Molecular characteristics of papillary thyroid carcinomas without \textit{BRAF} mutation or \textit{RET/PTC} rearrangement: relationship with clinico-pathological features

\textit{Stéphanie Durand}\textsuperscript{1,2}, \textit{Carole Ferraro-Peyret}\textsuperscript{1,3}, \textit{Mireille Jouflé}\textsuperscript{3}, \textit{Annie Chave}\textsuperscript{3}, \textit{Françoise Borson-Chazot}\textsuperscript{1,2}, \textit{Samia Selmi-Ruby}\textsuperscript{1,2} and \textit{Bernard Rousset}\textsuperscript{1,2,3}

\textsuperscript{1}Institut National de la Santé et de la Recherche Médicale, UMR 664, Lyon F-69372, France
\textsuperscript{2}Université Lyon 1, Faculté de Médecine Laennec, Lyon F-69372, France
\textsuperscript{3}Unité Fonctionnelle de Biologie Cellulaire, Hospices Civils de Lyon, Hôpital Edouard-Herriot, Lyon F-69437, France

(Correspondence should be addressed to B Rousset; Email: rousset@sante.univ-lyon1.fr)

\textbf{Abstract}

About 60–70\% of papillary thyroid carcinomas (PTC) present a \textit{BRAF}\textsuperscript{T1799A} gene mutation or a rearrangement of \textit{RET} gene (\textit{RET/PTC}). In this study, we examined whether PTC without \textit{BRAF}\textsuperscript{T1799A} mutation and without \textit{RET/PTC} rearrangement named PTC-ga(\textit{K}) were distinguishable from PTC-ga(\textit{C}) (with one or the other gene alteration) on the basis of gene expression characteristics. We analyzed the mutational state of 116 PTC and we compared gene expression profiles of PTC-ga(\textit{C}) and PTC-ga(\textit{K}) from data of a 200 gene macroarray and quantitative PCR. Seventy five PTC were PTC-ga(\textit{C}) and 41 were PTC-ga(\textit{K}). Unsupervised analyses of macroarray data by hierarchical clustering led to a complete segregation of PTC-ga(\textit{C}) and PTC-ga(\textit{K}). In a series of 42 genes previously recognized as PTC 'marker' genes, 22 were found to be expressed at a comparable level in PTC-ga(\textit{K}) and normal tissue. Thyroid-specific genes, \textit{TPO}, \textit{TG}, \textit{DIO1}, and \textit{DIO2} were under-expressed in PTC-ga(\textit{C}) but expressed at a normal level in PTC-ga(\textit{K}). A few genes including \textit{DUOX1} and \textit{DUOX2} were selectively dys-regulated in PTC-ga(\textit{K}) but expressed at a normal level in PTC-ga(\textit{C}). A few genes including \textit{DUOX1} and \textit{DUOX2} were selectively dys-regulated in PTC-ga(\textit{C}). Tumor grade of PTC-ga(\textit{K}) was lower than that of PTC-ga(\textit{C}). There was a strong association between the mutational state and histiotype of PTC; 81\% of PTC follicular variants were corresponded to PTC-ga(\textit{K}), whereas 84\% of PTC of classical form were PTC-ga(\textit{C}). In conclusion, we show that PTC without \textit{BRAF}\textsuperscript{T1799A} mutation or \textit{RET/PTC} rearrangement, mainly corresponding to follicular variants, maintain a thyroid differentiation expression level close to that of normal tissue and should be of better prognosis than PTC with one or the other gene alteration.

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\textbf{Introduction}

Papillary thyroid carcinomas (PTC), the most common forms of thyroid cancer, are characterized by two main gene alterations, either a rearrangement of \textit{RET} gene or a point mutation of \textit{BRAF} gene. As the result of a somatic chromosomal event, \textit{RET} gene (not expressed in thyroid epithelial cells) undergoes a rearrangement which leads to the fusion of its 3\textsuperscript{\textprime}part encoding the tyrosine kinase domain with the 5\textsuperscript{\textprime}part of different genes. The expression level of the resulting chimeric oncoproteins named RET/PTC depends on the newly acquired promoter (Fusco et al. 1987, Jhiang 2000). RET/PTC1 resulting from fusion with \textit{H4} (\textit{CCDC6}) gene (Grieco et al. 1990) and RET/PTC3 resulting from fusion with \textit{ELE1} gene (Santoro et al. 1994) are the most frequent rearranged forms. A RET/PTC gene rearrangement is found in 13–43\% of PTC (Kondo et al. 2006); this highly variable prevalence is related to several parameters including the detection method, geographical location of patients, and radiation exposure (Santoro et al. 1992, Lam et al. 1998). RET/PTC oncopgenic proteins primarily activate the...
MAPK pathway (Melillo et al. 2005, Mitsutake et al. 2005). A similar rearrangement involving NTRK1 gene (encoding another tyrosine kinase receptor) and different fusion partners leads to the expression of chimeric proteins with constitutive activity on different signaling cascades including the MAPK cascade. This genetic alteration has a low incidence in PTC. These different fusion partners leads to the expression of different levels of matrix-metalloproteases and a higher cell motility have been found in BRAFV600E-expressing cells as compared with RET/PTC3-expressing PCC13 cells (Mesa et al. 2006).

In vitro studies have shown that expression of RET/PTC (De Vita et al. 1998, Knauf et al. 2003) or BRAFV600E (Mitsutake et al. 2005, Liu et al. 2007) in PCC13 rat thyroid cells leads to a down-regulation of expression of thyroid-specific genes. A higher level of expression of matrix-metalloproteases and a higher cell motility have been found in BRAFV600E-expressing cells as compared with RET/PTC3-expressing PCC13 cells (Mesa et al. 2006).

PTC with the BRAFT1799A mutation exhibit a more advanced clinical stage (Namba et al. 2003, Nikiforova et al. 2003, Adeniran et al. 2006) and correspond to PTC at high risk of recurrence (Xing et al. 2005, Lupi et al. 2007). The invasive phenotype of PTC with the BRAFT1799A mutation is probably due to secondary genetic events linked to an increase in genome instability (Mitsutake et al. 2005). By contrast, carcinomas with a RET/PTC gene rearrangement rarely correspond to aggressive or undifferentiated carcinomas (Tallini et al. 1998, Adeniran et al. 2006).

Series of genes differentially expressed in PTC as compared with normal thyroid tissue and/or to other types of thyroid tumors have been proposed as ‘PTC markers’ i.e. genes potentially useful to develop diagnostic tools (Wasenius et al. 2003, Aldred et al. 2004, Finley et al. 2004, Mazzanti et al. 2004, Jarzab et al. 2005, Lubitz et al. 2006, Finn et al. 2007). By re-analyzing our macroarray gene expression data (Durand et al. 2008), we observed that the mutational state of PTC i.e. the presence or the absence of the BRAFT1799A mutation or the presence or the absence of a RET/PTC gene rearrangement was a parameter of sub-classification of these tumors by hierarchical clustering. This finding prompted us to perform a more detailed investigation of the similarities and differences in gene expression profiles of PTC without the BRAFT1799A mutation and without a RET/PTC rearrangement, named PTC-ga(−) and PTC with one or the other gene alteration, named PTC-ga(+) . Using both macroarray and quantitative PCR approaches and a rather large series of PTC (n=116), we have found that PTC-ga(−) and PTC-ga(+) exhibit marked differences in the level of expression of a number of genes previously designated as ‘PTC marker’ genes. In the present study, we document a relationship between the level of expression of thyroid differentiation-related genes and the mutational state of PTC.

Materials and methods

Human thyroid tissues and RNA preparation

Thyroid tissue samples were taken from the Lyon Thyroid Tumor Bank, previously described (Porra et al. 2005), which is a part of the Biological Resources Center (BRC) of the Lyon University Hospital. The rules of tissue collection by the BRC include the informed consent of patients. Specimens maintained in the bank consisted of fragments of thyroid tumor and normal thyroid tissue collected at the time of extemporaneous examination of surgical pieces from patients undergoing partial or total thyroidectomy. Tissue samples weighing 50–200 mg were frozen in liquid nitrogen and stored at −80 °C. Tumors were classified according to World Health Organization recommendations. This study, based on 116 PTC and 46 samples of normal thyroid tissue, was approved by the supervision interdisciplinary committee of the tumor bank and performed in accordance with protocols previously approved by the local human studies committee. Information about patients and tumors are provided in Table 1.

Thyroid tissue samples were used for transcript analyses by macroarray and/or real-time PCR. Total RNA isolated from tissue samples using the phenol-chloroform extraction procedure (Chomczynski & Sacchi 1987) was subsequently purified on silica column (provided by the RNeasy Minikit from Qiagen) followed by treatment according to the manufacturer’s protocol, to eliminate potential genomic DNA contamination. RNA integrity was controlled by microfluidic electrophoretic separation using the BioAnalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA).
None of the samples used in this study had a value of the 28S/18S rRNA ratio lower to 1.5.

**Preparation of cDNAs**

For macroarray analyses, cDNA probes were synthesized from 2 μg antisense RNA (prepared using the MessageAmp aRNA kit from Ambion, Austin, TX, USA; Van Gelder et al. 1990) by simultaneous reverse transcription and \( [\alpha-\text{33P}] \) deoxy-CTP labeling as previously described (Durand et al. 2008). For real-time PCR analyses, total RNA (1 μg) was retrotranscribed with the Moloney murine leukemia virus reverse transcriptase (Promega Corp) according to the manufacturer’s protocol.

**Detection of \( \text{BRAF}^{1799A} \) gene mutation**

The thymine to adenine transversion at nucleotide 1799 of the \( \text{BRAF} \) gene was detected by a real-time, allele-specific PCR method (Jarry et al. 2004) adapted to cDNA as previously described (Porra et al. 2005).

**Assessment of \( \text{RET}/\text{PTC} \) gene rearrangement**

\( \text{RET}/\text{PTC} \) gene rearrangement was detected from measurements of the tumor content in transcripts corresponding to the tyrosine kinase domain of \( \text{RET} \). Transcripts were assayed by quantitative PCR using the following primers: \( 5'\text{-GATCTCACAGGGGATG-CAGT-3'} \) and \( 5'\text{-CTGGCTTCACAGTGG-3'} \). The annealing temperature was 58 °C. When the tumor transcript content was higher than that of the paired normal tissue and/or higher than the average transcript content of a control group of normal thyroid tissue samples, it was concluded that the tumor contained the gene rearrangement. The specificity of the amplification was checked by fractionation of PCR end products by electrophoresis on 2% agarose gel and staining with ethidium bromide.

**Oligonucleotide macroarray**

An oligonucleotide-based macroarray of 200 genes was generated on nylon membrane (Durand et al. 2008). Genes building up the macroarray corresponded to genes previously reported to be differentially expressed in tumors and normal tissue or in benign and malignant thyroid tumors (list available at http://ifr62.univ-lyon1.fr/users/b_rousset/decanthyr2007/Table 2). Hybridization of \( [\text{33P}] \)-labeled cDNA onto the macroarray, image analyses and quantification of hybridization signals were performed as described (Durand et al. 2008).

**Statistical analyses of macroarray data**

Macroarray data (after normalization and log2 transformation) were analyzed by significance analysis of microarrays (SAM) software (http://www-stat.stanford.edu/~tibs/SAM) (Tusher et al. 2001). The criteria for SAM analyses were: i) a minimum twofold difference between two groups of samples, ii) a false discovery rate threshold of 5%, and iii) a \( q \) value lower than 0.05. Classification of samples according to gene expression data was performed by hierarchical clustering (http://rana.lbl.gov/EisenSoftware.htm) using Cluster software; results were displayed using TreeView software (Eisen et al. 1998).

**Quantification of transcripts by real-time PCR**

PCR was performed on a LightCycler (from Roche Diagnostics). Amplification of cDNAs was carried out in duplicate in a final volume of 10 μl containing the FastStart DNA Master SybrGreen (from Roche Diagnostics), 3–4 mM MgCl₂ (depending on the gene to amplify), 5 μM of forward and reverse primers and 2.5 ng of retro-transcribed RNA (except 18S rRNA which was amplified from 25 pg). Sequences of primers, positioning of primers on transcript sequence, size of amplicons, and temperature of hybridization are

### Table 1 Clinical and pathological characteristics of papillary thyroid carcinomas

<table>
<thead>
<tr>
<th>PTC</th>
<th>( N )</th>
<th>Age of patients (years)</th>
<th>Sex ratio</th>
<th>Tumor size (cm)</th>
<th>pT Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>All types</td>
<td>116</td>
<td>43.9 ± 1.6</td>
<td>88F/28M</td>
<td>2.7 ± 0.1</td>
<td>T1 50 T2 23 T3 25</td>
</tr>
<tr>
<td>( \text{BRAF}^{1799A} )</td>
<td>61</td>
<td>46.8 ± 2.0</td>
<td>49F/12M</td>
<td>2.5 ± 0.2</td>
<td>T1 20 T2 12 T3 18</td>
</tr>
<tr>
<td>( \text{RET}/\text{PTC} )</td>
<td>14</td>
<td>31.4 ± 3.8</td>
<td>11F/3M</td>
<td>2.3 ± 0.4</td>
<td>T1 5 T2 2</td>
</tr>
<tr>
<td>( \text{PTC-ga(+)} )</td>
<td>75 (*)</td>
<td>44.0 ± 1.9</td>
<td>60F/15M</td>
<td>2.5 ± 0.2</td>
<td>T1 25 T2 14 T3 21</td>
</tr>
<tr>
<td>( \text{PTC-ga(-)} )</td>
<td>41</td>
<td>43.8 ± 2.7</td>
<td>28F/13M</td>
<td>3.2 ± 0.2</td>
<td>T1 25 T2 9 T3 4</td>
</tr>
</tbody>
</table>

\( \text{PTC-ga(+)} \), PTC with the \( \text{BRAF}^{1799A} \) mutation or/and a \( \text{RET}/\text{PTC} \) gene rearrangement; \( \text{PTC-ga(-)} \), PTC without any of the two gene alterations. (*). Seven samples presented both the \( \text{BRAF}^{1799A} \) mutation and a \( \text{RET}/\text{PTC} \) rearrangement within the \( \text{PTC-ga(+)} \) group of tumors. F, Female; M, Male. Average values of the age of patients and the size of tumors are given with the S.E.M.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Gene ID</th>
<th>Biological process</th>
<th>References</th>
<th>ga(+) versus NT</th>
<th>ga(−) versus NT</th>
<th>ga(+) versus ga(−)</th>
<th>P value (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPINT1</td>
<td>Serine protease inhibitor, Kunitz type 1</td>
<td>6692</td>
<td>Proteolysis &amp; peptidolysis</td>
<td>E, I</td>
<td>2.8</td>
<td>1.8</td>
<td>1.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin 1</td>
<td>2335</td>
<td>Cell adhesion &amp; ECM</td>
<td>A, B, D, E, F, G, I</td>
<td>58.1</td>
<td>0.8</td>
<td>69.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CHI3L1</td>
<td>Chitinase 3-like 1 (cartilage glycoprotein-39)</td>
<td>1116</td>
<td>Cell adhesion &amp; ECM</td>
<td>A, E, F, I</td>
<td>35.6</td>
<td>1.7</td>
<td>21.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DUSP6</td>
<td>Dual specificity phosphatase 6</td>
<td>1848</td>
<td>Cell cycle &amp; apoptosis</td>
<td>A, F, I</td>
<td>23.2</td>
<td>1.7</td>
<td>13.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PRSS23</td>
<td>Protease, serine, 23</td>
<td>11098</td>
<td>Proteolysis &amp; peptidolysis</td>
<td>A, E, F, I</td>
<td>12.9</td>
<td>1.4</td>
<td>9.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PROS1</td>
<td>Protein S (alpha)</td>
<td>5627</td>
<td>Cell adhesion &amp; ECM</td>
<td>A, E, F, G, I</td>
<td>13.1</td>
<td>1.6</td>
<td>5.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SDC4</td>
<td>Syndecan 4 (amphiglycan, ryudocan)</td>
<td>6385</td>
<td>Cell adhesion &amp; ECM</td>
<td>A, D, E, I</td>
<td>7.7</td>
<td>1.5</td>
<td>5.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TIMP1</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
<td>7076</td>
<td>Cell adhesion &amp; ECM</td>
<td>A, B, D, F, G, H, I</td>
<td>5.9</td>
<td>0.5</td>
<td>12.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LAMB3</td>
<td>Laminin, beta 3</td>
<td>3914</td>
<td>Cell adhesion &amp; ECM</td>
<td>A, E, F, I</td>
<td>4.1</td>
<td>0.7</td>
<td>5.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KRT19</td>
<td>Keratin 19</td>
<td>3880</td>
<td>Cytoskeleton</td>
<td>A, D, F, G, I</td>
<td>5.9</td>
<td>1.0</td>
<td>6.0</td>
<td>&lt;0.001</td>
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<tr>
<td>CST6</td>
<td>Cystatin E/M</td>
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<td>Cell adhesion &amp; ECM</td>
<td>A, E, I</td>
<td>9.9</td>
<td>0.8</td>
<td>11.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MDK</td>
<td>Midkine (neurite growth-promoting factor 2)</td>
<td>4192</td>
<td>Cell cycle &amp; apoptosis</td>
<td>A, I</td>
<td>3.0</td>
<td>1.1</td>
<td>2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LGALS3</td>
<td>Lectin, galactoside-binding, soluble, 3 (galectin 3)</td>
<td>3958</td>
<td>Cell adhesion &amp; ECM</td>
<td>A, D, E, F, G, H, I</td>
<td>7.2</td>
<td>0.5</td>
<td>13.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gene symbol</td>
<td>Gene name</td>
<td>Gene ID</td>
<td>Biological process</td>
<td>References</td>
<td>Fold change</td>
<td>P value (t test)</td>
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<td></td>
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<tr>
<td>TPO</td>
<td>Thyroid peroxidase</td>
<td>7173</td>
<td>Thyroid metabolism</td>
<td>A, D, E, G, I</td>
<td>24.0</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.001</td>
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<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>596</td>
<td>Cell cycle &amp; apoptosis</td>
<td>A, I</td>
<td>4.6</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIO2</td>
<td>Deiodinase, iodothyronine, type II</td>
<td>1734</td>
<td>Thyroid metabolism</td>
<td>A, C, I</td>
<td>5.6</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.001</td>
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<tr>
<td>C11orf8</td>
<td>Chromosome 11 open reading frame 8</td>
<td>744</td>
<td>Unknown</td>
<td>A, C, H, I</td>
<td>14.5</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.001</td>
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<td></td>
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<tr>
<td>CITED2</td>
<td>Cbp/p300-interacting transactivator 2</td>
<td>10370</td>
<td>Transcription factor</td>
<td>A, I</td>
<td>3.1</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.01</td>
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<td></td>
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<tr>
<td>SLC26A4</td>
<td>Solute carrier family 26, member 4</td>
<td>5172</td>
<td>Thyroid metabolism</td>
<td>E, I</td>
<td>10.5</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.001</td>
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<td></td>
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<tr>
<td>ID4</td>
<td>Inhibitor of DNA binding 4</td>
<td>3400</td>
<td>Transcription factor</td>
<td>E, I</td>
<td>3.6</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.01</td>
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<tr>
<td>TG</td>
<td>Thyrogblobulin</td>
<td>7038</td>
<td>Thyroid metabolism</td>
<td>E, I</td>
<td>3.0</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.01</td>
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<td></td>
</tr>
<tr>
<td>DIO1</td>
<td>Deiodinase, iodothyronine, type I</td>
<td>1733</td>
<td>Thyroid metabolism</td>
<td>A, E, I</td>
<td>36.2</td>
<td>ga(+) versus NT &lt;0.001 ns &lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The 22 genes (identified by gene symbol, gene name, gene ID, and by a biological process they are involved in) correspond to genes reported to be either over-expressed (n=13) or under-expressed (n=9) in PTC as compared with normal tissue (NT) in at least two and up to six distinct studies identified by letters (A to I); A, Huang et al. 2000, B, Wasenius et al. 2003, C, Aldred et al. 2004, D, Finley et al. 2004, E, Mazzanti et al. 2004, F, Jarzab et al. 2005, G, Lubitz et al. 2006, Finn et al. 2007, I, Durand et al. 2008. The amplitude of changes (average fold change) in the expression level of each gene was calculated as the tumor (ga(+) or ga(−)) to NT signal intensity ratio in the case of up-regulated genes or the reverse for genes down-regulated in tumors. Variations in gene expression levels between PTC-ga(+) and PTC-ga(−) are given by ga(+) / ga(−) ratios. Statistical significance of differences in the level of expression of each gene (between the three groups of samples taken two by two) is given in the last columns of the table. For each of these 22 genes, the level of expression measured in PTC-ga(−) was not different from that measured in NT.
Figure 1 Subclassification of PTC by unsupervised hierarchical clustering from the 200-gene-macroarray expression data. Gene expression profiles of 23 PTC (17 PTC-ga(+) including 13 samples with the BRAFV600E mutation and four with a RET/PTC gene rearrangement and 6 PTC-ga(-)) and 28 normal thyroid tissue samples were subjected to treatment by Cluster and Treeview softwares. The 200-gene-macroarray data were first subjected to a filtration step consisting in the elimination of genes for which the s.d. of observed expression values was lower than 2. Sample classification by unsupervised hierarchical clustering was made on 113 genes using the un-centered correlation distance and average linkage method. On the upper part of the figure, samples are identified by an abbreviation (PTC or NT) preceded by the Tumor Bank running number. In the group of PTC-ga(+), PTC with a RET/PTC gene rearrangement are identified by an asterisk. Genes identified by their gene symbol appear on the right side of the figure. Each column gives the gene expression profile of a sample and each line indicates the variations in the level of expression of a given gene among tissue samples. Black and white colors indicate transcript levels above and below the median values respectively. The length of the branches on the tree forming the dendogram on the top of the figure reflects the degree of similarity between samples, longer the branch, larger the difference of gene expression.
provided in Supplementary Table 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/. PCR conditions included an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of amplification consisting of 15 s at 95 °C for denaturation, 6 s at the hybridization temperature (see Supplementary Table 1) for annealing and 9–11 s at 72 °C for the final extension step (depending on the size of amplicons). Fluorescence intensity measurements obtained at the end of each cycle were used to determine the crossing point value, i.e. the cycle number at which fluorescence was significantly greater than the background. The specificity of the PCR amplification was assessed by determination of the melting temperature of amplicons using a fusion program, consisting in a progressive temperature increase of 0.1 °C/s from 60 to 95 °C. Complementary DNA, corresponding to the different mRNAs to assay by quantitative PCR were generated by classical PCR and purified using the QIAquick purification kit (Qiagen) according to the manufacturer’s protocol. Amounts of cDNA corresponding to 10¹ to 10⁶ (10⁷ for FN1) copies were included in each PCR assay to generate calibration curves by plotting crossing point values as a function of cDNA copy number. Results were expressed in mRNA copies per microgram total RNA after normalization using 18S rRNA measurements. The normalization factor applied to a given sample corresponded to the 18S rRNA copy number measured in this sample divided by the average value of the 18S rRNA copy number of the series of samples which were reverse transcribed in the same run. Transcript concentration values were subjected to log transformation and statistical analyses were performed by Student t-test.

Western blot analyses

Thyroid tissue samples maintained at −80 °C were homogenized in ice-cold PBS (1 ml per 100 mg tissue) supplemented with protease inhibitors (aprotinin, leupeptin, and pepstatin, each at a concentration of 1 μg/ml) using a Teflon-glass Potter homogenizer. Homogenates were centrifuged at 100 000 g for 60 min at 4 °C to obtain crude thyroid membrane fractions. Protein was assayed by the Lowry method after solubilization in 0.1% sodium deoxycholate. Membrane proteins (30 μg protein) were fractionated by electrophoresis on 6% polyacrylamide gel in the presence of SDS and electro-transferred onto Immobilon P membrane (Millipore Corp., Bedford, MA, USA). After treatment with PBS supplemented with 5% non-fat dry milk and 0.2% Tween 20 (Sigma), membranes were incubated overnight at 4 °C with anti-DUOX rabbit antibodies provided by Dr F. Miot (IRIBHM, Brussels, Belgium; De Deken et al. 2000); the antiserum pre-adsorbed on liver acetone powder (10 mg/ml) was used at a 1/10 000 final dilution. After washings in PBS-0.2% Tween solution, membranes were incubated with goat anti-rabbit Ig conjugated to HRP (Bio-Rad Laboratories Inc.) for 1 h at room temperature. Immune complexes were detected by the Enhanced Chemiluminescence method using the chemiluminescent substrate from Amersham Pharmacia Biotech and exposed to Kodak X-OMAT AR films (Eastman Kodak Co). In a second step, the same membranes were incubated with a monoclonal antibody directed against the α-subunit of Na⁺K⁺ATPase; immune complexes were visualized using a biotinylated antimouse Ig antibody and streptavidin conjugated to alkaline phosphatase, as previously described (Trouttet-Masson et al. 2004).

Results

Sub-classification of PTC according to their mutational state

Expression data of 200 genes in 51 thyroid samples (23 PTC and 28 normal tissue (NT) samples) were first subjected to statistical treatment to eliminate unvarying genes. The unsupervised hierarchical clustering of the 51 samples based on data of the 112 remaining genes (Fig. 1) shows a complete separation of PTC and NT samples and, in addition, a classification of PTC according to their mutational state. The 17 PTC-ga(+) (PTC with the BRAF*T1799A mutation or a RET/PTC gene rearrangement) were totally separated from the 6 PTC-ga(−) (PTC without any of the two gene alterations). PTC-ga(+) and PTC-ga(−) were then considered as distinct groups for subsequent analyses of gene expression data by SAM. We found 82 and 41 genes differentially expressed (with a minimum twofold difference) in PTC-ga(+) versus NT and PTC-ga(−) versus NT respectively (Fig. 2A). It appeared that only 36 genes exhibited a similar change (increase for eight and decrease for 28) in both PTC-ga(+) and PTC-ga(−). Among the 82 genes differentially expressed in PTC-ga(+) versus NT, 46 were selectively either up- (n = 16) or down- (n = 30) regulated in PTC-ga(+). In the series of 41 genes differentially expressed in PTC-ga(−) versus NT, five genes were selectively dys-regulated in PTC-ga(−). Expression data from the 51 genes altered in PTC-ga(+) (n = 46) or PTC-ga(−) (n = 5) led to a complete separation of PTC-ga(+) and PTC-ga(−) by hierarchical clustering (Fig. 2B).
To validate macroarray measurements, transcripts of six genes were assayed by quantitative PCR on the same set of thyroid samples. As shown in Supplementary Figure 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/, hybridization signal intensities measured on macroarray plotted against mRNA copy number per μg RNA yielded correlation coefficients higher than 0.8. The amplitude of variations of expression of the six genes measured by microarray and by quantitative PCR was very similar, if not the same (see Supplementary Figure 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/).

Distinct molecular characteristics of PTC-ga(+) and PTC-ga(−)

Among the list of 46 genes selectively dys-regulated in PTC-ga(+), we extracted 22 of them (Table 2) which had been quoted as potential PTC ‘marker’ genes in, at least two and up to six, previous studies. Thirteen genes were highly over-expressed in PTC-ga(+) with tumor to NT ratio values ranging from three to more than 50 and nine genes were under-expressed with NT to tumor ratio ranging from 3 to 36. The level of expression of these 22 genes was comparable in PTC-ga(−) and NT.

The differences between PTC-ga(−) and the two tumor subtypes composing the group of PTC-ga(+) (PTC with the BRAFV600E mutation and PTC with a RET/PTC gene rearrangement) were further investigated by considering expression data from two set of genes either related to ‘cell adhesion and extracellular matrix’ (FN1, CHI3L1, PROS1, SDC4, TIMP1, LAMB3, CST6, LGALS3) and over-expressed in PTC or corresponding to thyroid-specific genes (TG, TPO, SLC26A4, DIO1, DIO2, FOXE1, PAX8) under-expressed in PTC. Data reported in Fig. 3 show that the level of expression of these genes (presented as log2 value of tumor to NT ratio) was remarkably similar in
PTC with the \(BRAF^{T1799A}\) mutation and PTC with a \(RET/PTC\) gene rearrangement but was different from that measured in PTC-ga(−). For example, the expression level of \(FN1\) and \(CHI3L1\) genes was in the normal range in PTC-ga(−) but was increased 20- to 40-fold in the two types of PTC forming the PTC-ga(+) group. Similarly, the expression level of \(TPO\) and \(DIOI\) was in the normal range in PTC-ga(−) but down-regulated by a factor of 20–40 in the two types of tumors grouped in PTC-ga(+) differences between PTC-ga(+) and PTC-ga(−) existed for other thyroid-specific genes: \(PAX8\) and \(FOXE1\) (bottom of Fig. 3) not considered as PTC ‘marker’ genes. \(PAX8\) and \(FOXE1\) transcript levels were decreased (about threefold) in PTC with the \(BRAF^{T1799A}\) mutation and in PTC with a \(RET/PTC\) gene rearrangement but were within the normal range in PTC-ga(−). Data from a previous study on \(NIS\) gene expression in PTC (Porra et al. 2005) were revisited after determination of the presence or absence of \(BRAF\) or \(RET/PTC\) gene alterations. \(NIS\) gene expression exhibited a 20-fold reduction in PTC-ga(+) and only a twofold decrease in PTC-ga(−) (data not shown).

The distinction between PTC-ga(+) and PTC-ga(−) from gene expression data was further ascertained using internal and external validation procedures. The internal approach consisted in the analyses of the expression level of a set of genes on a new and larger series of PTC (\(n=69\)) by quantitative PCR. The selected genes (taken from Table 2) were \(SDC4\), \(CHI3L1\), \(PROS1\), \(FN1\), \(TIMP1\), and \(C11ORF8\). Both individual sample values and mean values of PTC-ga(−), PTC with the \(BRAF^{T1799A}\) mutation and PTC with a \(RET/PTC\) rearrangement are presented in Fig. 4. Average gene expression levels were comparable in PTC with the \(BRAF^{T1799A}\) mutation and PTC with a \(RET/PTC\) rearrangement (with the exception of \(C11ORF8\) gene). As observed with a smaller series of samples (Fig. 3), there was a minimum 10-fold difference in gene expression levels between PTC-ga(−) and PTC-ga(+) and there was no difference between PTC-ga(−) and NT. For the external approach, we used Affymetrix microarray data from Giordano et al. (2005) (available online) to verify the relationship between gene alterations and changes in gene expression profiles in their series of PTC. We performed a hierarchical clustering analysis of their 55 thyroid samples (51 PTC and 4 NT) from expression data of the 51 genes, found to be differentially expressed in PTC-ga(+) and PTC-ga(−) in the present study. As it can be seen in Fig. 5 (and Supplementary Figure 3), PTC with the \(BRAF^{T1799A}\) mutation or with a \(RET/PTC\) rearrangement (36 of the 51 PTC) and PTC with a \(RAS\) mutation or ‘no mutation’ (15 of the 51 PTC) formed two separate clusters. It is worth to notice that a sample (only tested for \(RAS\) mutation), considered by the authors as a PTC with \(BRAF\) mutation from prediction analyses, was found within the group of PTC with \(BRAF\) mutation. Interestingly, PTC with a \(RAS\) mutation appeared to be grouped with PTC named PTC with ‘no mutation’ by Giordano and coworkers and with NT samples.

**Figure 3** Distinction between PTC-ga(+) and PTC-ga(−) and comparison between PTC with the \(BRAF^{T1799A}\) mutation and PTC with a RET/PTC rearrangement from expression data of two sets of genes either related to cell adhesion and extracellular matrix or involved in differentiated thyroid metabolism. Expression levels of genes related to cell adhesion and extracellular matrix (FN1 to LAMB3) or involved in differentiated thyroid metabolism (TPO to PAX8) in PTC classified according to their mutational state: presence of the \(BRAF^{T1799A}\) mutation (black symbols, \(n=13\)), presence of a RET/PTC rearrangement (grey symbols, \(n=4\)) or absence of these gene alterations, PTC-ga(−) (open symbols, \(n=6\)). Gene expression levels are expressed as \(\log_{2}\) value of tumor to NT ratio. Values of \(\log_{2}\) tumor to NT ratio equal to −1 and +1 indicate a 50% decrease and a twofold increase in gene expression level in the tumor as compared with NT respectively. Symbols and horizontal bars represent the mean and S.E.M.
Molecular characteristics of PTC-ga(−)

The partition of PTC according to their mutational state allowed to identify five genes selectively dys-regulated in PTC-ga(−) (Fig. 6A). Expression of SEPP1 gene encoding Selenoprotein P was decreased threefold in PTC-ga(−) as compared with NT. Expression levels of DUOX1 and DUOX2 genes encoding Dual oxidases (the main component of the NADPH-dependent H2O2 generating system of thyroid cells), TITF1 encoding thyroid transcription factor 1 and CCND2 encoding CyclinD2 were increased three to fourfold in PTC-ga(−) as compared with both NT and PTC-ga(+) . The up-regulation of DUOX1 and DUOX2 genes observed at the transcript level was verified at the protein level by western blot analyses of PTC-ga(−), PTC-ga(+) and paired NT samples (Fig. 6B). DUOX1 and DUOX2 proteins, identified by specific anti-DUOX antibodies (De Deken et al. 2000), migrated as a single band with an apparent molecular mass of ~180 kDa. The DUOX protein content of PTC-ga(−) was comparable with that of NT or decreased. In accordance with analyses at the transcript level, the DUOX protein content was higher in PTC-ga(+) than in paired NT.

Clinico-pathological features of PTC-ga(−)

In the present series of 116 PTC, PTC-ga(−) represented 35% of cases. The sex ratio and the age of patients was comparable in PTC-ga(−) and PTC-ga(+). The two groups of PTC differ on two parameters, the tumor size (3.2 cm in PTC-ga(−) versus 2.5 cm in PTC-ga(+)) and pT classification (Table 3); the tumor grade of PTC-ga(+) was higher than that of PTC-ga(−) (χ²-test, P<0.01). There was a highly statistically significant association of the mutational state of PTC with the tumor histiotype (P<0.0001); 84% of PTC of classical form were PTC-ga(−), whereas 81% of follicular variants of PTC corresponded to PTC-ga(−).

Discussion

We report that PTC without the BRAF^{T1799A} mutation and without a RET/PTC rearrangement, accounting for about one third of PTC, maintains a level of expression of thyroid differentiation close to that of normal tissue and exhibit favorable prognostic characteristics.

At each stage of the study, PTC without the BRAF^{T1799A} mutation or RET/PTC rearrangement (PTC-ga(−)) and PTC with one or the other gene alteration (PTC-ga(+)) behaved as distinct and homogeneous (in terms of gene expression levels) groups of samples. Within the group of PTC-ga(+), PTC with the BRAF^{T1799A} mutation and PTC with a RET/PTC rearrangement exhibited similar gene expression characteristics.
expression profiles. The PTC-ga(−) group was expected to be less homogeneous; indeed, it should be composed of tumors with unknown gene alteration and tumors with gene alterations of low incidence in PTC (Kondo et al. 2006) such as RAS gene mutation. From a principal components analysis (on the whole set of genes present on Affymetrix microarray), Giordano et al. (2005) reported that PTC with a RAS mutation were grouped close to PTC with no mutation. Using data from Giordano et al. (2005) corresponding to the set of genes (n = 51) differentially expressed in PTC-ga(+) and PTC-ga(−) and another statistical approach (supervised hierarchical clustering, Fig. 5), we found that PTC with RAS mutation were classified with PTC with no mutation and with NT samples.

Our macroarray and quantitative PCR data clearly show that genes quoted as PTC ‘marker’ genes in numerous studies are in fact good ‘marker’ genes for PTC-ga(+), representing the major type of PTC, but are not informative for PTC-ga(−). Among genes with unaltered expression level in PTC-ga(−), we found members of cell adhesion and extracellular matrix pathway, on the one hand, and genes involved in the differentiated thyroid metabolism, on the other hand. The former group of genes that includes FNI, CHI3L1, PROS1, SDC4, TIMP1, CST6, LGALS3, and LAMB3, exhibited an elevated expression (10–50-fold increase as compared with NT) in our series of PTC-ga(+) as in most published PTC series (Huang et al. 2001, Wasenius et al. 2003, Aldred et al. 2004, Finley et al. 2004, Jarzab et al. 2005, Lubitz et al. 2006, Finn et al. 2007) but no variation in PTC-ga(−). In a comparable but opposite way, genes with a function in thyroid differentiation: TG, TPO, NIS, DUOX1, DUOX2, SLCA26A4, DIO1, DIO2, FOXE, and PAX8 showed a decreased expression in our series of PTC-ga(+) as in most published PTC series, but no change (as compared with normal tissue) in PTC-ga(−). These data emphasize the need for a special attention in the constitution of groups of samples intended to serve as reference for the generation of tumor classifiers based on gene expression.

We identified a few genes expressed at a normal level in PTC-ga(+) but dys-regulated in PTC-ga(−). The selective increase in expression of DUOX genes in PTC-ga(−) shed light on the previous data from Lacroix et al. (2001) who reported that the DUOX transcript content of malignant thyroid tumors was highly variable. These authors found that DUOX expression was in the normal range or decreased in about 60% of carcinomas and increased (up to 10-fold) in the other cases. This variability in DUOX gene expression was likely related to the presence of PTC-ga(+) and PTC-ga(−) in their PTC sample series.

The sub-classification of PTC according to their mutational state led to the identification of 36 genes similarly altered in PTC-ga(+) and PTC-ga(−). Noteworthy, several genes, members of the top 12 ‘thyroid cancer’ markers issued from the meta-analysis of Griffith et al. (2006), exhibiting remarkable changes in their expression level in PTC (either over-expressed as FNI or under-expressed as TPO), do not belong to the list of 36 potential PTC ‘common markers’ presented in part as Supplementary Table 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/.
The association of subtypes of PTC defined from histological features with subgroups of PTC defined from gene alterations i.e. PTC of classical form (+ tall cell variants of PTC) with PTC-ga(+) on the one hand, and follicular variants of PTC with PTC-ga(−) on the other hand are in keeping with the present views in thyroid oncology (Kondo et al. 2006). We report a new relationship between subgroups of PTC defined from gene alterations and subclasses of PTC defined from thyroid-specific gene expression profiles. As illustrated in Supplementary Figure 4, which can be viewed online at http://erc.endocrinology-journals.org/supplemental, PTC-ga(+) corresponded to PTC with a marked loss of expression of thyroid differentiation and PTC-ga(−) to PTC with a thyroid differentiation state close to normal. The same result was obtained with expression data of Giordano et al. (2005) (data not shown).

One of the most interesting findings of this study deals with the relationship between the differentiation state and sub-classification of PTC. We found that PTC-ga(−), corresponding mainly to follicular variants of PTC are well-differentiated tumors as compared with PTC-ga(+), which are PTC of classical form in the majority of cases. A connection between the maintenance of thyroid follicular structure and the maintenance of thyroid functional activity in PTC is probable but still not demonstrated. Our data indicate that PTC-ga(−) tumoral tissue should maintain a definite (close to normal) capacity of trapping, organification, and retention of radioiodide which is a favorable condition for an efficient destruction of remnant cancer cells by radiation therapy after thyroidectomy. This assertion and a low tumor grade represent true indicators for a favorable prognosis in patients with PTC-ga(−). Our data are in keeping with and extend previous reports associating the BRAF mutation with i) loss of radioiodine avidity and failure of treatment of recurrences (Xing 2005, Riesco-Eizaguirre et al. 2006, Xing 2007), and ii) reduced expression of key genes (NIS, TG, TPO, PAX8) involved in iodine metabolism (Durante et al. 2007). In the study of Durante and coworkers, expression levels of thyroid-specific genes in BRAF-wild type PTC were intermediate between those found in normal tissue and in PTC with BRAF<sup>T1799A</sup> mutation. It is reasonable to think that the removal of PTC with a RET/PTC gene rearrangement from the group of BRAF-wild type PTC and their transfer to the group of PTC with BRAF<sup>T1799A</sup> mutation would have led to a shift of gene expression levels of other PTC i.e. PTC without BRAF mutation and without RET/PTC rearrangement, towards normal values as we observed.

In conclusion, the disclosure of marked differences in the level of expression of thyroid differentiation between PTC with and without the BRAF<sup>T1799A</sup> mutation, or a RET/PTC rearrangement is likely of importance for the patients’ outcome and offers new
arguments for a systematic determination of the \(BRAF\) and \(RET/PTC\) mutational state of PTC.

**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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