

Gene expression profiles of thymic neuroendocrine tumors (carcinoids) with ectopic ACTH syndrome reveal novel molecular mechanism

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Abstract

Although there has been increased knowledge about the molecular biology of neuroendocrine tumors (NETs), little is known about thymic carcinoids and even less about those with excessive hormone disorders, such as ectopic ACTH syndrome. This study was designed to gain insights into the molecular networks underlying the tumorigenesis of thymic carcinoids with ACTH secretion. By an approach integrating cDNA microarray and methods of computational biology, we compare gene expression profile between ACTH-producing thymic carcinoids and the normal thymus. In total, there are 63 biological categories increased and 108 decreased in thymic carcinoids. Cell proliferation was stimulated, which may explain the relatively uncontrolled cell growth of the tumor. Dysregulation of the Notch-signaling pathway was likely to be underlying the neuroendocrine features of this type of tumors. Moreover, inhibition of immunity and increased neuropeptide signaling molecules (POMC and its sorting molecule CPE) made the clinical manifestation reasonable and thus validated the array data. In conclusion, thymic carcinoids have a distinct gene expression pattern from the normal thymus, and they are characterized by deregulations of a series of biofunctions, which may be involved in the development of NETs. Hence, this study has provided not only a detailed comprehension of the molecular pathogenesis of thymic carcinoids with ectopic ACTH syndrome, but also a road map to approach thymic NETs at the system level.

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Introduction

Carcinoid tumors were first described as multiple tumors in the distal ileum at autopsy (Lubarsch 1888). The term 'karzinoide' was then used to describe a group of tumors that behaved in a more indolent fashion than typical adenocarcinomas. Carcinoid

tumors derived from the thymus were first documented in 1972 (Rosai & Higa 1972). It can be associated with ectopic ACTH syndrome or may be a component of multiple endocrine neoplasia syndrome type 1 (MEN1). Most carcinoids are benign in behavior, whereas thymic carcinoids display a much more

aggressive phenotype than those originating from other locations (Duh et al. 1987, Moran & Suster 2000, Gal et al. 2001, Kondo & Monden 2003, Tiffet et al. 2003). For the thymic carcinoids with ectopic ACTH syndrome, excessive ACTH production makes the tumors even more aggressive.

As to the potential molecular mechanism, efforts have been focused on the understanding of ectopic ACTH production, while the knowledge about the tumorigenesis is limited and most are genetic studies. There is one study which revealed chromosomal imbalances in ten neuroendocrine thymic tumors, including gains on chromosome Xp, 7p, 7q, 11q, 12q and 20q, and losses at 6q, 6p, 4q, 3p, 10q, 11q and 13q (Rieker et al. 2005). Loss of heterozygosity (LOH) at chromosome 1p has been reported in two thymic neuroendocrine tumors (NETs; Teh et al. 1998). Loss of chromosomes 3, 9p21-pter, Y and gain of chromosome 19p were discovered in one case (Leotlela et al. 2003). Although 25% of the reported thymic NETs are from MEN1 patients (Teh et al. 1998), LOH of the *MEN1* locus on chromosome 11q13 has not been reported in thymic NETs except in one patient. However, no study has yet been performed at the transcriptome level in thymic carcinoids.

To get insights into the molecular pathways responsible for the tumorigenesis of this type of neuroendocrine neoplasm, we conducted cDNA microarrays in five thymic carcinoids with ectopic ACTH secretion and six normal thymus controls.

Materials and methods

Patients

The study was approved by the Ruijin Hospital Ethics Committee for Human Research. Informed consent was obtained from each subject participating in the study after a full explanation of the purpose and nature of all procedures used. Five ACTH-producing human thymic carcinoid (ACs) tissues were obtained at surgery from patients with ectopic ACTH syndrome (Table 1), and AC5 was the recurrent tumor of AC4. The patients presented with a typical Cushing habitus accompanied by hyperpigmentation and hypokalemia. For all these four patients, the high-dose (8 mg) dexamethasone suppression test showed lack of suppression, computed tomography scanning documented anterior mediastinal masses, and the removed mediastinal tumors were confirmed as ACs by positive ACTH and NSE staining (Wang et al. 2006). The six noncancerous thymuses (NCs) were from independent individuals without ectopic ACTH syndrome.

Table 1 Demographic and biochemical findings in the six patients with ectopic ACTH syndrome

Case	Age (years)	Gender	K ⁺ (mmol/l)	HCO ₃ ⁻ (mmol/l)	PC (NR: 7–22 µg/ml)				ACTH (NR: 12–78 pg/ml)	2 mg DST	8 mg DST	Pathology	Tumors in other sites
					0800 h	1600 h	2400 h	UFC (NR: 20–90 µg/24 h)					
1	35	M	2.41	28.8	59.1	56.2	53.5	5473	114.7	-	-	Thymic carcinoid	-
2	29	M	2.62	31.3	39.5	41	40.3	3266	135	-	-	Thymic carcinoid	-
3	44	M	1.97	25.3	41.8	25.9	43.8	3859	536.3	-	-	Thymic carcinoid	-
4	35	F	2.13	33.5	37.6	38.5	28	932.3	765	-	-	Thymic carcinoid	Pituitary microadenoma, thyroid papillary carcinoma
5	41	F	12	3	22	35.7	31.6	14.1	1044.9	-	-	Thymic carcinoid	Pituitary microadenoma
6	44	F	2	3	34	17.4	13.6	12.5	597.4	-	-	Thymic carcinoid	-

PC, plasma cortisol; UFC, urine-free cortisol; DST, dexamethasone suppression test.

While we were preparing this manuscript, two additional ACTH-producing human thymic carcinoids from patients with ectopic ACTH syndrome were obtained (AC6 and AC7).

cDNA microarray

Total RNA was prepared using TRIzol (Life Technologies, Inc.), further purified with RNeasy column (Qiagen). For each of the 11 specimens, ~30 µg RNA was reversely transcribed into cDNA primed with oligo (dT) and labeled with Cy5-dCTP (Red fluorescent dye, R) using Superscript II reverse transcriptase (Life Technologies, Inc.), while the reference used in all hybridizations was prepared by pooling cDNA from six NCs and labeled with Cy3-dCTP (Green fluorescent dye, G). Microarrays with 12 630 cDNA clones representing 10 647 genes were fabricated in-house using a Generation III spotter (Amersham Biosciences). The cDNA clones were sequence verified and enriched with genes expressed in hematopoietic cells (Mao *et al.* 1998, Zhang *et al.* 2000). Among these, cDNA clones include commercial clones from Invitrogen. The scorecard plate including positive control, negative control, dynamic range control, ratio control, and housekeeping genes was spotted on each slide. Detailed information can be found at <http://www1.amershambiosciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&parentid=63004285&moduleid=165076#content>. Microarray slides were obtained from Full moon BioSystems (Sunnyvale, CA, USA). The clones were spotted in a final concentration of 200–400 fmol/µl in spotting buffer (50% DMSO) using 12 microspot pins to reach a complexity of 12 630 spots per slide. After spotting, the slides were u.v. cross-linked (400 mJ) and stored at room temperature (Zheng *et al.* 2005, Du *et al.* 2006).

Data mining

After normalization by intensity-dependent global LOWESS regression for each array, only those cDNAs whose expression was detected in at least 50% of both ACs and NCs were included for statistical analysis. After such preprocessing, 7081 well-measured cDNAs remained for further analysis. The preprocessed data sets were subjected to significance analysis of microarrays (SAM; Tusher *et al.* 2001) by using the unpaired two-class comparison for identifying differentially expressed cDNAs between NCs (NC1, NC2, NC3, NC4, NC5, and NC6) and ACs (AC1, AC2, AC3, AC4, and AC5). SAM utilizes a modified *t*-test statistic and sample-labeled permutations to evaluate statistical

significance measured by the false discovery rate (FDR; Storey & Tibshirani 2003), an estimate of the fraction of falsely significant genes. A significance threshold was expected to be at least 1.5-fold changes and FDR of 1%. Under such a threshold, 2409 significant cDNAs were identified. Furthermore, fold changes in transcript levels were calculated from the mean log₂ expression values of ACs group versus the mean of NCs group, and factitiously reviewed as additive specimen labeled as 'FC'.

As for gene clustering and visualization, a self organising map (SOM) software package implemented with the Matlab 6.5 environment was utilized to train log-transformed (base 2) ratios of intensities between 2409 significant cDNAs over 11 specimens as well as FC with eighty-one (9×9) neurons. Illustration of the SOM outputs was visualized by component plane presentation (CPP; Xiao *et al.* 2003, Fang *et al.* 2008), each presentation illustrating a sample-specific, genome-wide transcriptional map. The SOM outputs by CPP revealed distinct transcriptome profiles between two phenotypes, NCs versus ACs. Such profiling features might provide insights into the biology of thymic carcinoids with ectopic ACTH syndrome.

The search for enriched gene ontology (GO) functional categories in the lists of differentially expressed genes was conducted with MAPPFinder 2.0. The permuted *P* value was calculated by MAPPFinder 2.0 as a statistical measure of significance for gene expression in a given GO functional category. Output from the MAPPFinder was manually filtered to remove processes that represented the same genes (typically parent–child processes).

Real-time PCR

The SYBR Green assay contained 5 µl 2× SYBR Green Master Mix buffer (PE Biosystems, Warrington, UK), 0.1 µl forward and 0.1 µl reverse primer (20 mM), 1 µl cDNA, and 3.8 µl ddH₂O. PCR was carried out by a ABI PRISM 7900 system (Perkin–Elmer, Foster City, CA, USA) as follows: one cycle of 95 °C for 10 min (hot start) and 40 cycles of three steps (95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s). At the end of the amplification, a dissociation curve (melting curve) was built in the temperature range of 65–95 °C. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical adhesive covers (Applied Biosystems, Foster City, CA, USA). PCRs were performed in triplicate, and β-actin was coamplified to normalize the amount of RNA added to the reaction. All data were analyzed using the ABI PRISM SDS 2.0 software.

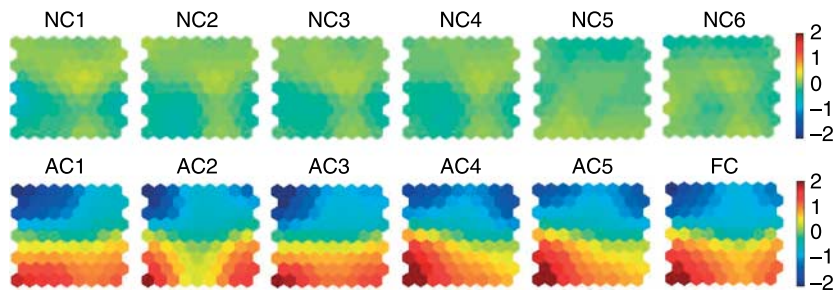


Figure 1 Component plane presentation-integrated self-organizing map (CPP-SOM) of thymic neuroendocrine tumors (carcinoids) with ectopic ACTH syndrome. Eighty-one (9×9) neurons were utilized to train log-transformed (base 2) ratios of intensities 2409 significant cDNAs over 11 specimens as well as ‘FC’. Each presentation illustrates a sample-specific, genome-wide transcriptional map in which up-regulated (red), down-regulated (blue), and moderately regulated (yellow and green) are well delineated. Color-coding index stands for log₂ ratios, with the brighter color to denote the higher value. Noncancerous thymus (NCs) and ACTH-secreting thymic carcinoid (ACs) displayed homologous transcriptome pattern respectively, while distinct between the two groups. FC factitiously represented the expression differences between mean ACs group versus the mean of NCs group.

Results

Transcriptome profiles of thymic NETs (carcinoids) with ectopic ACTH syndrome

To analyze the mechanisms underlying thymic carcinoids with ectopic ACTH syndrome, we performed transcriptome profiling on five samples (ACs) from thymic tumor patients with ectopic ACTH syndrome (Table 1) and six samples (NCs) of the NCs. After microarray hybridization and data acquisition, gene expression data were subjected to SAM to determine those genes with statistically significant differences. By using the criteria of FDR <1% and at least 1.5-fold changes, 2409 cDNAs were selected representing the transcriptome signatures of thymic carcinoids with ectopic ACTH syndrome. We then applied CPP-integrated SOM (CPP-SOM) for gene clustering and visualization of significant expression data. As shown in Fig. 1, CPP-SOM offers a global view of gene clustering, particularly with respect to the expression patterns. Genes mapped to the corner/edge areas of the map are mostly regulated, with red representing up-regulation and blue representing down-regulation. Also, each presentation of SOM illustrates a sample-specific transcriptome map, permitting direct comparisons of transcriptome differences between two phenotypes NCs versus ACs. Furthermore, gene expression-based sample relationships were visualized in the three-dimensional space captured by principal component analysis, as demonstrated in Fig. 2. Both of these analyses revealed homologous transcriptome profiles within NCs (and ACs), while distinct transcriptome profiles between NCs and ACs. It is of note that AC4 and AC5 shared a similar expression pattern (Fig. 1) and the closer

sample relationship (Fig. 2) when compared with other samples of ACs. It was consistent with the factor that AC5 was the recurrence of AC4, and also indicated that similar biological behavior could be reflected by similar expression profile.

To characterize the major biological processes (P), molecular functions (F), and cellular components (C), we used MAPPFinder (a component of GenMAPP version 2.0) to link gene expression data to the GO hierarchy. MAPPFinder produced a statistical list (based on permuted *P* value <0.05) of GO biological categories associated with the differentially

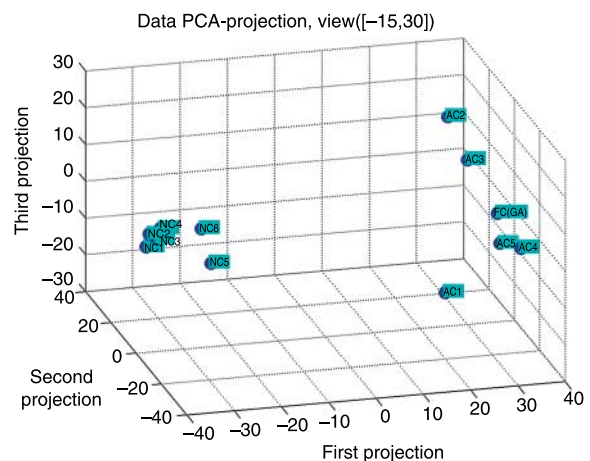


Figure 2 Visualization of sample relationships by principal component analysis (PCA). The three-dimensional plot illustrates PCA of 11 samples as well as FC illustrating the relationships based on 2409 significant cDNAs expression data. NC, noncancerous thymus; AC, ACTH-secreting thymic carcinoid. FC factitiously represented the expression differences between mean ACs group versus the mean of NCs group.

expressed genes, from which the most significant nonsynonymous categories with at least three genes changed were identified (permuted $P < 0.05$). In total, there are 63 biological categories increased and 108 decreased in thymic carcinoids. We then focused on those that might explain the tumor behavior and clinical presentations. After quantitative PCR confirming the aberrant expression of some of the potential candidate genes both in the four tumors using microarray data and two additional ACTH-producing thymic carcinoids (Fig. 3), an overview of the molecular pathology involvement was revealed and was addressed as follows.

Stimulation of cell proliferation pathway coordinating tumor phenotype

GO analysis revealed significantly increased regulation of cell proliferation. Table 2 demonstrated all the genes related to proliferation and those significantly deregulated in thymic carcinoids, with *CDC25B* the most up-regulated, *CTBP1* the most down-regulated, and also the deregulated Wnt/ β -catenin-signaling pathway (Fig. 3A and B).

Wnt and Notch pathways coordinating the characteristics of neuroendocrine differentiation

Among the aberrant expression of genes involved in cell differentiation, the Wnt-signaling pathway was revealed as being tightly associated with thymic carcinoids (Table 3). *CTNNB1*, as previously described, was present at higher levels in AC patients. The expression of β -catenin target genes, *MYC* (*c-Myc*) and *CCND1* (*cyclin D1*), was also examined in AC tissues. *CCND1* expression was consistently high in AC tumors and *c-Myc* expression changed modestly (Fig. 3B). *PPP2CB*, which was up-regulated on average about three times (Fig. 3C), encodes the phosphatase 2A (PP2A) catalytic subunit. Consistently lower expression of *NOTCH2*, the encoding gene for one of the Notch family members, was observed in our thymic carcinoid compared with the normal thymus (Fig. 3C).

Molecular pathways coordinating the clinical manifestation of thymic carcinoids with ectopic ACTH syndrome

Unlike the etiological pathways described above, some other pathways revealed in our data might be a consequence of the tumors. The up-regulated

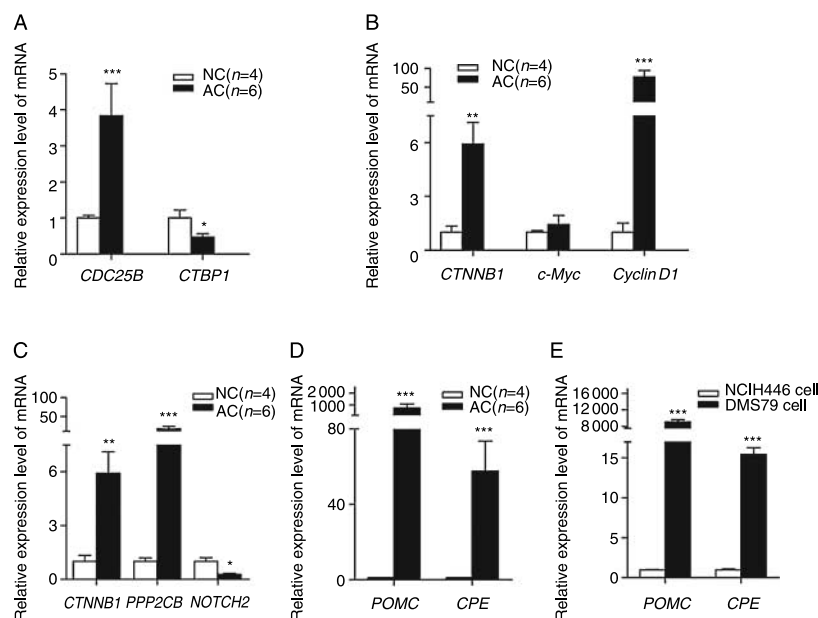


Figure 3 Quantitative PCR revealed consistent alteration with the array data in the thymic carcinoid tissues. (A) Increased expression of *CDC25B* and decreased expression of *CTBP1* in AC patients ($n=6$) compared with NC patients ($n=4$). (B) β -Catenin and its downstream target genes expression was up-regulated in AC patients ($n=6$). (C) Decreased expression of *NOTCH2* and Wnt pathway molecules in AC patients ($n=6$). (D) *POMC* and *CPE* gene expression increased dramatically in AC patients ($n=6$). (E) *POMC* and *CPE* displayed much higher expression level on DMS79 cell compared with NCIH446 cell. Gene expression level was normalized to the corresponding levels of β -actin mRNA. Data are represented as the mean \pm s.e.m. Unpaired Student's *t*-test was used for evaluation of statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. NC, noncancerous thymus; AC, ACTH-secreting thymic carcinoid.

Table 2 Enriched functional category of genes involved in regulation of cell proliferation

Genbank	Symbol	FC	AC1	AC2	AC3	AC4	AC5	FDR (%)
NM_021874	<i>CDC25B</i>	3.73	2.92	4.55	5.40	1.47	3.34	0.02
NM_006454	<i>MXD4</i>	2.20	1.88	2.48	2.22	2.23	2.13	0.04
NM_003641	<i>IFITM1</i>	1.71	1.31	1.83	1.73	1.64	1.51	0.03
NM_003029	<i>SHC1</i>	1.62	1.79	0.50	1.39	1.16	1.26	0.03
NM_001904	<i>CTNNB1</i>	1.54	-0.36	2.49	0.41	1.29	1.86	0.29
NM_002032	<i>FTH1</i>	1.49	1.95	1.39	1.29	1.43	1.31	0.03
AF018081	<i>COL18A1</i>	1.32	-0.37	-0.03	2.27	1.87	1.92	0.29
NM_005655	<i>TIEG</i>	1.09	0.94	0.65	0.78	1.55	1.21	0.20
NM_000508	<i>FGA</i>	1.02	0.82	1.59	1.03	1.94	0.28	0.22
NM_002831	<i>PTPN6</i>	-0.97	-0.40	-0.69	-1.13	-0.96	-1.00	0.24
AV654571	<i>PCAF</i>	-1.01	-0.08	-1.39	-0.62	-1.29	-1.24	0.53
NM_004235	<i>KLF4</i>	-1.13	-0.70	-0.73	-1.14	-1.38	-0.73	0.44
NM_006019	<i>TCIRG1</i>	-1.14	-1.13	-0.25	-0.90	-1.32	-1.05	0.46
NM_005620	<i>S100A11</i>	-1.24	-1.92	-1.27	-2.63	-1.95	-1.30	0.13
NM_016418	<i>NF2</i>	-1.24	-0.25	-1.28	-0.59	-1.40	-1.37	0.44
NM_003144	<i>SSR1</i>	-1.29	-0.46	-2.02	-0.72	-1.44	-1.42	0.40
NM_005746	<i>PBEF1</i>	-1.32	-1.73	-0.76	-1.12	-1.50	-1.41	0.41
NM_005962	<i>MXI1</i>	-2.00	-1.17	-3.00	-2.77	-2.22	-1.69	0.25
NM_001328	<i>CTBP1</i>	-3.38	-1.65	-4.80	-6.03	-2.27	-2.13	0.09

AC, ACTH-secreting thymic carcinoid.

neuropeptide-signaling pathway is among them (Table 4). *POMC*, the ACTH encoding gene, was strikingly up-regulated by more than four times in these patients. *CPE* with the corresponding protein carboxypeptidase E was increased by more than three times (Fig. 3D). We also examined *POMC* and *CPE* expression in DMS79 and NCIH446 cell lines, which respectively represent human ACTH-producing small cell lung cancer and nonACTH-producing small cell lung cancer. In accordance with the results of the patients, DMS79 cell line displayed high levels of *POMC* and *CPE* expression (Fig. 3E), which to some extent compensate with the display on the patients.

Discussions

As a type of neoplasm, thymic carcinoids are also characterized by uncontrolled cell proliferation, although with relatively benign features. *CDC25B* is a member of the *CDC25* family of phosphatases, which primarily targets cyclin B-Cdk1 and controls the entry into mitosis. Both *in vitro* and *in vivo* studies have confirmed an oncogenic role for *CDC25* (Kristjansdottir & Rudolph 2004, Boutros et al. 2007), especially the clear and consistent trend associating *CDC25* with breast cancer (Galaktionov et al. 1995, Ma et al. 2001). As to those tumors with neuroendocrine origin, it was reported that only

Table 3 Enriched functional category of genes involved in regulation of cell differentiation

Genebank	Symbol	FC	AC1	AC2	AC3	AC4	AC5	FDR (%)
NM_004156	<i>PPP2CB</i>	2.64	2.24	1.53	2.84	2.87	2.70	0.01
D80010	<i>LPIN1</i>	1.92	2.62	0.57	1.37	2.32	2.24	0.02
NM_014707	<i>HDAC9</i>	1.86	1.75	2.16	1.85	1.53	1.74	0.01
NM_014863	<i>GALNAC4S-6ST</i>	1.52	2.11	1.92	1.58	0.56	0.48	0.01
NM_012443	<i>SPAG6</i>	1.34	1.04	1.00	0.60	1.64	1.56	0.01
NM_016645	<i>NEUGRIN</i>	1.31	1.58	1.49	1.62	0.51	0.73	0.01
NM_000214	<i>JAG1</i>	1.11	1.10	-0.37	1.10	1.71	1.66	0.13
AB044088	<i>BHLHB3</i>	1.08	0.46	1.35	1.28	0.27	0.91	0.24
NM_001242	<i>TNFRSF7</i>	-1.11	0.12	-1.31	0.02	-0.92	-0.81	0.24
AF208043	<i>IFI16</i>	-1.17	0.33	-1.53	-2.06	-1.55	-1.53	0.06
NM_021103	<i>TMSB10</i>	-1.31	-2.52	-0.44	-0.90	-1.39	-1.39	0.01
AJ227863	<i>MBNL1</i>	-1.50	-0.73	-1.87	-1.61	-1.34	-1.52	0.01
NM_024408	<i>NOTCH2</i>	-1.58	-1.17	-2.47	-0.92	-1.85	-1.54	0.01

AC, ACTH-secreting thymic carcinoid.

Table 4 Enriched functional category of genes involved in regulation of neuropeptide signaling pathway

Genebank	Symbol	FC	AC1	AC2	AC3	AC4	AC5	FDR (%)
NM_000939	<i>POMC</i>	4.27	2.92	4.55	5.40	1.47	3.34	0.06
NM_001873	<i>CPE</i>	3.29	3.07	3.99	3.28	3.06	2.99	0.02
AL161973	<i>MLLT4</i>	1.65	1.20	1.23	0.53	2.15	1.77	0.02
AF307080	<i>LPHN3</i>	1.11	0.18	1.45	0.36	1.78	1.03	0.10
NM_012302	<i>LPHN2</i>	0.99	0.81	-0.23	0.02	0.40	1.04	0.10
AB018301	<i>GPR116</i>	0.82	0.35	0.05	1.16	1.47	1.40	0.29
AF478567	<i>PDZGEF2</i>	-0.92	-0.90	-0.66	-0.97	-0.49	-0.50	0.12
NM_016340	<i>PDZGEF2</i>	-0.94	-0.32	-0.69	-0.69	-0.39	-0.37	0.08
BC000316	<i>RAB2L</i>	-1.00	-0.76	-0.72	-0.92	-1.10	-1.49	0.12
NM_001703	<i>BAI2</i>	-1.10	-1.22	-0.77	-1.03	-1.16	-1.14	0.05
NM_004761	<i>RAB2L</i>	-1.11	-1.39	-0.86	-1.06	-0.86	-1.05	0.05
NM_014737	<i>RASSF2</i>	-1.17	-1.25	-1.24	-1.61	-1.32	-1.10	0.02

AC, ACTH-secreting thymic carcinoid.

CDC25-positive medullary thyroid carcinoma showed a significantly worse disease-free survival rate than those without expression (Ito *et al.* 2005). *CTBP1*, which is the most down-regulated gene in our study of thymic carcinoids, encodes a phosphoprotein belongs to CTBP family. CTBP proteins are conserved among vertebrates as well as invertebrates and function as transcriptional corepressors. Possibly by binding and modulating *EIA* (Delouis *et al.* 2005), *Evi-1* oncogene (Senyuk *et al.* 2002), *BRCA1* (Izutsu *et al.* 2001), and *P53* (Paliwal *et al.* 2006), *CTBPs* are tightly involved in oncogenesis in humans. Loss of *CTBP* expression has been reported in malignant melanoma and knock-out of wild-type *CTBP* was associated with progression of human melanoma (Poser *et al.* 2002).

The canonical Wnt-signaling pathway has a critical role in cell fate determination (e.g. the decision to proliferate or differentiate; Reya & Clevers 2005). *CTNNB1*/β-catenin (β-catenin), which is the central and essential component in the Wnt-signaling cascade, was up-regulated more than five times in AC patients. Aberrant activation of β-catenin promotes cell proliferation and initiates colorectal tumorigenesis (van de Wetering *et al.* 2002). More recently, Kim *et al.* (2008) reported that of 51 solid pseudopapillary neoplasm cases, 94.4% were positive for nuclear β-catenin, which indicates that the Wnt/β-catenin pathway might take part in NETs tumorigenesis. To search for more evidence of Wnt pathway activation, we examined the β-catenin target genes *MYC* (*c-Myc*) and *CCND1* (*cyclin D1*) expression in AC tissues. We found consistently high level of *cyclin D1* in AC tumors, which is required for cell cycle G₁/S transition and affects cell cycle progression. However, *c-Myc* expression changed only modestly. This difference may be attributed in part to tissue-specific characteristics of Wnt target genes. Besides these extremely

deregulated genes, 11 other genes displayed increased expression and 10 decreased. It is then speculated that up-regulation of genes like *CDC25B*, Wnt/β-catenin and down-regulation of genes like *CTBP* indicated higher modulation of cell growth in thymic carcinoids.

Compared with common tumors derived from epithelial cells, thymic carcinoids display remarkable neuroendocrine differentiation. NETs are a very heterogeneous group arising from neuroendocrine cells, which are distributed in many tissues and organs. Considering the morphological and physiological similarity, it is most likely that there is a specific common genetic switch underlying this large group of specific tumors (Barakat *et al.* 2004), while little has been discovered yet. As we described above, the Wnt/β-catenin pathway not only promotes cell proliferation but also affects cell differentiation. It has been implicated in neuroendocrine differentiation, such as endocrine cell development in the anterior lobe of pituitary (Treier *et al.* 1998) and neuroendocrine transdifferentiation of prostate cancer cells (Yang *et al.* 2005). Higher expression of *CTNNB1* in AC patients indicated its potential participation in neuroendocrine differentiation of thymic carcinoids. *Xenopus* studies confirmed a positive role for the PP2A catalytic subunit in Wnt signal transduction (Ratcliffe *et al.* 2000). The Notch family is another evolutionarily conserved signaling pathway that controls cellular differentiation. *NOTCH2*, the encoding gene for one of the family members, is down-regulated in the differentiated corticotrope (Raetzman *et al.* 2004), and persistent expression of *NOTCH2* could delay gonadotrope differentiation (Raetzman *et al.* 2006). The authors considered that the absence of Notch signaling may be important to permit differentiation, which might also be the case in our thymic carcinoids, since consistently lower expression was observed

compared with the normal thymus. Although other evidence is necessary to establish the roles of Wnt and Notch pathways in the neuroendocrine features of thymic carcinoids, our data has provided a clue.

CPE has been found to be involved in pulmonary NETs (He *et al.* 2004), pituitary adenomas (Fan *et al.* 2002), and insulinoma (Wang *et al.* 2004). Suppression of pathways involved in the immune response, inflammatory response, antigen processing, and immune cell activation explained the immunosuppression status of such patients.

From our data, it is not possible to reflect the aggressive biologic behavior of thymic carcinoids, because three out of four patients demonstrated benign features and are still well after follow-up for 5–6 years, and only one suffered recurrence 4 years after the first surgery. Consistently, the functional category associated with cell motility was down-regulated in thymic carcinoids.

Ectopic ACTH syndrome caused by nonpituitary NETs has been known for several decades, and numerous studies have been trying to clarify the molecular basis of POMC deregulation. This included large-scale gene expression analysis in bronchial carcinoids (Pascual-Le Tallec *et al.* 2002) and DMS-79 cells (Turney *et al.* 2004). To the best of our knowledge, this is the first study focusing on the tumorigenesis of this type of tumor.

In summary, thymic carcinoids have a distinct expression pattern compared with the normal thymus. They are characterized by deregulations of many biofunctions including abnormal proliferation and differentiation signals, which may be involved in the development of NETs. Other abnormalities like activation of neuropeptide signaling and inhibition of immune response might explain the hormone disorder and immunity defects evident in ectopic ACTH syndrome.

Declaration of interest

There is no conflict of interest that would prejudice the impartiality of the research reported. Any financial or potential conflict of interest is fully declared within the text of the article.

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