Hypomethylation in the promoter region of POMC gene correlates with ectopic overexpression in thymic carcinoids

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Abstract

The ectopic ACTH syndrome is caused by abnormal expression of the POMC gene product arising from non-pituitary tumors in response to the ectopic activation of the pituitary-specific promoter of this gene. It has been proved that methylation of the CpG island in the promoter region is associated with silencing of some genes. Using bisulphite sequencing, we identified hypermethylation in the 5′ promoter region of the POMC gene in three normal thymuses and one large cell lung cancer, and hypomethylation in five thymic carcinoid tumors resected from patients with ectopic ACTH syndrome. The region undergoing hypermethylation was narrowed to coordinates −417 to −260 of the POMC promoter. Furthermore, we observed that the levels of POMC expression correlated with the methylation density at −417 to −260 bp across the E2 transcription factor binding region of the POMC promoter. It is concluded that hypomethylation of the POMC promoter in thymic carcinoids correlates with POMC overexpression and the ectopic ACTH syndrome.

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Introduction

A large number of peptide hormones are produced in tumors outside the specialized endocrine gland, resulting in ectopic hormone syndromes. The tissues of origin from which the tumors arise usually do not produce such hormones physiologically, suggesting a common mechanism for initiating ectopic hormone gene expression. The ectopic adrenocorticotropic hormone (ACTH) syndrome is one of the most common ectopic hormone syndromes, and is caused by, among others, small cell lung cancers and thymic carcinoids (Beuschlein & Hammer 2002). It has been known that DNA methylation of CpG islands is a developmentally regulated process that acts in concert with other regulatory mechanisms to control gene expression (Yang *et al.* 2003, Popiela *et al.* 2004), including tissue-specific gene expression (Paroush *et al.* 1990, Kirillov *et al.* 1996). The CpG islands of the pro-opiomelanocortin (POMC) promoter region was first described by Gardiner-Gardin & Frommer (1994). CpG islands are usually unmethylated in tissues where the gene of interest is physiologically expressed. In contrast, in the pituitary-specific promoter of the POMC gene they are methylated in non-expressing tissues (Newell-Price *et al.* 2001). POMC promoter activity has been widely studied in the small cell lung cancer cell line, DMS-79. In contrast, the relationship between POMC promoter activity and its methylation status has not been reported in ACTH-secreting thymic carcinoids.

In this study, we analyzed the pituitary promoter region of the POMC gene in tissues generated from normal thymuses and ACTH-secreting thymic carcinoids. We demonstrated that CpG islands in the POMC promoter region were hypomethylated in thymic tissues derived from thymic carcinoid tumors of patients with the ectopic ACTH syndrome, whereas they were hypermethylated in the normal thymuses from which ACTH-secreting tumors arose, suggesting a positive correlation between CpG island hypomethylation and POMC promoter activity in thymic carcinoids.

Materials and Methods

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections were deparaffinized in xylene and rehydrated through descending ethanol to distilled water. Endogenous peroxidase was quenched by incubating the slides in 3% H_2O_2 for 10 min at room temperature. The primary antibodies used were

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mouse monoclonal anti-ACTH antibodies at 1:1000 dilution (Dako Corporation, Carpinteria, CA, USA).

RNA extraction and quantitation of POMC gene expression

RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was heated at 70 °C for 5 min and placed on ice for 5 min. A mix of 5 μl M-MLV RT 5 × reaction buffer, 10 mM of each of the four dNTPs, 200 U of M-MLV transcriptase enzyme (H–), 25 pg/μl of oligo (dT) primer and 20 U of RNase inhibitor (all from Promega, Madison, WI, USA) was added to each sample, followed by incubation at 40 °C for 60 min and 70 °C for 15 min. Two μl of cDNA products was amplified with 1 unit of Ampli Taq Gold (PE Applied Biosystems, Foster, CA, USA) and the specific primers for POMC spanning different exons. β-actin-specific primers were used as an internal control. The sequences of the primers used to amplify the POMC gene (+72 ~ +612) are as follows: forward 5′-AGCCTCCCGAGACAGAG CCT-3′ and reverse 5′-GTTAGGGTACACCTTCAC TGGG-3′. Reactions were carried out in a PTC-225 DNA Engine Tetrad (MJ Research Inc., Waltham, MA, USA) thermocycler. Denaturation was performed at 95 °C for 8 min, followed by 45 s at 95 °C, annealing for 45 s at 58 °C and extension for 1 min at 72 °C for 30 cycles. These conditions were chosen to avoid reaching a plateau phase at the end of the amplification. The PCR products were loaded onto 1·5% agarose gels and visualized by ethidium bromide under UV light.

In order to quantify the POMC transcripts among the thymic carcinoids, real-time PCR was performed using double stranded DNA dye SYBR Green PCR Master Mix (PE Biosystems, Warrington, Cheshire, UK) on the ABI PRISM 7900 system (Perkin-Elmer, Boston, MA, USA). The reaction of the SYBR Green assay contained 5 μl of 2 × SYBR Green Master Mix buffer, 0·1 μl forward and 0·1 μl reverse primers (20 mM), 1 μl cDNA and 3·8 μl double distilled H_2O. PCR was carried out 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 plotted in the temperature range 65–95 °C. All amplifications and detections were carried out in a MicroAmp optical 384-well reaction plate with optical adhesive covers (Applied Biosystems). PCRs were performed in triplicate and β-actin was co-amplified to normalize the amount of RNA added to the reaction. All data were analyzed using the ABI PRISM SDS 2·0 software (Applied Biosystems, Foster, CA, USA). Primer sequences were as follows; POMC (+124 ~ +362): forward 5′-AAGATGCCGAG ATCGTGCTG-3′ and reverse 5′-ATGACGTACTTCC GGGGGTTC-3′; β-actin: forward 5′-CATCCTCACC CTGAAGTACCC-3′ and reverse 5′-AGCCTGGATAG CAACGTACATG-3′.

DNA extraction and sequencing of the POMC promoter

The thymic carcinoid tissues were digested with proteinase K (Merck, KGaA, Darmstadt, Germany) and DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). Amplification of the POMC promoter (nucleotides –700 to + 254) was obtained with the forward primer 5′-CCACAGGGAAAGCACTTC-3′ and the reverse primer 5′-GGCTTCTCATGCCGCAGT-3′, using the same conditions described for the RT-PCR. PCR products were purified by gel extraction (Omega BioTek, Doraville, GA, USA) and directly sequenced by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Bisulphite modification and sequencing

Five hundred ng of genomic DNA were digested with PstI overnight, denatured at 100°C for 5 min and treated with 0·3 M NaOH for 15 min. The denatured DNA was mixed with 2 volumes of 2% (w/v) melted low melting point (LMP) agarose (FMC, Rockland, ME, USA). Five μm beads were formed in ice-cold mineral oil. The beads were then treated with a 2·5 M bisulphite solution on ice for 30 min, followed by incubation at 50 °C for 6 hours. The bisulphite solution was freshly prepared by mixing 7·6 g sodium bisulphate (Sigma, St. Louis MO, USA) in 10 ml H_2O and 3 ml fresh 2 M NaOH and 0·22 g hydroquinone (Sigma) in 2 ml H_2O. Beads were washed in 1 × TE buffer (pH 8·0), treated with 500 μl 0·2 M NaOH, and washed again with 1 x TE buffer (pH 8·0). Bisulphite-converted DNA in the beads was amplified by nested-PCR using the forward primer 5′-GGTTTT A GGGGAGTAGTGTAA-3′, and the reverse primer 5′-CTACGACCCTTACCTATCTC-3′ (-566 ~ +98); nest-forward 5′-ATAATGGGGAA ATCTGGAGGT-3′, nest-reverse 5′-CGAACTCTTCT TCCCCTCCTT-3′ (-436 ~ +30). PCR products were visualized on 1% agarose gel, recovered and sequenced by both direct sequencing and subclone sequencing using ABI PRISM 3100 Genetic Analyzer.

Informed consent was obtained from every participant and the study was approved by the hospital ethical board.

Results

Immunohistochemistry

The ectopic ACTH syndrome was diagnosed in patients who presented with enlargement of the mediastinum, as detected by computed tomography, elevated plasma cortisol and ACTH, and lack of ACTH suppression by 8 mg dexamethasone. ACTH staining was positive in each of the five thymic carcinoid tumors resected from patients with the ectopic ACTH syndrome (Fig. 1).

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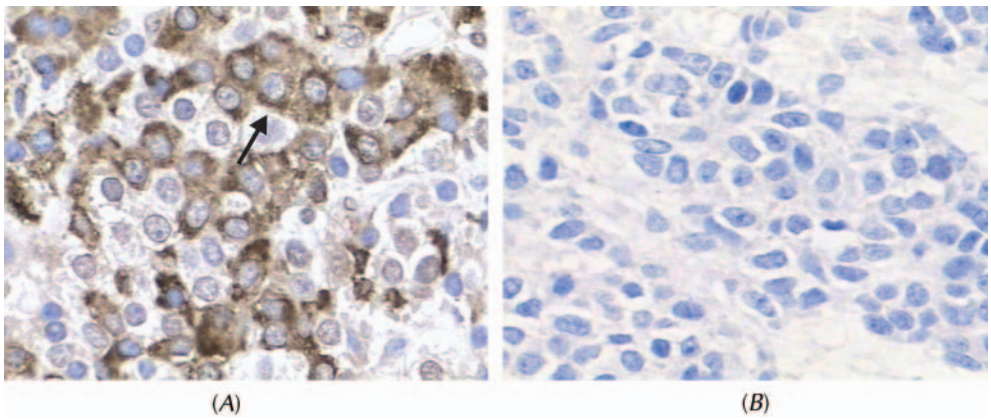

Figure 1 ACTH immunohistochemisty. (A) ACTH was stained in each of the five thymic carcinoids. The arrow indicates brown ACTH particles. (B) ACTH was not stained in the normal thymus. (Magnification × 400)

Quantification of POMC gene expression

RT-PCR showed POMC overexpression in all five thymic carcinoids (TC1–5), whereas no POMC expression was observed in three normal thymuses (N1–3) and one large cell lung cancer (Fig. 2). Real time PCR demonstrated that POMC transcripts were expressed at levels at least 1000 times greater in four out of five thymic carcinoids compared to normal thymus. Among these four carcinoids, those from patients 4 (TC4) and 5 (TC5) stood out because they showed the highest and lowest levels of POMC expression respectively. POMC expression was minimal in the RNA exctracted from the patient with large cell lung cancer (Fig. 3A and B).

Hypomethylation of the POMC promoter in thymic carcinoids

The CpG islands of the POMC promoter region were predicted using the WebGene computer program (http://www.itab.mi.cnr.it/webgene/). This analysis showed a region from –486 to +1290 bp as a CpG island with a GC content of 59% and a ratio of observed:expected CpG of 0·78. Direct DNA sequencing after bisulphite treatment demonstrated hypermethylation in the POMC promoter region in normal thymic tissues compared to thymic carcinoids (Fig. 4). However, because the base-calling program from ABI PRISM cannot be used for quantification of the amount of methylation observed on direct sequencing of the PCR products, we performed subclone sequencing of individual molecules amplified from –436 to +30 bp across the pituitary-specific promoter region of the POMC gene. This analysis demonstrated that hypomethylation was principally located at –417 to –260 bp in the thymic carcinoids (Fig. 5).

Discussion

The POMC gene has three transcripts, the shortest one of which is about 800 nucleotides long. This transcript lacks exons 1 and 2 and is expressed in many normal adult

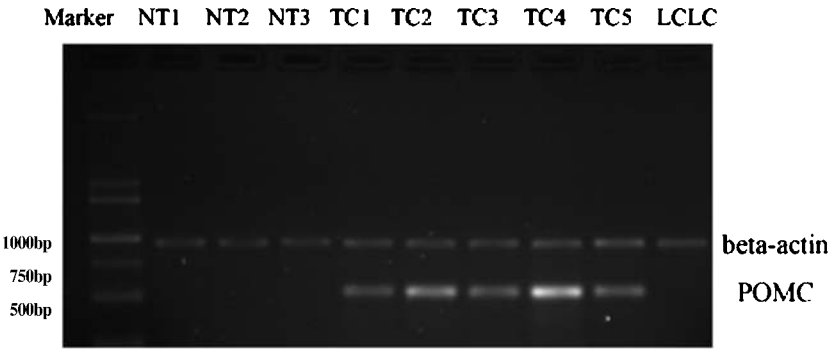

Figure 2 RT-PCR of POMC. POMC was overexpressed in the thymic carcinoids removed from five patients with ectopic ACTH syndromes (TC1–5), whereas POMC was not expressed in three normal thymuses (NT1–3) and one large cell lung cancer (LCLC).

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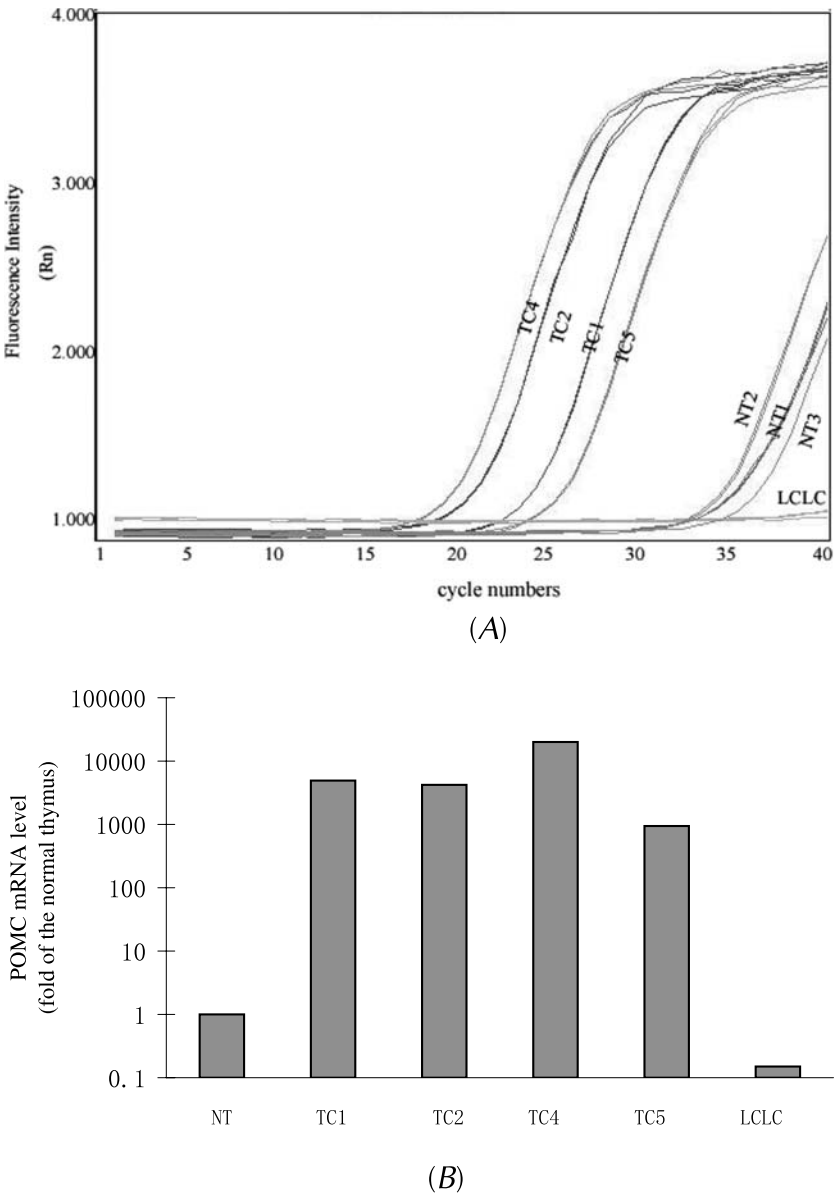

Figure 3 Real-time PCR for quantification of POMC expression. (A) Amplification profiles of POMC in four thymic carcinoids (TC), three normal thymuses (NT) and one large cell lung cancer (LCLC). (B) qPCR analysis was normalized to the corresponding levels of β-actin mRNA. POMC expression levels are indicated as an arbitrary ratio of the mean value of three normal thymuses. In the thymic carcinoids POMC transcripts were present at a level at least 1,000-fold more than in the normal thymuses. Among the thymic carcinoids, POMC expression was highest in TC4 and lowest in TC5 respectively.

tissues, such as the adrenal glands, the thymus and the testes (Lacaze-Masmonteil *et al.* 1987). The longest transcript is 1450 nucleotides and is expressed in non-pituitary tumors (De Keyzer *et al.* 1989). The 800 nucleotide transcript cannot encode the bioactive protein as it lacks a signal peptide necessary for membrane translocation and precursor processing. The transcripts expressed in the thymic carcinoids of our patients were bioactive and measured 1200 nucleotides in length (Rees *et al.* 2002), which is in agreement with our previous study (Wang *et al.* 2003).

It has been reported that ectopic ACTH production is due to abnormal changes of epigenetic or regulatory factors of the POMC gene (de Keyzer *et al.* 1997,

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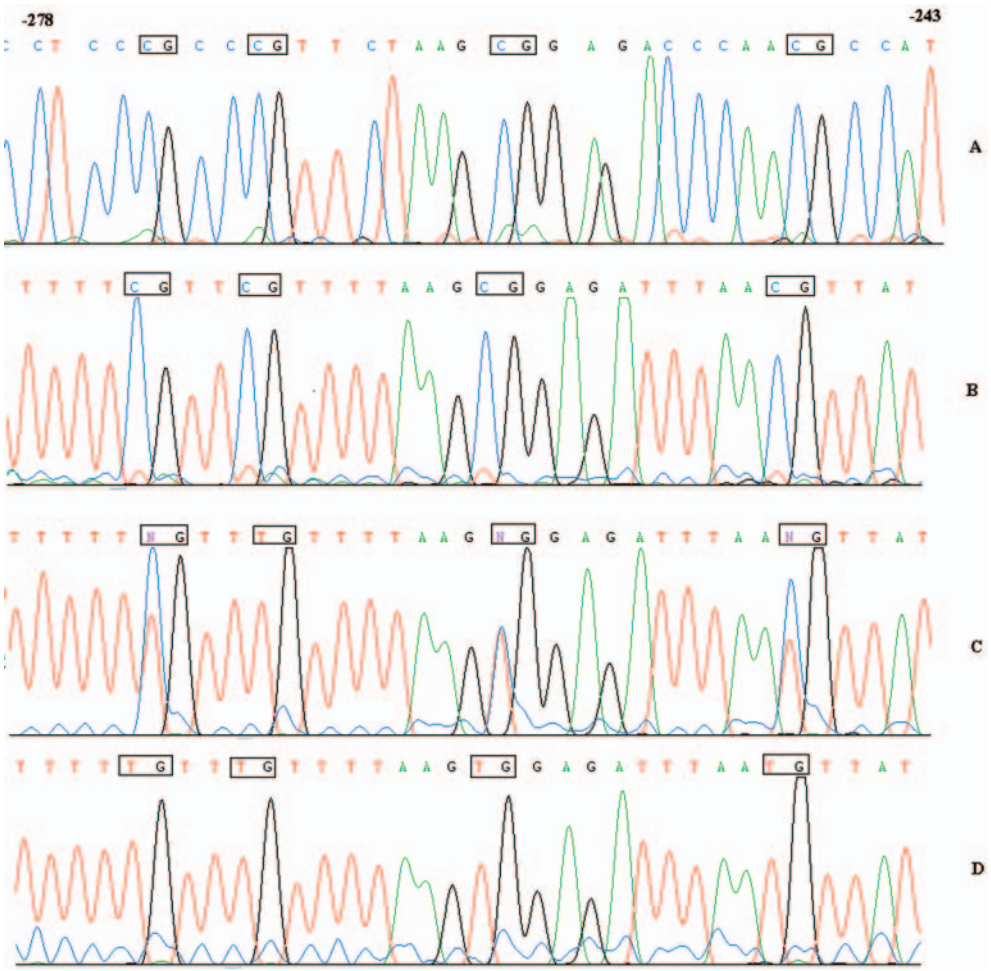

Figure 4 Bisulphite treatment and sequencing. Cytosine (C) was transformed to thymine (T) with bisulphite treatment unless C was methylated. Four CpG sites between –278 and –243 bp in the POMC promoter region were demonstrated. Row A represents sequences before bisulphite treatment and rows B, C and D represent the corresponding sequences after bisulphite treatment in one large cell lung cancer, one normal thymus and one thymic carcinoid respectively. Complete methylation was observed in the large cell lung cancer, partial methylation in the normal thymus and hypomethylation in the thymic carcinoid.

Picon *et al.* 1999*a*, *b*, Newell-Price *et al.* 2001). Functional analysis showed that the +21 to –417 region conferred full promoter activity in both DMS-79 and AtT-20 cells (Picon *et al.* 1995). This region is located in a CpG island of 1776 bp. Direct sequencing following bisulphite treatment revealed hypermethylation of this CpG islands at –260 to –417 bp in normal thymus. In contrast, no hypermethylation could be detected in the DNA derived from the thymic carcinoids. To rule out the possibility that genomic DNA hypomethylation is a cancer-specific feature, we further analyzed DNA extracted from a large cell lung cancer that did not express POMC and showed complete methylation of all CpG sites in the POMC promoter region. DNA hypermethylation was also found in the POMC promoter of a pituitary adenoma, malignant insulinoma and normal lung lacking POMC expression, whereas hypomethylation was identified in the DMS-79 cell line, bronchial carcinoids and pituitary corticotroph adenoma expressing POMC (Newell-Price *et al.* 2001). Usually the methylated CpG islands in the promoter regions block transcription initiation by binding methylated CpG binding proteins, and transcription repressors (Nan *et al.* 1998). In our study, POMC was overexpressed in all ACTH-secreting thymic carcinoid tumors. TC4 stood out because there was an extremely elevated expression of POMC, and at the same time there was a completely unmethylated POMC promoter. In contrast, there was almost no expression of POMC in the presence of a hypermethylated POMC promoter in the tissue extracted from a large cell lung cancer. POMC expression

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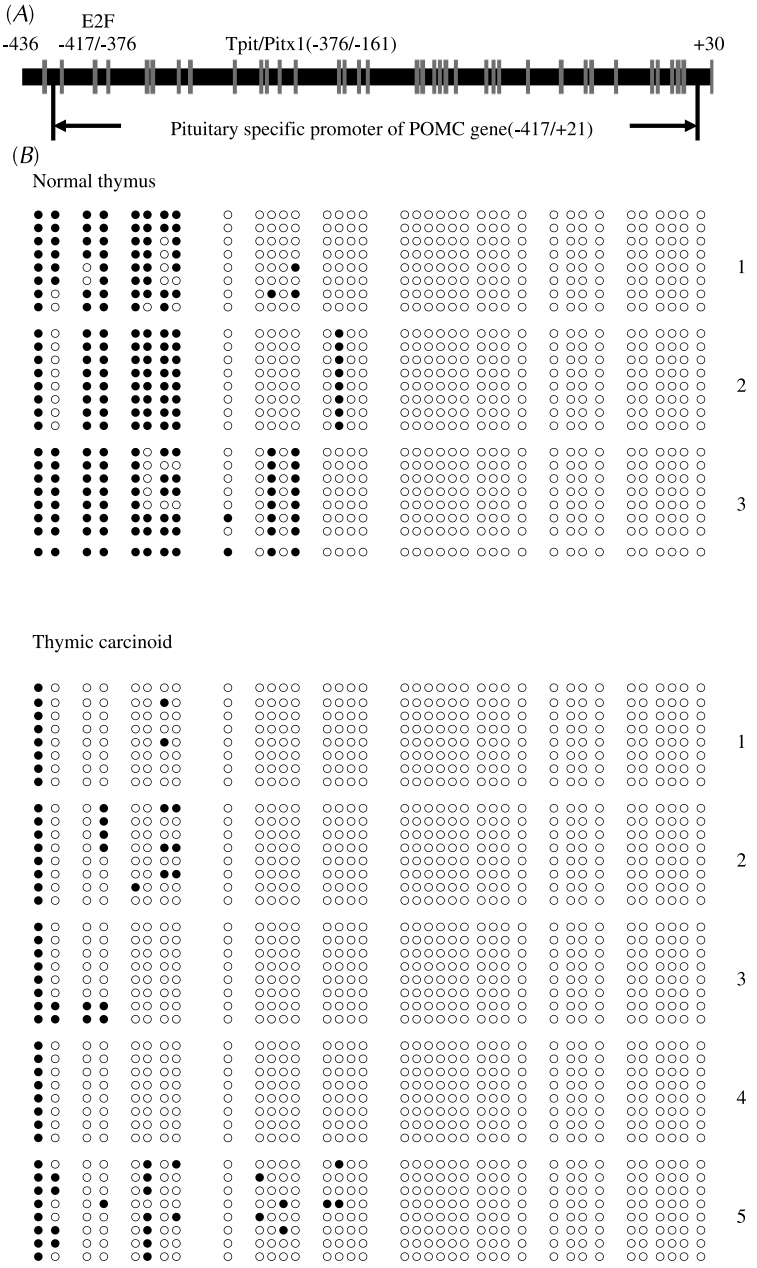

Figure 5 Subclone squencing of CpG islands in the POMC promoter. (A) represents the CpG island to scale, with vertical lines representing individual CpG dinucleotides. (B) represents CpG methylation density to scale, with each row representing a single clone; 8 clones were selected for sequencing from each of three normal thymuses (1, 2 and 3) and five thymic carcinoids (1, 2, 3, 4, 5). Each open circle represents an unmethylated CpG site, each closed circle represents a methylated CpG site. The region from –417 to –260 was hypomethylated or even unmethylated in the five thymic carcinoids compared to the three normal thymuses. The region downstream of –290 (–260 ~ +30) in the POMC promoter was thoroughly unmethylated in both thymic carcinoids and normal thymuses.

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was compared among four thymic carcinoids by real time PCR. Expression levels decreased in the order TC4<TC1<TC2<TC5 in a way that paralleled methylation density decline at −417 to −260 bp in the POMC promoter region.

In conclusion, overexpression of the POMC gene was related to the degree of its promoter hypomethylation. In addition, the density of the POMC promoter methylation is an important factor determining the degree of gene expression. A C/T polymorphism was identified in the POMC transcript in one thymic carcinoid, suggesting that the expression of the POMC gene was not allele specific. It is speculated that hypomethylation is not allele specific either.

Among the four domains previously defined in the human POMC promoter, domain IV (−376 to −417) had the distinctive property of being active in DMS-79 cells but not in AtT-20 cells (Picon *et al.* 1995). It was reported that E2 transcription factor binding was required for the activity of domain IV in DMS-79 cells and contributed to the expression of the POMC gene in small cell lung cancer cells (Picon *et al.* 1999*a*). The area of POMC promoter methylation of greatest density in the thymic carcinoid tumors was also located in the E2F binding region(−417 to −260 bp), thereby implicating the importance of methylation of this region in regulating POMC gene expression.

We also propose that other ectopic hormone syndromes, including the syndrome of inappropriate antidiuretic hormone production, non-islet cell tumour hypoglycaemia, humoral hypercalcemia of malignancy and ectopic human chorionic gonadotropin syndromes etc . . . might be associated with CpG island hypomethylation in the promoter region. If this was really the case, target methylation of the CpG islands in the promoter regions would be a promising approach to treat ectopic hormone syndromes, especially in the cases of occult tumors.

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