

# Comparative genomic hybridization analysis of thymic neuroendocrine tumors

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**Thymic neuroendocrine (carcinoid) tumors are a rare neoplasm of the anterior mediastinum. The tumors frequently exhibit a wide spectrum of histology and appear to follow a more aggressive behavior than their nonthymic counterparts. Given the differing clinicopathologic manifestations, thymic neuroendocrine tumors may also possess different cytogenetic abnormalities from those that occur in foregut carcinoid tumors. In this study, we employed comparative genomic hybridization to detect genomic instability in 10 sporadic thymic neuroendocrine tumors and one multiple endocrine neoplasia type 1 (MEN1)-associated case. Gross chromosomal imbalances were found in nine cases, including gains of chromosomal material on regions X, 8, 18 and 20p and losses on 3, 6, 9q, 13q and 11q. We did not observe deletion at locus 11q13 where the *MEN1* gene is located. These findings were essentially dissimilar to those reported in sporadic and MEN1-associated foregut carcinoid tumors. Consequently, we consider that a distinctive cytogenetic mechanism is at work in the development of thymic neuroendocrine tumors, which is different from that of foregut carcinoid tumors. *Modern Pathology* (2005) 18, 358–364, advance online publication, 23 July 2004; doi:10.1038/modpathol.3800246**

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The term *thymic carcinoid tumor* was first introduced by Rosai and Higa<sup>1</sup> to denote a group of thymic neoplasms that histologically resemble the carcinoid tumors of other systems. Later series of studies demonstrated that this group of neoplasms frequently displays a diversity of morphologic features ranging from typical carcinoid as those seen in the bronchopulmonary and digestive tracts, to poorly differentiated small-cell carcinoma. In addition, many such tumors behave more aggressively than the conventional nonthymic carcinoid tumors. Reportedly around half of the cases invaded the surrounding mediastinal structures, and 30–40% metastasized.<sup>2–4</sup> Consequently, Moran and Suster<sup>3</sup> proposed to designate the entity as *thymic neuroendocrine carcinoma* with a three-tiered (low, intermediate and high) grading system, which is roughly equivalent to the classic carcinoid, atypical carcinoid and small-cell undifferentiated

carcinoma of the World Health Organization (WHO) classification.<sup>5</sup>

Owing to differing pathologic and clinical presentation, we postulate a different cytogenetic oncogenesis between the thymic and foregut neuroendocrine tumors. Nevertheless, few cytogenetic studies specifically addressing the thymic neuroendocrine tumors are available. So far, only a total of 12 cases in three reports of multiple endocrine neoplasia type 1 syndrome (MEN1)-associated thymic neuroendocrine tumors were examined by using loss of heterozygosity (LOH) study at the 11q13 locus where *MEN1* gene is located.<sup>6–8</sup> The cytogenetic and molecular events underlying the development of thymic neuroendocrine tumor remain largely unknown. In this study, we aim to characterize the chromosomal aberrances in thymic neuroendocrine tumors with comparative genomic hybridization (CGH) analysis and compare our findings with those of previous reports.

## Materials and methods

### Case Selection

We retrieved 11 cases of thymic neuroendocrine tumors from the surgical pathology archive at Taipei

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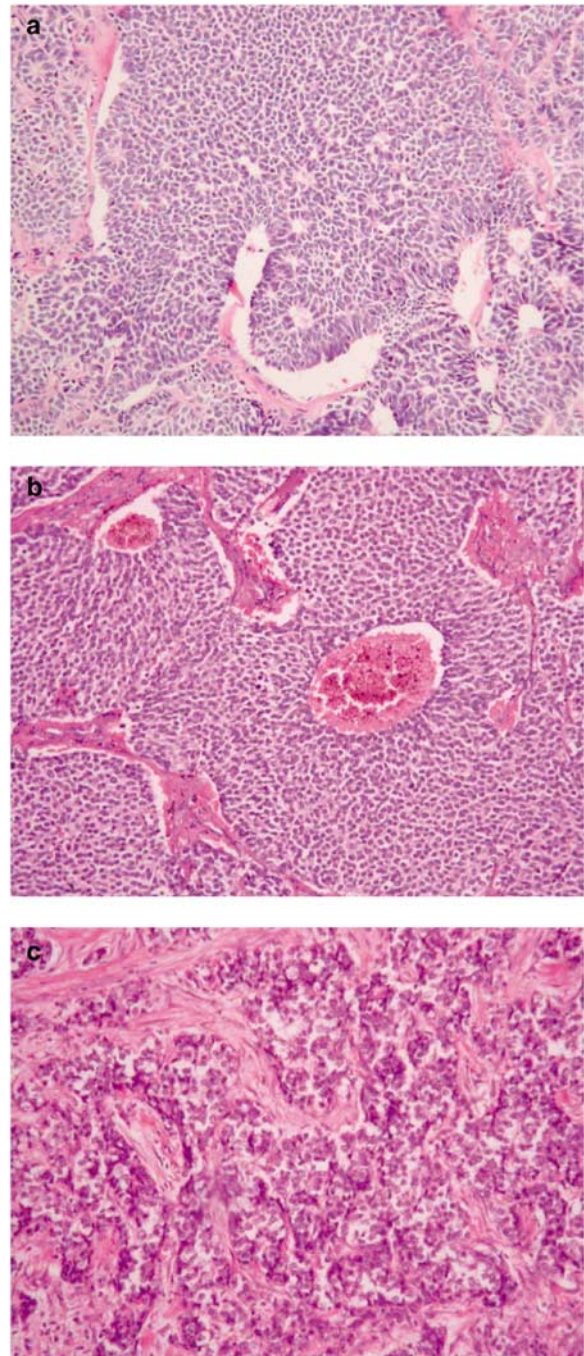
Veterans General Hospital from 1985 to 2000. The specimens were obtained through autopsy, radical surgery, tumor removal and debulking. Small biopsies unsuitable for further evaluation were excluded. These cases had complete clinicopathologic evaluations, including image studies to ascertain the mediastinal primary and absence of pulmonary lesions, and to rule out metastatic diseases.

The hematoxylin and eosin slides of each case were reviewed and subtyped as low-grade, intermediate-grade and high-grade neuroendocrine tumors (Figure 1) based on the criteria proposed by Moran and Suster.<sup>3</sup> Briefly, the low-grade (well-differentiated) tumor is composed of uniform cells in prominent organoid pattern with mild atypism and rare necrosis and mitoses. The intermediate-grade (moderately differentiated) tumor displays increased mitotic activity, solid growth and necrosis. The high-grade (poorly differentiated) tumor is characterized by solid infiltrative masses with marked atypism, extensive necrosis and high mitotic count. Immunohistochemical stains for epithelial markers and neuroendocrine markers (eg chromogranin A, synaptophysin, CD56) were performed to support the diagnosis.

The tumors were staged according to the WHO classification of thymic tumors<sup>5</sup> as Stage I, *encapsulated*; stage II, *minimally invasive*, that is, surrounded by a capsule which is microscopically penetrated by tumor growth or which invades the mediastinal fat; stage III, *widely invasive*, that is, spreading by direct extension into adjacent structures such as pericardium, large vessels, or lung; and stage IV, *with implants or metastases*.

### DNA Extraction

Since no frozen specimens were available for this rare tumor, we adopted a highly effective method to extract DNA from formalin-fixed, paraffin-embedded samples as previously described.<sup>9</sup> Briefly, portions of the tumors from the specimens were identified under the microscope by a pathologist (CC Pan) and were then cut into small pieces to include as little paraffin as possible, then incubated in xylene at 45°C for 1 h, and washed twice with 70% ethanol. After complete drying in air, 1 ml of lysis buffer (100 mM Tris-HCl, pH 7.8; 5 mM ethylenediaminetetraacetic acid; 0.2% sodium dodecyl sulfate and 200 mM NaCl) supplemented with proteinase K at a final concentration of 0.3 mg/ml was added and incubated at 55°C for 72 h; additional proteinase K (10 ml of 20 mg/ml stock solution) was added at 24 and 48 h. DNA was then extracted by using the standard phenol-chloroform-isoamyl alcohol method. DNA samples were quantified using a DyNA Quant 200 fluorometer (Hofer, Pharmacia Biotech, San Francisco, CA, USA).



**Figure 1** Histology of thymic neuroendocrine (carcinoid) tumor. (a) Low-grade tumor shows uniform cells in organoid pattern without necrosis. (b) Intermediate-grade tumor reveals more solid areas and punctate necroses. (c) High-grade tumor consists of infiltrative masses of small anaplastic cells.

### CGH

Amounts of 500 ng of DNA extracted from the tumor samples or normal placenta were each labeled with fluorescein-conjugated 12-dUTP or Spectrum Red-conjugated dUTP by nick translation. The sizes of the resulting probes ranged typically from 500 to 2000 base pairs. Slides containing metaphase

chromosomes were prepared using blood from volunteers. Peripheral blood lymphocytes were cultured and stimulated with phytohemagglutinin according to standard procedures. For DNA hybridization, the metaphase chromosome templates were denatured at 76°C for 3 min in 70% deionized formamide and  $2 \times$  SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), then dehydrated by gradient ethanol and air-dried. Amounts of 200 ng of the labeled probes from both tumor and control samples were then ethanol-precipitated with 10  $\mu$ g of human Cot-1 DNA and dissolved in 10  $\mu$ l hybridization solution (Master Mix 1.0: 70% formamide, 14.3% dextran sulfate and  $2.8 \times$  SSC) to form the probe mixture. The mixed probes were heat-denatured at 76°C for 7 min and competitively hybridized to the metaphase chromosome templates at 37°C for 36–72 h in a humid chamber. After hybridization, the slides were washed and counterstained with 0.1 mg/ml 4,6-diamidino-2-phenylindole (DAPI).

The CGH hybridization patterns were then typically analyzed using QUIPS XL genetic workstation (Vysis, Downers Grove, IL, USA) that included programs for both image acquisition and analysis. The fluorescent images were first observed under a microscope equipped with standard fluorescence system (Axioscope, Carl Zeiss, Germany) and a cooled-CCD camera (Photometrics, Tucson, AZ, USA). Images from at least 6–10 complete assemblies of metaphase chromosome were captured using the SmartCapture software of the QUIPS program. To facilitate sequential observation and perfect registration of images from three different fluorescence channels, all excitation filters were mounted and controlled by a Ludl filter wheel (Ludl Electronic Products, Hawthorne, NY, USA). Also, a triple-dichroid mirror and a triple-bandpass emission filter (Chroma Technology, Brattleboro, VT, USA) were used in the microscope to avoid manual switching when changing fluorescence channels. The blue fluorescence images of DAPI-stained chromosomes were used for autokaryotyping, whereas red and green images from competitive hybridization staining provided the signals for deriving the intensity ratio profile between normal and tumor samples. Genomic aberrations such as gains (amplification) or losses (deletion) at certain chromosome regions were defined when the mean green (from tumor part) to red (from normal part) ratios is above 1.2 or below 0.8, respectively.

### Statistical Analysis

We adopted two-tailed Fisher's exact test to assess the correlation of genetic aberrances with clinicopathologic features. The incidence of 11q13 loss was compared with the accumulated data from prior CGH studies on foregut carcinoids by two-tailed Fisher's exact test. We made the comparison because prior CGH studies of foregut carcinoids<sup>10–12</sup>

disclosed a high incidence of allelic losses at this locus where the *MEN1* gene is located. The computation was achieved using the Stata program (Stata Corp., TX, USA, 1997).

## Results

The patients ranged in age from 32 to 74 years (mean: 53.8 years). There was a male predominance (M:F = 10:1) partly owing to the patient pattern at the institute, where male patients predominate. One patient (No. 8) had a recurrence 2 years after the primary tumor. Both the primary and recurrent tumors were submitted for CGH analyses. One patient (No. 7) had a synchronous pancreatic neuroendocrine tumor, adrenocortical hyperplasia and parathyroid hyperplasia, suggesting MEN1. The patient also had familial history of MEN1. The pancreatic neuroendocrine tumor was assessed for CGH in parallel. None of the patients had Cushing's syndrome or other paraneoplastic syndromes.

Most of cases were either minimally invasive (stage II) or widely invasive (stage III). Histologically, three of 11 cases were classified as low grade, eight as intermediate grade and one as high grade.

Follow-up data could be obtained for all of the patients. Five patients died of the disease (survival times: 11–52 months; mean: 31.8 months). The other patients were alive without evidence of disease during the follow-up duration (follow-up times: 20–158 months; mean: 91.7 months).

### CGH

Genomic alterations were detected in nine cases. Gains were observed in chromosome regions X (four cases), 8 (three cases), 18 (two cases) and 20p (one case). Losses were observed in chromosomes 6 (two cases), 13q (two cases), 3 p (one case), 9q (one case) and 11q (one case) (Figures 2 and 3). The genomic aberrations of the recurrent tumor of case no. 8 were nearly identical to those of the primary tumor excepting the addition of a gain on the X chromosome in the recurrent tumor. The chromosomal abnormalities detected in the thymic and pancreatic neuroendocrine tumors of the patient with MEN1 (case no. 7) were entirely different. The clinicopathologic features and CGH findings are tabulated in Table 1.

### Statistical Analysis

No statistical significance could be found between the CGH findings and clinicopathologic features including stage, grade and survival ( $P > 0.05$ ). A meta-analysis of prior CGH studies<sup>10–12</sup> on foregut carcinoids showed 25 of 69 (36%) cases as having an 11q13 loss. On the contrary, none of our cases showed deletion at 11q13. The difference regarding

the incidences of an 11q13 loss was statistically significant between foregut and thymic neuroendocrine tumors ( $P = 0.0144$ ).

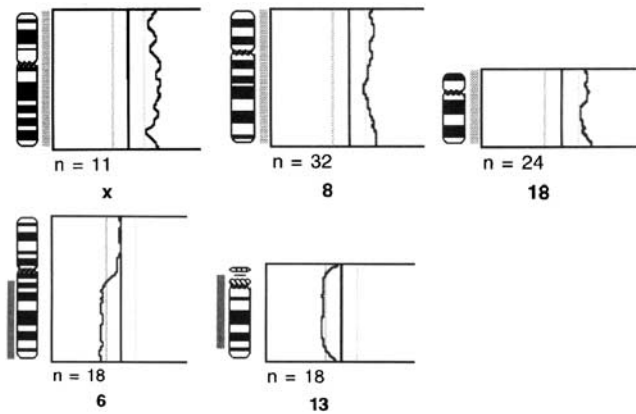
## Discussion

CGH is an efficient tool to provide a profile of the entire tumor genome, though it may not be sensitive enough to discern small chromosomal instability. It can be applied to archival formalin-fixed, paraffin-embedded tissue; thus it makes possible a cytogenetic examination for rare diseases, when fresh

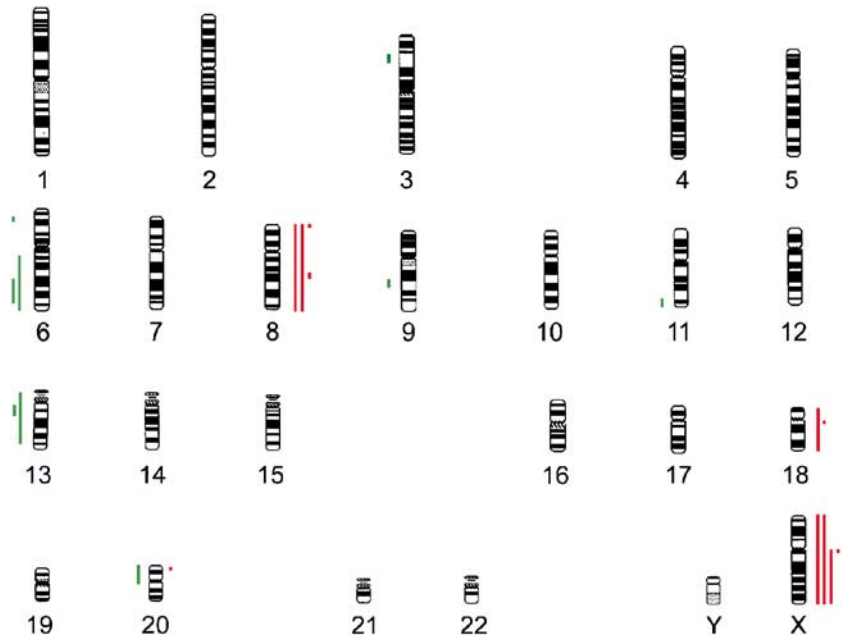
tissue is hardly attainable. Using the same protocol, we have successfully performed CGH on the paraffin specimens of other kinds of cancer.<sup>9,13</sup> In this study, we delineated the genetic alterations of thymic neuroendocrine tumor—a rare and hitherto poorly characterized neoplasm. To our knowledge, our study is the first CGH analysis on thymic neuroendocrine tumors.

Two limitations intrinsic to this study should be addressed. First, due to the rarity of the neoplasm, the case numbers were low. The limited cases did not allow a statistically significant statement with regard to the genetic differences among histologic subtypes and clinical stages. The low number of cases analyzed in the present study also hampered drawing conclusions on genetic alterations in these tumors. Second, even though we selected samples in which the tumor cells accounted for more than 80% of cell population, there was still a small population of normal cells that might confound the results. Despite the limitations, we still have successfully detected a few gross chromosomal imbalances in nine of 11 cases. Nevertheless, the few genetic alterations are well in line with the recently published data of Goto *et al*<sup>14</sup> on thymic neuroendocrine tumors showing diploidy in most of the cases they studied.

We did not find any deletion at the locus 11q13 where the *MEN1* gene is located, and we only detected deletion on 11q24–25 in one case (case 6). Contrarily, prior CGH and LOH studies disclosed a high incidence of allelic losses on 11q in both sporadic and *MEN1*-associated neuroendocrine tumors of the bronchopulmonary<sup>10–12,15–18</sup> and the digestive systems.<sup>19–23</sup> The rate of 11q13 LOH was



**Figure 2** Representative examples of genomic imbalances on chromosomes X, 8, 18, 6 and 13 detected by CGH. The chromosomal ideograms are shown along with the mean ratio profiles. *N* indicates number of metaphases analyzed. Lower/upper thresholds are the ratio values of 0.8 and 1.2. Gains of chromosomal material are shown as a bar on the right side of the ideogram; losses are depicted along the left side.



**Figure 3** Compilation of CGH analysis of the chromosomal aberrations in nine thymic neuroendocrine (carcinoid) tumors. Vertical lines on the right side of each chromosome ideogram represent gains. Vertical lines on the left side represent losses.

**Table 1** Clinicopathologic features and CGH findings

No.	Gender	Age (years)	Stage	Grade	Status	Survival (months)	Chromosomal gain	Chromosomal loss
1	M	65	2	Low	DOD	52	Nil	Nil
2	M	32	1	Low	AW	107	8p, 8q	Nil
3	M	51	2	Low	AW	156	X	Nil
4	M	71	2	Intermediate	DOD	35	18p, 18q	13q12–14
5	M	74	2	Intermediate	DOD	11	18q11–12.1	Nil
6	M	39	3	Intermediate	DOD	24	8p, 8q	3p21, 9q21–22, 11q24–25
7	M	42	2	Intermediate	DOD	40	8p23, 8q21.1–23, Xq	Nil
7p	—	—	—	—	—	—	Nil	16p12–13.1
8	M	52	3	Intermediate	AW	84	Nil	6q13–27, 20p, 20q11
8r	—	—	1	Intermediate	—	—	Xq11–13	6q13–27, 20p, 20q11
9	F	60	2	Intermediate	AW	25	Nil	Nil
10	M	41	3	Intermediate	AW	20	X	Nil
11	M	65	3	High	AW	158	20p12–13	6p22–23, 6q22–25, 13q11–33

DOD: died of disease; AW: alive and well; 7p: pancreatic neuroendocrine tumor of no. 7; 8r: recurrent thymic neuroendocrine tumor of no. 8.

reported as high as 78% in foregut carcinoid tumors.<sup>17</sup> Our CGH data are in agreement with the findings in previously reported LOH studies on 12 MEN1-associated thymic neuroendocrine tumors, all of which demonstrated retention of heterozygosity at the locus 11q13.<sup>6–8</sup> The absence of 11q alterations in thymic neuroendocrine tumors supports the distinction from their presumed foregut counterparts at the cytogenetic level despite overlapping histologic features.

On the other hand, we observed a few cytogenetic aberrations not usually present in the foregut neuroendocrine tumors. Two cases had variable lengths of genetic losses on chromosome 6. Interestingly, chromosome 6 has been reported to be a frequent target of deletion in thymoma and thymic carcinoma.<sup>24–26</sup> Two cases had 13q loss. Allelic loss on 13q have been observed in thymic epithelial tumors<sup>25,26</sup> and non-small-cell lung carcinomas.<sup>27,28</sup> The tumor suppressor genes mapped to the 13q12–14 regions include the retinoblastoma gene and the hereditary breast cancer susceptibility gene (*BRCA2*). To date, there have been no studies addressing those genes in thymic neuroendocrine tumors. We consider these findings as having certain histogenetic implications, but it requires recruiting more cases for further investigation.

Regarding the chromosomal gains, four cases had gain on X chromosome and three cases had gain on chromosome 8. The amplification spanned over the whole chromosome in some of cases. We suspect a trisomy X or 8 in those cases. Karyotyping or fluorescent *in situ* hybridization would be helpful to validate this speculation. Gain of the entire or partial copy of X chromosome have been found at high frequency in endocrine tumors such as pituitary adenoma<sup>29</sup> and adrenocortical adenoma,<sup>30</sup> as well as several solid and hematopoietic malignancies.<sup>31–33</sup> There is growing evidence that a few X-linked genes are involved in the development of neoplasia.<sup>34</sup> Trisomy 8 frequently occurs in myelocytic malignancies<sup>35</sup> and breast cancers.<sup>36,37</sup> Multiplication of 8q has also been described in cancers of

the breast and the liver.<sup>38,39</sup> The candidate oncogene on 8q includes *c-myc*, which has yet to be investigated in thymic neuroendocrine tumors.

Finally, one case exhibited complete amplification of the chromosome 18, and one case showed partial gain on the long arm. Gain of 18q has been reported in neuroendocrine carcinomas of lung and it is presumed to correlate to the expression of oncogene *BCL2* that is located at 18q21.<sup>12,28</sup> Supplementing that hypothesis, a recent study also demonstrated overexpression of *BCL2* protein in a portion of thymic neuroendocrine tumors.<sup>40</sup>

In conclusion, we identified chromosomal abnormalities chiefly involving chromosome 6, 8, 13, 18 and X in thymic neuroendocrine tumors. Based on our findings, in conjunction with previous ones, we favor a fundamental molecular divergence between thymic neuroendocrine tumors and foregut carcinoid tumors. Owing to the limited sample size, we are unable to obtain statistical significances concerning the correlation between cytogenetic changes and clinicopathologic parameters such as stage, grade and survival; however, our data could still offer new insights into the underlying cytogenetic mechanism of thymic neuroendocrine tumors and open an avenue for further researches regarding this peculiar neoplasm.

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