Gene expression profiling associated with the progression to poorly differentiated thyroid carcinomas

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BACKGROUND: Poorly differentiated thyroid carcinomas (PDTC) represent a heterogeneous, aggressive entity, presenting features that suggest a progression from well-differentiated carcinomas. To elucidate the mechanisms underlying such progression and identify novel therapeutic targets, we assessed the genome-wide expression in normal and tumour thyroid tissues.

METHODS: Microarray analyses of 24 thyroid carcinomas – 7 classic papillary, 8 follicular variants of papillary (fvPTC), 4 follicular (FTC) and 5 PDTC – were performed and correlated with RAS, BRAF, RET/PTC and PAX8-PPARG alterations. Selected genes were validated by quantitative RT–PCR in an independent set of 28 thyroid tumours.

RESULTS: Unsupervised analyses showed that gene expression similarity was higher between PDTC and fvPTC, particularly for tumours harbouring RAS mutations. Poorly differentiated thyroid carcinomas presented molecular signatures related to cell proliferation, poor prognosis, spindle assembly checkpoint and cell adhesion. Compared with normal tissues, PTC had 307 out of 494 (60%) genes over-expressed, FTC had 137 out of 171 (80%) genes under-expressed, whereas PDTC had 92 out of 107 (86%) genes under-expressed, suggesting that gene downregulation is involved in tumour dedifferentiation. Significant UHRF1 and ITIH5 deregulated gene expression in PDTC, relatively to normal tissues, was confirmed by quantitative RT–PCR.

CONCLUSION: Our findings suggest that fvPTC are possible precursors of PDTC. Furthermore, UHRF1 and ITIH5 have a potential therapeutic/prognostic value for aggressive thyroid tumours.

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Well-differentiated thyroid carcinomas (WDTCs), such as papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), are usually treated successfully with surgery and radioactive iodine; however, poorly differentiated thyroid carcinoma (PDTC) and anaplastic (or undifferentiated) thyroid carcinoma (ATC) can behave aggressively with no effective form of treatment (Patel and Shaha, 2006).

Previous reports suggest a model of progression from WDTC to PDTC and to ATC. PDTC show limited follicular cell differentiation and are, both morphologically and behaviourally, positioned between well- and undifferentiated carcinomas (DeLellis et al, 2004). Indeed, cases of WDTC containing areas of poor- or undifferentiation, as well as, cases of PDTC/ATC containing well-differentiated areas, have been widely detected (Lam et al, 2000). Progression is further suggested by the sequential increase in chromosomal abnormalities from WDTC to PDTC and ATC (Wreesmann et al, 2002; Rodrigues et al, 2004). Mutations in the RAS and BRAF genes also support a model of tumour progression, as the frequency of these events in PDTC is midway between well-differentiated and undifferentiated carcinomas, rather than being randomly distributed (Garcia-Rostan et al, 2003; Nikiforova et al, 2003). Other alterations, such as tumour suppressor TP53 mutations, are specifically found in PDTC and ATC, and are often associated with RAS or BRAF mutations (Quiros et al, 2005; Wang et al, 2007), suggesting an accumulation of events during progression. Nevertheless, it is not clear whether PDTC derive from either PTC or FTC, or whether they arise de novo. In addition, the genetic and epigenetic mechanisms underlying the process remain ill defined.

Genome-wide expression analysis has been successfully used to identify molecular signatures, improving the diagnosis and prognosis of several types of tumours (Quackenbush, 2006). For thyroid neoplasias, one of the earliest reports of genome-wide expression analysis described a consistent gene expression profile that distinguished PTC from normal cells...
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RNA processing and hybridisation

RNA samples were processed following the one-cycle eukaryotic target labelling protocol from Affymetrix, and were hybridised using the HG-U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). Hybridisation results were scanned using the GeneChip Scanner 3000 and stored in the GeneChip Operating Software.

Microarray data analysis

Partek Genomics Suite Software (Partek Inc, St Louis, MO, USA) was used for unsupervised analyses. First, array data were normalised and the expression levels were determined applying the robust multi-array average method (Irizarry et al, 2003), and data were corrected for non-biological factors. Samples were represented three dimensionally, according to the expression levels of all probe sets, by principal components analysis (PCA). Probe-sets data were also used to obtain a dendogram of the samples, by hierarchical clustering, with the Pearson correlation coefficient.

DNA-ChipAnalyzer (dChip) 2006 software (Li and Wong, 2001) was used to obtain differentially expressed genes between groups. Arrays were normalised with the invariant set normalisation method and expression levels were calculated by model-based expression analysis with perfect match-only model. Probe sets that were absent in all samples or those that did not change across samples (coefficient of variation lower than 0.2 and higher than 10) were eliminated from further analysis. Probe sets were considered to be differentially expressed between two groups when the lower 90% limit of the confidence interval of the fold change (ratio of the expression level in the two groups) was equal or higher than twofold, with an unpaired t-test considered significant at \( P \leq 0.01 \). Onto-Express from Onto-Tools package (Khatri et al, 2002) was used to classify genes differentially expressed according to their biological role.

Gene set enrichment analysis (GSEA) software (Subramanian et al, 2005) was used to determine whether members of defined groups of genes, which share common features (gene sets), are preferentially placed towards the top or the bottom of a list of genes. In this list, genes were ranked according to the differential expression between two sample groups. This method was applied using two catalogues of gene sets: one whose products are involved in specific pathways/functions and another defined by expression neighbourhoods, which indicates molecular signatures associated with cancer-related genes. Statistical significance was estimated by a nominal \( P \) value obtained by phenotype permutation. \( P \) values were corrected for multiple hypothesis testing using false discovery rate and family wise-error rate (FWER). Gene sets were considered significant at \( P \leq 0.05 \) and with FWER \( \leq 0.25 \).

First-strand cDNA synthesis

cDNA was synthesised from 1 \( \mu g \) of total RNA (for cDNA sequence analysis) or 2 \( \mu g \) of total RNA (for quantitative RT – PCR), at 37°C for 90 min, using random primer \( p(dN)\_6 \) (Roche Diagnostics Corporation, Indianapolis, IN, USA) and SuperScript II reverse transcriptase (Invitrogen, Paisley, UK).

Mutational analysis of the RAS, BRAF genes and PAX8-PPARG, RET/PTC rearrangements

Mutational analysis was undertaken using cDNA from the tumour samples of the microarray set. PTC were screened for BRAF mutations and rearrangements of RET/PTC and, in addition, follicular variants were also analysed for RAS mutations and PAX8-PPARG rearrangements. FTC were screened for RAS and PAX8-PPARG rearrangements. PTC were analysed for BRAF, RAS and PAX8-PPARG genes. Primers were designed to amplify exons

Materials and methods

Tissue samples

Both tumour and normal thyroid tissue samples were obtained at time of surgery, and were immediately frozen in liquid nitrogen. Histological classifications followed the criteria described in World Health Organization (WHO) classification of thyroid tumours (DeLellis et al, 2004). All samples were obtained with permission, and the project was approved by our institution ethical committee.

The microarray sample set consisted of a total of 24 tumour samples – 5 PTD, 7 classic PTC (cPTC), 8 follicular variants of PTC (fvPTC) and 4 FTC (Supplementary Table 1). A pool of human thyroid total RNA obtained from 65 Caucasian individuals with 18–61 years old, whom died from sudden death (BD Bioscience, Franklin Lakes, NJ, USA), and 2 normal tissue samples taken from the opposite lobe of thyroid tumours, were also processed.

An independent sample set, consisting of five PTD, seven cPTC, seven fvPTC, nine PTC and six normal thyroid tissues taken from the opposite lobe of thyroid tumours, was used for quantitative real-time RT–PCR. Expression was also studied in 2 pooled tumour samples with RNA Integrity Number equal or higher than 7.7.

Total RNA isolation/extraction

Total RNA was extracted and purified using the RNeasy Mini kit (Quiagen, Hamburg, GmbH, Germany) according to the manufacturer’s protocol, and quantified by UV spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was assessed by micro capillary electrophoresis (Agilent 2100 Bioanalyzer, Santa Clara, CA, USA) and samples with RNA Integrity Number equal or higher than 7.7 were selected for microarray analysis.

(Huang et al, 2001). Most of the genes identified in this work were corroborated in subsequent studies. Gene expression studies have also been used to differentiate benign from malignant thyroid tumours, and correlate gene expression patterns with specific mutations or rearrangements in PTC and FTC (for review, see Eszlinger et al, 2007).

To our knowledge, only two studies have addressed the genome-wide expression of PTD. One of these studies compared gene expression of PTD and ATC cell lines to normal thyroid tissue, and showed that these cells presented largely altered expression profiles that have been associated with the cancer process (Rodrigues et al, 2007). Although the authors confirmed some of the abnormal expressed genes in primary tumours, it has been shown that immortal cell lines may not fully reflect the functional aspects of the tumours, and that some molecular processes might be specifically acquired during the immortalisation step (Dairkee et al, 2004). In the other study, which used WDT, PTD and ATC primary tumours, deregulation of different molecular pathways, such as the MAPK signalling pathway, focal adhesion and cell motility, cell proliferation and cell-cycle progression, was associated with dedifferentiation in PTD and ATC (Montero-Conde et al, 2008).

In this study, we used the array platform GeneChip Human Genome U133 Plus 2.0 (HG-U133 Plus 2.0) to analyse the expression of a wide range of genes (> 30 000) in well- and poorly differentiated thyroid tumours and, to correlate, for the first time, gene expression with BRAF, RAS, RET/PTC and PAX8-PPARG alterations.

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of five normal thyroid tissues was used as calibrator to determine the expression level equal, or higher, than two-fold in a group

The result of mutational analysis for each tumour specimen is shown.

Global gene expression similarity between the 27 samples was calculated as mean ± standard error of the mean (s.e.m.). Comparisons between sample groups were performed using the Pearson or the Spearman correlations (for a non-Gaussian distribution). The samples distribution was not Gaussian or variances between groups were not equal. Correlations of quantitative RT–PCR data with other variables were performed using the Pearson or the Spearman correlations (for a non-Gaussian distribution). The correlations and differences between group means were considered significant at P<0.05.

**RESULTS**

**Mutation screening**

Tumour samples were screened for mutations in MAPK pathway effectors, which are frequently mutated in thyroid cancer (Supplementary Table 1). The BRAF V600E substitution was only present in cPTC, accounting for 57.1% (four out of seven) of the cases. On the other hand, mutations of N- or KRAS genes were observed in 50% (four out of eight) of the fvPTC and in 40% (two out of five) of PDTC. All FTC (n = 4) were RAS negative. The PAX8-PPARG fusion gene was found in 12.5% (one out of eight) of fvPTC and in 25% (one out of four) of FTC. No RET/PTC1, -2 or -3 rearrangements were identified in FTC. However, as other rearrangements involving the RET (Giampi et al, 2007) or NTRK1 (Pierotti and Greco, 2006) genes have also been described in FTC, we specifically analysed RET and NTRK1 microarray mRNA expression in FTC negative for mutations. In one cPTC (sample 2 – Supplementary Table 1), a 20-fold increase in RET expression was detected in comparison to the other samples. FISH confirmed the presence of a RET/PTC rearrangement in 37% (71 out of 194) of these tumour cells (data not shown).

**Unsupervised analyses for global gene expression profiling**

We carried out unsupervised analyses to examine the relationship between gene expression, tumour histotype and mutational status. Global gene expression similarity between the 27 samples was examined using hierarchical clustering (Figure 1). As represented on the dendrogram, distinct profiles separated FTC from the other tumours. Interestingly, a case of fvPTC diffuse (or multinodular) with a PAX8-PPARG rearrangement clustered with FTC. Different molecular signatures were present in the FTC sub-set: cPTC formed a separate sub-group from fvPTC and, among these PTC subtypes, samples with RAS/BRAF mutations were separated from samples without mutations. By PCA of data for all probe sets, which represents the samples three dimensionally according to the global gene expression profile, the FTC and the fvPTC diffuse (or multinodular) also formed a group apart from the other tumours and normal thyroid samples, which tended to cluster together (Supplementary Figure 1; Supplementary Movie 1). PDTC samples, particularly those with RAS mutations, clustered with PTC in both representations.

**Genes differentially expressed between tumours and normal tissue**

Differentially expressed genes were defined as those with an expression level equal, or higher, than two-fold in a group relatively to another, with a P value ≤0.01. We compared each...
tumour group (cPTC, fvPTC, FTC and PDTC) with the three normal thyroid samples, and we found over-expression of about 60% of probe sets for both cPTC and fvPTC, whereas in FTC and PDTC, about 80% of the probe sets were under-expressed (Figure 2). PDTC had 92 downregulated genes relatively to normal tissues (Supplementary Table 2), but only 15 out of the 107 genes differentially expressed were over-expressed (Table 1).

The biological processes mainly represented by the probe sets differentially expressed between thyroid tumours and normal tissues were the signal transduction, cell adhesion, regulation of transcription and cell proliferation/cell cycle (Supplementary Figure 2). We were also able to identify 11 probe sets that were under-expressed in all tumours comparatively to normal tissues (Table 2).

**Genes specific for PDTC**

The PDTC group had 3, 8 and 1 over-expressed probe sets and 11, 154 and 59 under-expressed probe sets compared with FTC, cPTC and fvPTC, respectively (data not shown). Only two probe sets were consistently under-expressed in the PDTC tumour set comparatively to the WDTC (Table 1).

**GSEA for PDTC**

GSEA is another method for interpreting gene expression data that focus on groups of genes sharing common biological function, chromosomal location or regulation. This approach can show important effects on pathways, which might be missed in single-chromosomal location or regulation. This approach can show focus on groups of genes sharing common biological function, which green and red correspond, respectively to a lower and a higher expression than the mean value for the gene, in all samples being compared. On the other hand, differences in the PBK expression were not statistically significant (Figures 3C and D). *UHRF1* expression was decreased in all tumours samples relatively to normal tissue samples, but the highest expression levels were detected in PDTC. Differences in the *UHRF1* expression (Figure 3B) between PDTC and normal tissue were statistically significant (11.77 ± 0.36 vs 1.77 ± 0.36; *P* < 0.01), even if only considering the expression in the independent set (14.30 ± 5.49 vs 1.77 ± 0.36; *P* < 0.05) (Figure 3A). On the other hand, differences in the PBK expression were not statistically significant (Figures 3C and D). *ITIH5* expression was decreased in all tumours samples relatively to normal tissues (Figures 3E and F). Statistically significant differences were detected in all tumour groups relatively to normal samples, except in cPTC (PDTC: 0.22 ± 0.17, *P* < 0.001; FTC: 0.15 ± 0.05, *P* < 0.001; fvPTC: 0.22 ± 0.03, *P* < 0.05 vs normal: 1.00 ± 0.10). Differences were also significant when considering the independent set of samples (PDTC: 0.41 ± 0.33,

**Validation of microarray gene expression**

Real-Time RT–PCR was performed to validate three genes differentially expressed between PDTC and normal thyroid samples: ubiquitin-like, containing PHD and RING finger domains, 1 (*UHRF1*), PDZ-binding kinase (*PBK*) and inter-alpha (globulin) inhibitor H5 (*ITIH5*). This validation was processed in an independent sample set of 28 tumours (Figure 3). *UHRF1* and *PBK* had increased expression in all tumour samples relatively to normal tissue samples, but the highest expression levels were detected in PDTC. Differences in the *UHRF1* expression (Figure 3B) between PDTC and normal tissue were statistically significant (11.77 ± 0.36 vs 1.77 ± 0.36; *P* < 0.01), even if only considering the expression in the independent set (14.30 ± 5.49 vs 1.77 ± 0.36; *P* < 0.05) (Figure 3A). On the other hand, differences in the PBK expression were not statistically significant (Figures 3C and D). *ITIH5* expression was decreased in all tumours samples relatively to normal tissues (Figures 3E and F). Statistically significant differences were detected in all tumour groups relatively to normal samples, except in cPTC (PDTC: 0.22 ± 0.17, *P* < 0.001; FTC: 0.15 ± 0.05, *P* < 0.001; fvPTC: 0.22 ± 0.03, *P* < 0.05 vs normal: 1.00 ± 0.10). Differences were also significant when considering the independent set of samples (PDTC: 0.41 ± 0.33,

**Figure 2** Expression profile of the genes differentially expressed between tumours and normal tissues. Each tumour histology was compared with the normal thyroid tissues and expression levels of the genes differentially expressed were represented. Expression levels are indicated by colour intensities in which green and red correspond, respectively, to a lower and a higher expression than the mean value for the gene, in all samples being compared. On the left of each profile, the number of under- and over-expressed genes in the tumour set is shown. At the bottom, the total number of differentially expressed genes is indicated. Only one probe set was considered for each gene. cPTC=classic papillary thyroid carcinoma; FTC=follicular thyroid carcinoma; fvPTC=follicular variant of papillary thyroid carcinoma; PDTC=poorly differentiated thyroid carcinoma.
**Table 1** Main characteristics of differentially expressed genes in poorly differentiated tumours

| Probe set     | Gene name in array HG-U133 Plus 2.0 | Official symbol | Accession number | Biological process | LBFC | P-value |
|---------------|-------------------------------------|-----------------|------------------|-------------------|------|---------|
| 204170_s_at   | CDC28 protein kinase regulatory subunit 2 | CKS2            | NM_001827        | Cell cycle        | 5.43 | 4.68E-03|
| 219148_at     | PDZ binding kinase                  | PAK             | NM_018492        | Cell cycle        | 4.29 | 8.04E-03|
| 230503_s_at   | KIAA0101                            | KIAA0101        | NM_014736        | Cell cycle        | 3.46 | 5.80E-03|
| 205034_at     | Cyclin E2                           | CONE2           | NM_004702        | Cell cycle        | 2.95 | 6.21E-03|
| 202975_s_at   | Rho-related BTB domain containing 3 | RHOBTB3         | N21138           | —                 | 2.94 | 9.42E-03|
| 218096_at     | I2-acylglycerol-3-phosphate O-acetyltransferase | AGPAT5         | NM_018361        | Phospholipid metabolism | 2.76 | 4.23E-03|
| 225655_at     | Ubiquitin-like, containing PHD and RING finger domains, 1 | UHRF1           | AK025578         | Cell cycle        | 2.61 | 8.96E-03|
| 229551_s_at   | Zinc-finger protein 367             | ZNF367          | N62196           | Transcription regulation | 2.59 | 6.36E-04|
| 220608_s_at   | Homo sapiens PRO1914 protein (PRO1914) | ZNF770          | NM_014106        | Transcription regulation | 2.58 | 7.33E-04|
| 222848_at     | Leucine zipper protein FKCG14       | CENPK           | BC000549         | Transcription regulation | 2.53 | 4.85E-04|
| 224726_at     | Mindbomb homolog 1 (Drosophila)     | MIB1            | W80418           | Notch signaling   | 2.38 | 1.98E-04|
| 220145_at     | ASAP                                | MAP9            | NM_024286        | Cell cycle        | 2.07 | 6.36E-03|
| 218819_at     | DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26 | INT56           | NM_012141        | DNA DBS processing | 2.05 | 7.46E-03|
| 235609_at     | BRCA1 interacting protein C-terminal helicase 1 | BRIP1           | BF56791          | —                 | 2.03 | 2.24E-03|
| 203007_s_at   | Lysoospholipase l                   | LPLAI           | AF077198         | —                 | —    | —       |

Genes over-expressed in PDTC vs normal thyroids

Abbreviations: DBS = double-strand breaks; LBFC = lower bound of fold change; snRNA = small nuclear RNA. *Assigned in EntrezGene. Information taken from Online Mendelian Inheritance in Man (OMIM) or from EntrezGene. P-values for differences in mean expression between groups were calculated using an unpaired t-test.

**Table 2** Main characteristics of differentially expressed genes in the four thyroid tumour histotypes vs normal thyroid tissues

| Probe set     | Gene name in array HG-U133 Plus 2.0 | Official symbol | Accession number | Biological process | LBFC | P-value |
|---------------|-------------------------------------|-----------------|------------------|-------------------|------|---------|
| 205382_s_at   | D component of complement (adipin)  | CFD             | NM_001928        | Immune response   | −9.31| 4.74E-03|
| 204606_at     | Chemokine (C-C motif) ligand 21     | CCL21           | NM_002989        | Inflammatory response | −7.57| 5.72E-03|
| 235849_at     | Hypothetical protein MGC45780      | SCAR5           | BE787752         | Immune response   | −6.38| 9.25E-03|
| 205350_at     | Cellular retinoid acid binding protein 1 | CRABP1         | NM_004378        | Retinoic acid metabolism | −6.13| 5.13E-03|
| 203060_s_at   | 3'-phosphoadenosine 5'-phosphosulfate synthase 2 | PAPS2          | AF074331         | Sulfur metabolism | −5.98| 8.55E-04|
| 212713_at     | Microfilament-associated protein 4  | MFAP4           | R72286           | Cell adhesion     | −4.20| 3.81E-04|
| 1556427_s_at  | Similar to hypothetical protein     | LOC221091       | AL834319         | —                 | −4.07| 5.57E-03|
| 219777_at     | Zinc-finger protein, multitype 2    | ZFP2M           | NM_012082        | Transcription regulation | −4.03| 7.60E-03|
| 205413_at     | Chromosome 11 open reading frame 8  | MAPED2          | NM_001584        | Nervous system development | −3.59| 9.00E-05|
| 206201_s_at   | Mesenchyme homeobox box 2 (growth arrest-specific homeobox) | MEOX2          | NM_005924        | Development       | −2.92| 5.85E-03|
| 217255_at     | Olfactomedin-like 1                 | OLFM1           | AW305097         | Cell proliferation | −2.64| 2.34E-04|

Abbreviation: LBFC = lower bound of fold change. *Assigned in EntrezGene. Information taken from Online Mendelian Inheritance in Man (OMIM) or from EntrezGene. P-values for differences in mean expression between tumours and normal tissues were calculated using an unpaired t-test.

P < 0.05; FTC: 0.14 ± 0.07, P < 0.001 vs normal: 1.00 ± 0.10). Additionally, we assessed UHRF1 and ITIH5 expressions in two PDTC cell lines. No significant increase in UHRF1 expression was detected (mean ± standard deviation: 1.86 ± 0.16 and 1.43 ± 0.16 vs 1.77 ± 0.36). On the other hand, ITIH5 expression was undetectable (Ct values > 37) in both cell lines. Correlation of quantitative RT-PCR data with the expression levels obtained in the microarray analysis was statistically significant (Pearson correlation r = 0.61 for UHRF1 with P = 0.0015; Spearman correlation r = 0.70 for PBK with P = 0.0001; Pearson correlation r = 0.94 for ITIH5 with P < 0.0001). There was no correlation of gene expression with tumour size, gender or age, except for ITIH5, whose decreased expression was associated with larger tumours (Spearman correlation r = −0.33 with P = 0.02).
Table 3  Gene sets enriched in the poorly differentiated vs the well-differentiated groups

| Gene set name | Gene set description | Nominal P-value | FDR | FWER | Reference |
|---------------|----------------------|-----------------|-----|------|-----------|
| **Functional-defined gene sets** | | | | | |
| LEE_TCELLS3_UP | Enriched in both intrathymic T progenitor cells and CD3+CD4+CD8+ thymocytes | 1.01E-02 | 0.95 | 0.43 | Lee et al (2004) |
| YU_CMYC_UP | C-Myc activated genes | 6.93E-03 | 0.56 | 0.47 | Yu et al (2005) |
| GREENBAUM_E2A_UP | Upregulated in E2A-deficient pre-B-cell lines | 5.14E-03 | 0.44 | 0.51 | Greenbaum et al (2004) |
| VANTVEER_BREAST_OUTCOME_GOOD_VS_POOR_DN | Poor prognosis marker genes in breast cancer | 7.61E-03 | 0.38 | 0.55 | van’t Veer et al (2002) |
| MANALO_HYPOXIA_DN | Genes downregulated in human pulmonary endothelial cells under hypoxic conditions | 8.89E-03 | 0.31 | 0.55 | Manalo et al (2005) |
| ADIP_DIFF_CLUSTERS | Strongly upregulated at 24 h during differentiation of 3T3-L1 fibroblasts into adipocytes | 2.40E-02 | 0.32 | 0.62 | Burton et al (2002) |
| CANCER_UNDIFFERENTIATED_META_UP | Genes commonly upregulated in undifferentiated cancer relative to well-differentiated cancer | 1.64E-02 | 0.30 | 0.64 | Rhodes et al (2004) |
| HUMAN_TISSUE_TESTIS | Genes expressed specifically in human testis tissue | 1.15E-02 | 0.34 | 0.70 | Su et al (2002) |
| ZHAN_MM_CD138_PR_VS_REST | Top ranked over-expressed genes in proliferation subgroup of bone marrow plasma cells from multiple myeloma patients | 9.83E-03 | 0.31 | 0.70 | Zhan et al (2006) |
| CROONQUIST_IL6_STARVE_UP | Genes upregulated in multiple myeloma cells exposed to cytokine IL6 vs IL6-starved cells | 7.21E-03 | 0.29 | 0.71 | Croonquist et al (2003) |
| BRCA_PROGNOSIS_NEG | Negatively correlated with metastasis and poor prognosis in breast cancer | 1.37E-02 | 0.28 | 0.73 | Van’t Veer et al (2002) |
| P21_ANY_DN | Downregulated after ectopic expression of p21 (CDKN1A) in ovarian cancer cell line | 2.12E-02 | 0.27 | 0.74 | Wu et al (2002) |
| DOX_RESIST_GASTRIC_UP | Upregulated in gastric cancer cell lines resistant to doxorubicin, compared with parental chemoresistant lines | 1.94E-02 | 0.31 | 0.80 | Kang et al (2004) |
| CROONQUIST_IL6_RAS_DN | Genes downregulated in multiple myeloma cells exposed to IL6 vs NRAS activating mutations cells | 1.73E-02 | 0.29 | 0.80 | Croonquist et al (2003) |
| REN_E2F1_TARGETS | E2F1 targets in primary fibroblast WI-38 | 2.84E-02 | 0.28 | 0.81 | Ren et al (2002) |
| BREAST_DUCTAL_CARCINOMA_GENES | Genes upregulated in high tumour grade breast tumours progressing from pre-invasive ductal carcinoma in situ to invasive ductal carcinoma | 2.43E-02 | 0.30 | 0.84 | * |
| SERUM_FIBROBLASTCELLCYCLE | Cell-cycle-dependent genes, regulated after exposure to serum in a variety of human fibroblast cell lines | 5.04E-02 | 0.28 | 0.85 | Chang et al (2004) |
| BRENTANI_CELL_CYCLE | Cancer-related genes involved in the cell cycle | 1.87E-02 | 0.28 | 0.86 | Brentani et al (2003) |
| SHEPARD_CRASH_AND_BURN_MUT_VS_WT_DN | Genes upregulated in wild-type zebrafish compared with the B-Myb loss-of-function mutants | 2.13E-02 | 0.28 | 0.86 | Shepard et al (2005) |
| GOLDRATH_CELL_CYCLE | Cell-cycle genes induced during antigen activation of CD8+ T cells | 2.50E-02 | 0.27 | 0.87 | Goldrath et al (2004) |

Expression neighbourhoods-defined gene sets

| Gene set name | Gene set description | Nominal P-value | FDR | FWER | Reference |
|---------------|----------------------|-----------------|-----|------|-----------|
| GNF2_CKS1B | Expression neighbourhood of CKS1B in the GNF2 expression compendium | 0.00 | 0.04 | 0.02 | * |
| MORF_BUB1 | Expression neighbourhood of BUB1 in the MORF expression compendium | 4.74E-03 | 0.14 | 0.10 | * |
| MORF_BUB1B | Expression neighbourhood of BUB1B in the MORF expression compendium | 0.00 | 0.20 | 0.19 | * |
| GNF2_ESPL1 | Expression neighbourhood of ESPL1 in the GNF2 expression compendium | 9.59E-03 | 0.18 | 0.21 | * |

Abbreviations: BUB1 = budding uninhibited by benzimidazoles 1 homolog (yeast); BUB1B = budding uninhibited by benzimidazoles 1 homolog beta (yeast); CD = cluster of differentiation; IL6 = interleukin 6; CDKN1A = cyclin-dependent kinase inhibitor 1A (p21, Cip1); CKS1B = CDC28 protein kinase regulatory subunit 1B; ESPL1 = extra spindle pole bodies homolog 1 (S. cerevisiae); FDR = false discovery rate; FWER = family wise-error rate. *Assigned in Molecular Signatures Database (www.broad.mit.edu/gsea/msigdb/index.jsp). References are supplied as supplementary data.
DISCUSSION

The more aggressive thyroid carcinomas (PDTC and ATC) have high malignant potential and it is not yet clear whether they arise from pre-existing indolent WDTC or whether they arise de novo. Some PDTC cases bear areas of pre-existing PTC and have a significant prevalence of BRAF mutations (Nikiforova et al., 2003). Others, instead of BRAF, frequently display RAS mutations (Garcia-Rostan et al., 2003), which are typically detected in follicular thyroid adenomas, FTC and fvPTC. Comparative Genomic Hybridisation (CGH) studies showed that among 11 copy number changes present in PTC, 8 were also present in the PDTC set, thus suggesting common genetic pathways (Wreesmann et al., 2002).

In our work, we were able to identify distinct gene expression profiles among different thyroid tumour histotypes. Our results suggest that PDTC have a gene expression profile closer to PTC, in particular to the follicular variant, than to FTC. In fact, for PDTC harbouring RAS mutations, a clear similarity to the gene profile of RAS-mutated fvPTC was observed. Interestingly, these RAS-mutated PDTC, presented papillary like nuclei. In keeping with

Figure 3  Expression of the UHRF1, PBK and ITIH5 genes in different tumour histotypes, assessed by quantitative RT–PCR. Relative mRNA levels for UHRF1 (A, B), PBK (C, D) and ITIH5 (E, F) were assessed in an independent sample set (left panel) and in the entire sample set, comprising the microarray and the independent sample sets (right panel). Expression levels were normalised with the GAPDH expression and determined relatively to a calibrator. Error bars denote ± s.e.m. The P values for difference in mean expression between groups were performed using the Kruskal–Wallis with Dunn’s Multiple Comparison test. These genes could not be assayed in one cPTC from the microarray set, PBK could not be evaluated in one PDTC sample, as well as, ITIH5 in one fvPTC and one cPTC from microarray set. cPTC = classic papillary thyroid carcinoma; FTC = follicular thyroid carcinoma; fvPTC = follicular variant of papillary thyroid carcinoma; PDTC = poorly differentiated thyroid carcinoma; NT = normal thyroid tissue.
our findings, it has been observed that fvPTC, in contrast to CPTC, are more frequently aneuploid (Wreesmann et al., 2004), a feature common in PDTC. Therefore, fvPTC are likely to be precursors of PDTC, particularly those cases harbouring RAS mutations.

We also analysed the differential gene expression between tumours and normal thyroid tissues. PTC cases had slightly more over- than under-expressed probe sets, confirming previous reports (Huang et al., 2001). On the other hand, FTC and PDTC had clearly a predominance of downregulated probe sets, which is also in accordance with others (Aldred et al., 2003; Rodrigues et al., 2007). Studies have shown that allelic losses are clearly more frequent in FTC and PDTC than in FTC (Ward et al., 1998; Rodrigues et al., 2004). This could account for the differences in gene expression as a genomic lesion could, theoretically, under-expression of genes. Epigenetic mechanisms, such as DNA hypermethylation, are also likely to explain these expression profiles. Indeed, increased frequency of hypermethylated CpG islands is a common alteration in tumour progression.

Eleven probe sets were simultaneously under-expressed in all tumours relatively to normal tissues, suggesting that these genes may have important suppressor activity in thyroid tumourigenesis. Among these, metallophosphoesterase domain containing 2 (MPPED2) and cellular retinoic acid-binding protein 1 (CRABP1), under-expression have already been observed in thyroid tumours (Griffith et al., 2006).

As observed earlier in other genome-wide studies, comparing clinically aggressive PTC with differentiated FTC cases (Fluge et al., 2006) or comparing FTC to PDTC with normal tissue (Rodrigues et al., 2007) or with WDTC (Montero-Conde et al., 2008), we found that many of the genes differentially upregulated in PDTC relatively to normal tissues were associated with the cell-cycle, indicating that the deregulation of this process is crucial in the progression to more aggressive thyroid tumours. In particular, we identified genes with major roles in mitosis, such as CDC28 protein kinase regulatory subunit 2 (CKS2) and cyclin E2 (CCNE2), which have been reported as over-expressed in various types of tumours (Gudas et al., 1999; Scriidel et al., 2008).

We selected three genes for real-time RT-PCR analysis, which were shown in the microarray analysis, to be differentially over-expressed (PBK and UHRF1) and under-expressed (ITIH5) between PDTC and normal thyroid tissues. UHRF1 and ITIH5 expression levels were statistically different between PDTC and normal thyroid samples. Although not statistically significant, PBK had higher expression levels in PDTC compared with normal thyroid. Therefore, PBK is also a potential therapeutic target, as it encodes a mitotic protein, member of MAPK kinases family, which was found to be over-expressed in haematological (Nandi et al., 2004) and breast tumours (Park et al., 2006), and in PDTC cell lines (Rodrigues et al., 2007). UHRF1 over-expression has been already reported in lung (Jenkins et al., 2005) and breast (Hopfner et al., 2000) cancers. By conventional CGH analyses, the chromosomal locus 19p13.3, where UHRF1 is located, was identified as a common region of chromosomal gains in Hürthle cell thyroid neoplasms (Wada et al., 2002) and recently, using array-CGH, gains in the chromosomal region were also observed (Li et al., 2008). UHRF1 encodes a nuclear protein that transcriptionally regulates TOP2A (Hopfner et al., 2000), an enzyme that catalyses the breaking and rejoining of DNA strands, during transcription. Interestingly, TOP2A showed a five-fold increase in PDTC vs normal tissues (P = 0.02). In addition, UHRF1 is known to regulate the retinoblastoma protein (Jeanblanc et al., 2005) and is involved in the DNA damage response (Jenkins et al., 2005). More recently, an essential role for UHRF1 in the control of DNA methyltransferase 1 (DNMT1), the protein responsible for DNA methylation maintenance in mammalian cells, has also been reported (Bostick et al., 2007; Sharif et al., 2007). UHRF1 and DNMT1 interactions have been shown to be involved in VEGF regulation, a major pro-angiogenic protein (Achour et al., 2008).

Classification according to biological functions showed that about 8.5% of the genes differentially expressed between PDTC and normal tissues were downregulated and all related to cell adhesion. Interestingly, by further analysis, we found that under-expressed genes were mainly related to the cell membrane, encoding receptors, transmembranar or extracellular proteins. In agreement with this, the ITIH5 gene, which may have an essential function in cell attachment and invasion, was under-expressed in PDTC tumours. ITIH5 is a recently discovered member of the inter-alpha (globulin) inhibitor heavy chains (ITIH) gene family (Himmelfarb et al., 2004). In particular, the main function of ITIH is based on their covalent linkage to hyaluronic acid, the major component of the extracellular matrix. Therefore, deregulation of ITIH gene expression affects the stability of the extracellular matrix and so, may promote tumour invasion and metastasis (Bost et al., 1998). Accordingly, ITIH genes have been shown to be downregulated in a variety of human tumours and have been proposed as tumour suppressor or metastasis repressor genes (Hamm et al., 2008). ITIH5 downregulation in breast cancer, caused by promoter hypermethylation, is associated with poorer clinical outcome, and reduced protein expression was proved to be a bad prognostic marker in invasive node-negative patients (Veeck et al., 2008). In fact, we found that lower expression of ITIH5 was statistically associated with larger tumours, as well as with more aggressive cases, such as PDTC (reaching undetectable levels in the two PDTC cell lines). Interestingly, we could also observe that extensively invasive FTC had lower ITIH expression levels than minimally invasive FTCs.

Compared with WDTC, PDTC had enriched gene sets (represented by over-expressed genes) associated earlier with cell cycle and poor prognosis signatures. Interestingly, one of these sets corresponded to a meta-signature of genes differentially over-expressed in undifferentiated relatively to well-differentiated cancers of different tissues (Rhodes et al., 2004). Among the most represented genes in these sets were cell-cycle regulators (CDC2, CCNB2, CDKN3 and TOP2A), as well as genes with a role in the structure of the kinetochore and in the mitotic spindle assembly checkpoint (MSAC) (CENPA, BUB1 and MAD2L1). We also found statistically significant molecular signatures associated with the BUB1 and BUB1 β (yeast) (BUB1B) genes. Some of these genes were reported earlier to be over-expressed in advanced cases of thyroid tumours (Montero-Conde et al., 2008; Wada et al., 2008). These observations indicate that PDTC may have abnormalities in MSAC or in the attachment of kinetochores, which may compromise mitotic fidelity and contribute to chromosomal instability. Accordingly, we observed earlier (Banito et al., 2007) that four out of the five PDTC analysed in this study were aneuploid.

In the analysis of genes differentially expressed between PDTC and WDTC, we only identified two genes. One of these, the 3-phosphoinositide-dependent protein kinase-1 gene (PDK1), encodes a protein responsible for protein kinase B or AKT activation. PI3K/AKT pathway has a central role in regulation of apoptosis, proliferation and cell-cycle progression and its abnormally expression is frequently found in a great number of thyroid tumours (Shinohara et al., 2007). Unexpectedly, and contrary to other cancer types, we observed that PDK1 gene was under-expressed in PDTC.

The identification of molecular mechanisms involved in tumour progression is important in the design of new strategies for treating aggressive neoplasias, such as PDTC and ATC. For instance, over-expression of UHRF1 in PDTC samples, a protein that seems to be essential for DNM1 function, indicates that UHRF1 targeting may offer a new therapeutic approach for PDTC cases. On the other hand, ITIH5 downregulation may be an essential mechanism in thyroid tumourigenesis, especially in tumour metastasis. In addition, and similarly to breast cancer, ITIH5 may prove to be a useful prognostic marker.
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