly for 8505C. There was no statistical difference in the percentage of EdU labeled cells for HTori-3 cells.

We next investigated the role of PC on the migration and invasion properties of the three cell lines. These were evaluated by using migration or invasion chambers and by counting the cells which had crossed the specific membranes. SiPC transfection altered the migration and/or the invasion abilities of the cells: the number of migrating cells was reduced by one half for the 3 cell lines after transfection of siPC (Figure 4d), and the number of invading cells decreased about 4 times for TPC1 cells, and about 2 times for 8505C cells. No effect on invasion was observed in HTori-3 cells (Figure 4e).

Pyruvate carboxylase is involved in the replenishment of the TCA cycle in thyroid cell lines.

The three cell lines were then used to investigate the role of PC in energy cancer metabolism (Figure 5). We used the Seahorse Technology to measure O_2 consumption rates (OCR), and different substrates were given to the cells to evaluate their contribution to oxidative metabolism: glucose, lactate, pyruvate, glucose + glutamine, or glucose + glutamine +/- BPTES, a glutaminase inhibitor. O_2 consumption was higher when siCTRL transfected cells received lactate or pyruvate than glucose, and was even more important with glucose + glutamine, suggesting that glutamine is a central nutrient for anaplerosis in the 3 cell lines. The OCR was markedly reduced following siPC transfection regardless of the substrate, suggesting that PC participates in the replenishment of the TCA cycle. Despite the lower level of PC expression in HTori-3 cell line than in the 2 other cell lines (Figure 4b), we also observed a decrease of OCR in these cells. This was less pronounced when the cells were fed with glucose + glutamine, suggesting that glutamine alone is sufficient. To understand how the cells are taking advantage of anaplerotic resources, we treated them with BPTES. This treatment did not affect O_2 consumption in siCTRL transfected cells, but strongly decreased it in PC knockdown cells. This suggests that PC and GLS are codependent, i.e. if one of the 2 major anaplerosis pathways is affected, the other takes over.

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Thyroid cancer cells have an increased oxidative metabolism compared to stromal cells

The overexpression of PC in PTC and its participation in the replenishment of the TCA cycle, coupled with the increased expression of many other metabolic proteins, suggests an increased oxidative metabolism in this tumor type. However, PTC are heterogeneous tumors containing stromal cells, mainly CAFs, and we have previously shed light on the important role of this stroma in tumor expansion (21). Within this context, since our "omic" studies were performed with bulk tissues, we decided to investigate the cellular distribution of several upregulated metabolic proteins in the cancer and in the stromal cells of PTC, and more specifically their expression in CAFs. We performed immunostainings of PC, PCK2, PDHα1, GLS, ECH1, PCCB, LDHA/B, as well as of additional proteins absent in our MS/MS data but known to play important roles in metabolism: PDK, MCT1/4, GLUT1, HK2 (Figure 6).

PC overexpression was specific to cancer thyrocytes (Figure 6a), as were two other overexpressed enzymes linked to the metabolism of oxaloacetate: phosphoenolpyruvate carboxykinase 2 (PCK2) (Figure 6a) and pyruvate dehydrogenase (PDH) (Figure 6c), the latter being responsible for pyruvate decarboxylation into acetyl-CoA, an allosteric activator of PC. These data suggest that anaplerosis occurs in PTC with PC mediating oxaloacetate entrance into the TCA cycle. Interestingly, pyruvate dehydrogenase kinase (PDK), which inhibits PDH and is described in many cancers as overexpressed, was not deregulated in PTC (Figure 6c). Protein levels for PDHα1 and PDK were also analyzed by Western blotting: PDHα1 was strongly upregulated in 2/8 samples and weakly upregulated in 4/8 samples, while PDK levels remained constant in most of the samples (data not shown). In addition, GLS was also specifically increased in tumor cells, suggesting activity of the major alternate anaplerotic reaction (Figure 6a). Two enzymes implicated in β-oxidation of lipids leading to acetyl-CoA production were analyzed: 3-5, 2-4, dienoyl-CoA isomerase (ECH1) and propionyl-CoA carboxylase B subunit (PCCB). Both were also specifically upregulated in the cancer cells, suggesting an increased production of acetyl-CoA in these cells (Figure 6a).

LDH showed increased expression in cancer cells and in CAFs, and this was observed for LDHA and LDHB (Figure 6b). Since LDH is a tetramer composed of two different subunits (LDH-M and LDH-H, respectively encoded by the *LDHA* and *LDHB* genes) present in variable

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combinations, these results could be explained by the existence of mixed isoforms of LDH, all recognized by the specific anti-LDHA and anti-LDHB antibodies. The monocarboxylate transporter MCT1, mediating the uptake of lactate, was only overexpressed in the cancer cells while MCT4, triggering the export of lactate, was found in tumor cells and was also weakly expressed in CAFs in some areas (Figure 6b). Finally, we analyzed the expression of HK2 and of the glucose transporter GLUT1. As depicted in Figure 6b, both enzymes were specifically overexpressed in the thyroid cancer cells. HK2 showed a heterogeneous expression across the entire tumor with some tumor cells expressing higher levels of this enzyme.

DISCUSSION

Scientific domains in cancer research are clear and easily understandable as long as they are kept distinct. However, as soon as concepts and findings in one domain are extended to another, difficulties and discrepancies appear. Whereas the cause and initial genetic mechanism of a particular tumor may be well defined, its phenotype and even genotype evolve in space and time, resulting in spatial and temporal heterogeneity. Experimentally, studies on whole tissues (e.g. omics studies) ignore the morphological and metabolic heterogeneity of the tumor. On the other hand, focused experimental studies *in vitro* (e.g. cancer cells, cell lines) and *in vivo* ignore tissue complexity (30).

With regard to cancer metabolism, much has been learned about the versatility of *in vitro* models depending on the culturing conditions, for instance the existence of both the Warburg and the reverse Warburg effects, the alternative use of substrates depending on their availability, the roles of lactate and H^+ as metabolites and signals (31). On the other hand, the description of cancers *in vivo* is based mostly on observations at one point in time (“snapshots”). It is therefore interesting to investigate which situation exists *in vivo* in human cancers. For this, global measurements (genetics, mRNA and protein expression, etc.) should be complemented by methods allowing to define spatial and time distributions of properties. In this study, we have tried to integrate some fundamental experimental concepts on tumor energy metabolism, in order to define the spatial characteristics of metabolism, the Warburg effect, and anaplerosis of the well-defined,

most frequent, human thyroid cancer, PTC. This could provide a few conceptual links between experimental and physiopathological concepts.

For decades, the hypothesis of Otto Warburg orientated cancer research to better understand how tumors work, and to find new treatments. According to the concept of the Warburg effect, tumors have an increased glycolysis and a drastically reduced oxidative mitochondrial activity, which led to the development of therapies specifically targeting the glycolytic pathway (32, 33). However, several studies demonstrated that the TCA cycle is still functional in some tumors and produces catabolic precursors for their proliferative needs (6). In this study, we show that PTC have a very active TCA cycle, which contrasts with the Warburg model, and this activity is not mutually exclusive with glycolysis, since both metabolic pathways are active.

As energy metabolism in PTC is poorly characterized, we decided to investigate the metabolic alterations in these tumors. A proteomic analysis of 5 PTC revealed upregulation of a high number of proteins involved in metabolism, which were further characterized. Although the number of samples analyzed is too low to draw definite conclusions, these deregulations seem to be amplified according to tumor stage, suggesting a correlation between increased energy metabolism and tumor aggressiveness. Accordingly, Nahm et al. showed that the expression of glycolysis-related proteins correlates with poorer prognosis (17). PC appeared to be overexpressed at both the mRNA and protein level, a new finding in the field. This enzyme that allows pyruvate to be carboxylated into oxaloacetate, funneled in the TCA cycle, was only recently described as deregulated in lung and breast cancers (34, 35). This suggests that pyruvate is not systematically transformed into lactate according to the Warburg effect, but could be an essential resource to maintain the oxidative mitochondrial metabolism. We first defined the functional role of PC in thyroid cell lines. Two commonly used thyroid cancer cell lines, TPC1 and 8505C (23, 25), and HTori-3 cells, non-tumorigenic SV40-immortalized human thyrocytes (24) were used for this purpose. Although TPC1 and 8505C cells respectively derive from a PTC and an ATC, these cell lines have evolved into a common, dedifferentiated phenotype (23, 25). Thus, TPC1 and 8505C cells were not chosen as a relevant model for the corresponding *in vivo* tumor, but rather as experimental *in vitro* models for thyroid cancer. Both cell types showed decreased proliferation, migration, invasion and mitochondrial respiration rates

following PC knockdown, suggesting a role of PC in generating energy and metabolic precursors through the pyruvate conversion into oxaloacetate and its consumption by the TCA cycle. No changes were observed in the proliferation and invasion rates in HTori-3 cells following PC knockdown, but their migration rate and their oxygen consumption were reduced. The non-tumorigenic character and the non-cancer origin of HTori-3 cells might explain these differences.

Cellular heterogeneity is an emerging concept in interpreting cancer biology, and PTC are indeed heterogeneous tumors, containing thyroid cells, stromal cells such as cancer-associated fibroblasts, lymphocytes and endothelial cells. To further explore the increased oxidative mitochondrial metabolism in PTC, and to analyze the contribution of the different cell types, we investigated the cellular distribution of several metabolic proteins in the cancer and in the stromal cells by IHC. PC, PCK2 and PDHα1, three enzymes related to the metabolism of oxaloacetate, were specifically overexpressed in cancerous thyrocytes, whereas the expression of PDK, an inhibitor of PDH, remained unchanged. PC and PDH, by producing respectively oxaloacetate and acetyl-CoA, itself an allosteric activator of PC, contribute to replenish the TCA cycle. PCK2, the mitochondrial isoform of PCK, although well-known for its role in gluconeogenesis, underlines the importance of the TCA cycle in absence of glucose (36). The overexpression of PDH, observed in 75% of the samples, and the absence of deregulation of PDK contrast with the findings described in other cancers, such as non-small cell lung carcinomas, where the repression of the PDH/PDK pathway has been associated with aerobic glycolysis (Warburg effect) (37). Taken together, our data support the presence of an actively functioning TCA cycle in PTC, involving pyruvate metabolism. In addition, they also suggest that acetyl-CoA can be produced from lipid degradation since several enzymes involved in this pathway showed an increased expression, as measured by MS/MS. Among them, the specific overexpression in cancer cells of ECH1 and PCCB was confirmed by IHC. This is in agreement with other studies reporting a role of lipid degradation in cancers, such as in triple negative breast cancer, following stress conditions (38, 39).

The other important anaplerotic reaction able to replenish the TCA cycle, i.e. glutaminolysis, also appears to sustain the energy metabolism in PTC. Indeed, an

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overexpression of GLS was observed in the cancerous cells of the PTC and glutamine was identified as an essential nutrient in thyroid cell lines tested for oxidative respiration. Inhibiting glutaminase by BPTES highlights the existence of a compensatory relationship between GLS and PC, already described for glioblastoma cells in culture and in xenografts (12), in addition to the anaplerotic role of GLS.

All the enzymes involved in oxidative metabolism that were studied were specifically overexpressed in thyroid cancer cells. Cancer-associated fibroblasts have been described in other cancers such as breast and brain cancers to feed cancerous cells by producing lactate into the tumor microenvironment (10, 40). Our data show that the LDH enzymes are present in cancerous cells and in CAFs, but do not allow to definitively conclude on the metabolism of lactate. Regarding the lactate transporters, MCT1 (uptake of lactate) is overexpressed in the tumor cells, while MCT4 (export of lactate) is present in tumor cells but also weakly expressed in CAFs in some areas. This confirms other studies on thyroid cancer (22), and suggests that lactate is released from cancerous cells and from CAFs in some areas of the tissue, and is taken up by other cancerous cells, allowing production of ATP through mitochondrial oxidative phosphorylation. It supports the metabolic model of the reverse Warburg effect between cancerous cells and CAFs, as well as the existence of a metabolic symbiosis between cancer cells that are not equal with regard to oxygen availability (41). Finally, we investigated the origin of pyruvate, which could derive from glucose but also from lactate. Our proteomic data revealed an overall upregulation of the glycolytic enzymes in the 5 analyzed PTC. We performed IHC with GLUT1 and HK2 antibodies, and we observed that both enzymes were specifically overexpressed in tumor cells, confirming a previous report (17). The overexpression of GLUT1 is consistent with the observation that, in differentiated thyroid carcinomas, glucose uptake in $BRAF^{V600E}$ positive tumors was higher than in BRAF wild type tumors (16). In addition, the overall survival of patients with high GLUT1 expression is reduced (17). Altogether, our results suggest that, in PTC, glucose would be distributed between the TCA cycle and the production of lactate, as it was reported in KRAS-mutated non-small cell lung cancers (NSCLC) (20), in glioblastomas (42) and in bladder cancer (43).

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In conclusion, PC is a key enzyme in thyroid cancer cells, at the intersection between glycolysis and the TCA cycle, playing an important role in anaplerosis. The distribution of other overexpressed enzymes showed that the TCA cycle is very active in tumor cells. However, our results give only a snapshot of the Warburg effect, metabolism, and anaplerosis in PTC at the time of tissue collection. They do not consider the evolution of the described temporal heterogeneity. For this, further *in vivo* studies, e.g. a ^{13}C-tracer analysis with labeled glucose and lactate, could provide information about carbon trafficking inside the tumors (44). Hypoxia should also be investigated in PTC to identify areas with distinct metabolic adaptations. Exploring the expression pattern of the different metabolic proteins comparing central and peripheral tumor areas might also bring interesting information regarding tumor heterogeneity. Such knowledge could give a basis for the rational use of new therapeutic tools targeting tumor metabolism (14, 33). However, this concept implies that all the cells exhibit the same pattern at the same time, which in the case of metabolism, is not true (30, 45). Moreover, cancer cells are able to adapt to changing conditions by shifting from one metabolic pattern to another.

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CM, JD and ASt conceived the experiments; ASt and CC carried out the experiments; CM, ASt interpreted the results and wrote the manuscript; GD, CC, OF helped with data analysis; GA collected patient samples; ASp, LC and DL collected and reviewed the histopathological slices of the thyroid cancers; RW performed the MS/MS analysis.

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AUTHOR DISCLOSURE STATEMENT

Protocols have been approved and consent by the Ethics Committee of the J. Bordet Institute (protocol number: 1978), in accordance with the Declaration of Helsinki.

No competing financial interests exist.

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FIGURE LEGENDS

	Protein	Name	DB number	PTC-a	PTC-b	PTC-c	PTC-d	PTC-e
GLYCOLYSIS	HK1	Hexokinase 1	E7ENR4	1,2	1,7	2,8	0,8	-0,1
	GPI	Glucose-6-phosphate isomerase	B4DG39	0,7	0,2	1,7	-0,7	1,3
	PFKP	Phosphofructokinase, platelet	Q5VSR7	-2,2	1,5	3,5	1,1	0,4
	ALDOA	Fructose-bisphosphate aldolase	B7Z3K9	0,7	1,1	1,0	0,8	0,6
	TPI	Triosephosphate isomerase	Q53HE2	0,9	1,6	2,4	1,8	0,8
	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	E7EUT4	1,1	1,6	1,7	1,3	1,2
	PGK1	Phosphoglycerate kinase 1	A8K4W6	0,5	1,7	-2,1	0,9	-0,3
	PGM	Phosphoglycerate mutase	Q53G35	-0,1	-0,8	1,0	0,5	3,3
	ENO2	Enolase 2	Q6FHV6	1,0	1,1	1,9	1,4	0,0
	PKM2	Pyruvate kinase	B4DUU6	1,3	1,6	1,6	1,3	-0,7
TCA CYCLE	PC	Pyruvate carboxylase	E9PS68	3,0	3,7	3,2	2,7	2,2
	PDHA1	Pyruvate dehydrogenase subunit A1	A5YVE9	3,7	3,0	1,8	1,1	1,6
	PDHB	Pyruvate dehydrogenase subunit B	B4DDD7	1,0	0,2	2,4	0,9	1,5
	DLAT	Dihydrolipoamide S-acetyltransferase	Q86YI5	0,8	2,8	6,1	1,4	1,3
	PDHX	Pyruvate dehydrogenase complex, component X	B2R673	0,3	-0,1	2,7	1,2	3,3
	PCK2	Pyruvate carboxykinase	Q6IB91	1,1	5,4	2,6	1,2	0,8
	CS	Citrate synthase	B4DJV2	2,0	2,4	2,3	2,0	1,2
	ACO2	Aconitase 2	A2A274	0,5	4,0	2,0	0,3	1,7
	IDH2	Isocitrate dehydrogenase 2	Q53GL5	1,3	2,4	1,7	0,2	2,5
	FH	Fumarate hydratase	B1ANK7	1,9	0,6	3,3	2,1	2,2
	SUCLG2	Succinate-CoA ligase subunit beta	C9JVT2	2,1	5,3	4,8	1,9	2,0
	SDHB	Succinate dehydrogenase complex subunit B	Q0QEY7	1,0	2,2	4,0	6,7	1,7
	MDH2	Malate dehydrogenase 2	Q6FHZ0	-1,9	-0,1	2,0	0,5	0,0
OTHERS	ME	Malic enzyme	B2R8J2	-0,1	2,0	4,0	4,9	2,8
	MDH1	Malate dehydrogenase 1	F5H098	0,8	0,7	1,1	0,2	1,0
	CTP	Mitochondrial citrate transport protein	Q6LAP8	1,0	3,1	2,3	2,1	4,0
	PCCA	Propionyl Coenzyme A carboxylase, alpha	B2RDE0	1,6	1,1	2,3	1,7	3,2
	PCCB	Propionyl Coenzyme A carboxylase, bêta	E7EX59	0,0	3,5	4,3	-0,9	3,2
	ECH1	3,5-delta 2,4-dienoyl-CoA isomerase, mitochondrial	B4DVS4	5,4	2,9	1,8	1,1	5,2
	LDHA	L-lactate dehydrogenase A	B7Z5E3	1,4	1,4	1,7	0,6	1,2
	LDHB	L-lactate dehydrogenase B	Q5U077	1,0	-0,2	1,3	0,4	1,2
	GLS	Glutaminase	A8K132	-1,9	1,9	5,8	3,1	4,3

Figure 1: **Protein expression levels of metabolic enzymes or proteins measured by MS/MS analysis, and presented in log2 of expression ratios (tumor/normal)**. DB number = accession number in Uniprot database. Cutoff of the expression ratios in log2 is 1; (red: expression level ≥ 1; green: expression level ≤ -1).

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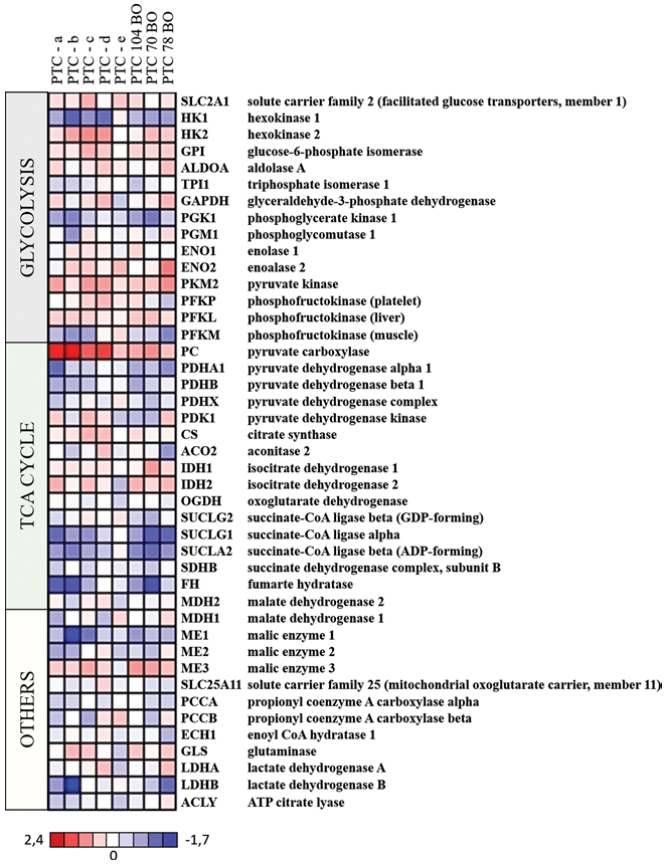

Figure 2: Heatmap of mRNA expression levels of metabolic proteins. Expression levels were obtained by Affymetrix microarrays and are presented in log2 of expression ratios (tumor/normal). Genes were grouped by metabolic pathways: glycolysis, TCA cycle et "others". PTCa-e were the 5 PTC analyzed by MS/MS spectrometry.

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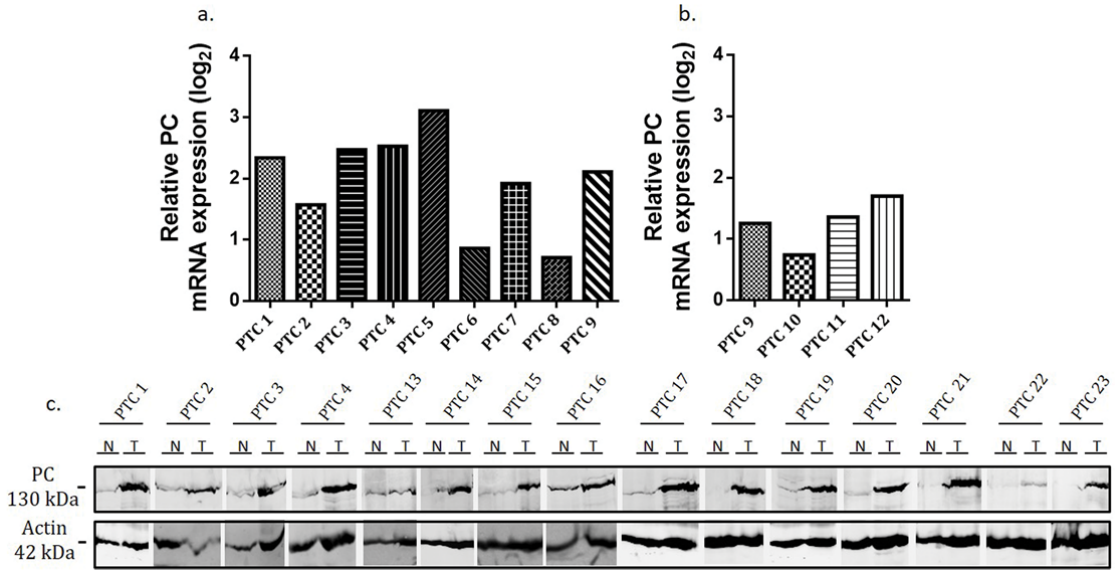

Figure 3: Pyruvate carboxylase expression in PTC. (a,b) : Upregulation of PC mRNA was measured by qRT-PCR, and the results are presented as log2 of expression ratios (a: tumor versus normal adjacent tissue; b: tumor versus a pool of 22 normal tissues). The expressions were normalized with the housekeeping genes NEDD8 et TTC1. (c) The upregulation of PC was validated at the protein level by Western blotting (N: normal; T: tumor).

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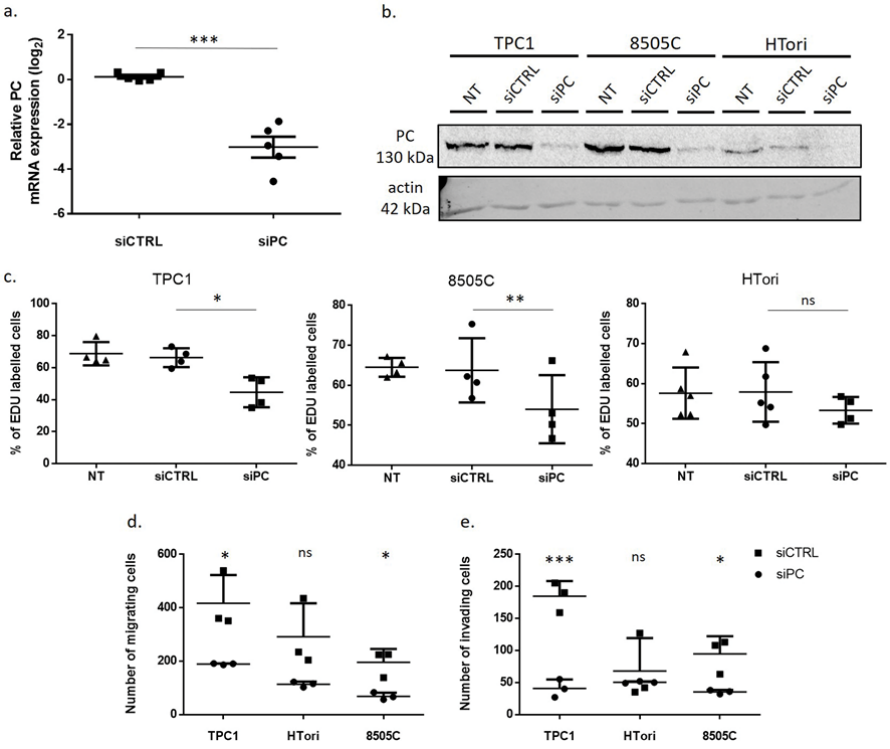

<u>Figure 4</u>: PC knockdown following siRNA transfection and functional effects in thyroid cell lines. Transfection of siRNA against PC (siPC) was performed in 3 thyroid cell lines (TPC1, 8505C et HTori). Cell lines were also transfected with a negative control siRNA (siCTRL) or not transfected (NT). PC expression was measured (a) at the mRNA level by qRT-PCR after 48 hours in TPC1 cells and (b) at the protein level by Western blotting after 72 hours in the three cell lines (n=5). The mRNA expressions are represented with the mean ± SD, and the t-test showed a significant difference between transfected conditions (siCTRL vs siPC); ***: p-value < 0.001. (c) Cell lines were labelled with EdU 72 after siRNA transfection and the percentage of EdU positive cells among 10 000 cells was analyzed by flow cytometry (N=4). Results are represented with mean ± SD, and a student paired t-test showed a significant difference between transfected conditions (siCTRL vs siPC); *: p-value = 0,0247; **: p-value = 0,017; ns: not significant. (d)(e) Cells were seeded in migration or invasion chambers. FBS was used as chemoattractant. The cells were counted after (d) migration through the porous membrane, (e) invasion through the matrigel membrane (N=3). Results are represented with mean ± SD, and a t-test showed a significant difference between transfection conditions (siCTRL vs siPC) for TPC1 and 8505C cells; *: p-value < 0.05; **: p-value < 0.001; ns: not significant.

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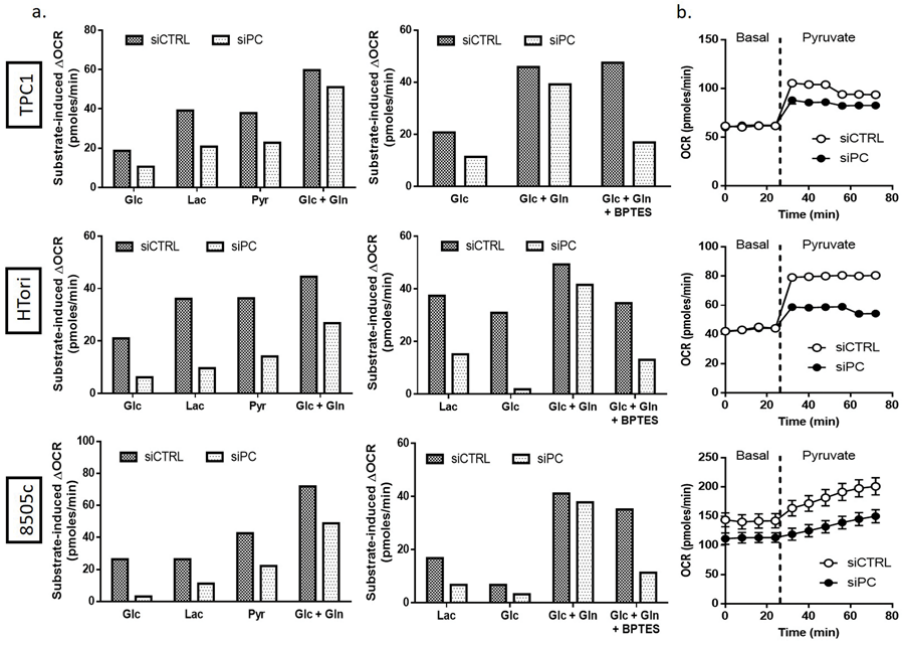

<u>Figure 5</u>: Oxygen consumption rates measurements following PC knockdown. TPC1, HTori and 8505C cells were transfected with siPC or siCTRL 72h before OCR measurements, which were taken before and after substrate addition. Substrates: Glc: glucose, Lac: lactate, Pyr: pyruvate, Gln: glutamine. BPTES: glutaminase inhibitor. (a) ΔOCR data for one representative experiment of 2 independent ones (2 biological replicates) and 6 measurements by experiment (6 technical replicates). (b) OCR over time data for one representative experiment with 6 measurements (6 technical replicates) for each time point before and after the addition of pyruvate.

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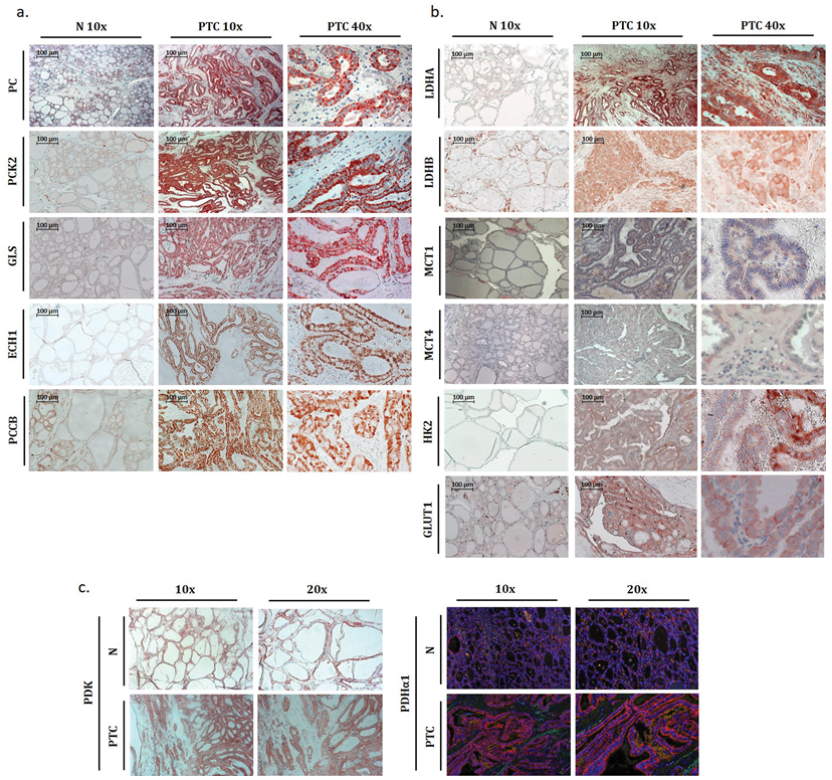

Figure 6: Expression of different metabolic enzymes and transporters in PTC and their normal adjacent tissues (N). (a)(b) Immunostainings were performed on paraffin sections with antibodies against the following proteins: PC, PCK2, GLS, ECH1, PCCB, LDHA, LDHB, MCT1, MCT4, HK2, GLUT1, PDHα1, and PDK (N=4). A negative control (no primary antibody) has been performed to confirm the specificity of each antibody. (c) Expression levels of PDHα1 and PDK were measured by Western blotting. N: normal, T: tumor.

Table S1: Patient information, clinical data, and presence of BRAF-mutation for the 5 PTC analyzed by MS/MS. W: woman, M: man, TNM: tumor lymph nodes metastasis, mut: mutation

Table S2: Identified proteins by MS/MS with a more than 2 fold deregulated expression in the 5 PTC compared to normal tissues. Green: log2 ratios of underexpressed proteins in tumors; Red: log2 ratios of overexpressed proteins in tumors.

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SUPPLEMENTARY DATA

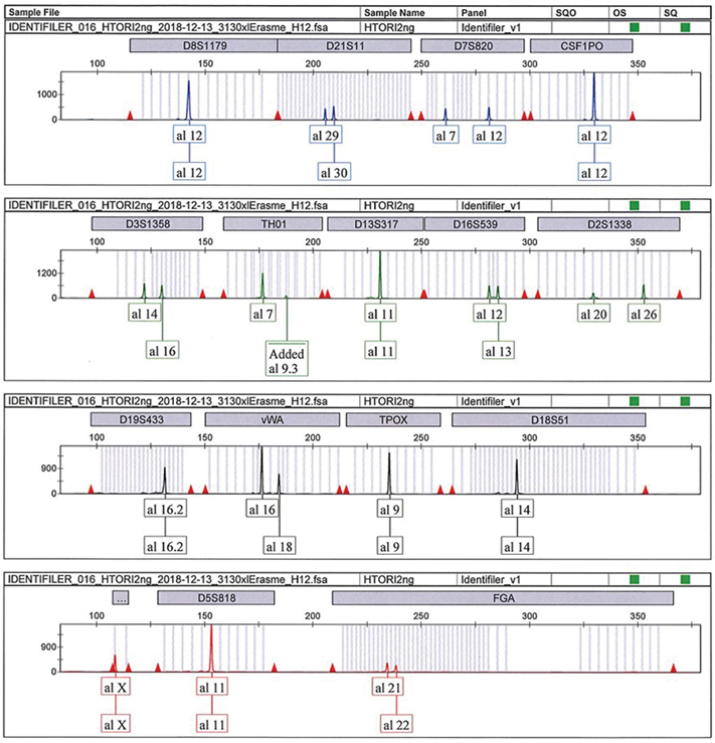

Figure S: DNA profiling by STR analysis of the HTori-3 cell line.