DNA Copy Number Changes in Thyroid Medullary Carcinomas Determined by Comparative Genomic Hybridization

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Medullary thyroid carcinoma (MTC) is a relatively rare tumor, first described in 1959,1 it develops in the calcitonin-producing C-cells of the thyroid gland. This tumor is very distinct in its clinico-pathologic, biochemical, and genetic characteristics. The typical clinical course is more favorable than for anaplastic carcinoma, but worse than for papillary or follicular carcinomas.2-4 Approximately 50% of the MTCs involve the cervical and para-tracheal lymph nodes at the time of diagnosis and are resistant to radio-iodine therapy. Early detection and radical resection are critical for curative treatment.

The RET proto-oncogene (RET), at 10q, has been identified as the disease related gene in virtually all cases of multiple endocrine neoplasm (MEN) 2, and familial MTC cases and in 25-33% of sporadic cases of MTC.3 However, apart from activating RET mutations, which seem to be an important event in the development of many if not most, MTCs, little is known about other genetic aberrations in these tumors. In the present study, comparative genomic hybridization (CGH) was used to screen 29 patients for DNA copy number changes in an effort to identify additional genetic events involved in development of MTC.

MATERIALS AND METHODS

Patients

Twenty-nine cases of MTC were retrieved from the surgical file at Asan Medical Center, dated from January 1996 to June 2004. Patients were chosen for the analysis based on the availability of paraffin-embedded tissue. Clinical and follow-up data (gender, age, calcitonin level, familial and medical histories, and recurrence or metastasis) were obtained from the medical records.
The histological sections were re-examined and the pathological stage was reclassified according to the American Joint Committee on Cancer staging (AJCC) 6th edition.\(^5\)

**Tissue microarray**

From each tumor, three 5 mm diameter-cores of representative areas were selected, then manually embedded in new paraffin blocks using a skin biopsy needle. Sections were cut to 5 μm thickness from the tissue microarray blocks for histochemical (Congo-red) and immunohistochemical (calcitonin, chromogranin A and CEA) staining.

**DNA extraction**

Tumor cells were collected after removal of the stroma by scraping the material with a clean scalpel from three unstained slide sections of 10-μm thickness. The slides were deparaffinized in xylene (three times, for 5 min each) and 100% ethanol (twice, for 5 min each). The tumor cells were transferred to a microcentrifuge tube containing digestion buffer, and high-molecular weight DNA was extracted as previously described.\(^6\) DNA from peripheral blood lymphocytes obtained from healthy male and female donors was extracted according to standard procedure and was used for reference in the CGH analyses.

**Comparative genomic hybridization**

CGH was performed as described elsewhere.\(^7,8\) Normal DNA was extracted from peripheral blood from healthy donors and was labeled with Texas-Red-dUTP (Dupont, Boston, MA, USA) in a standard nick-translation reaction. The tumor DNA was labeled with a mixture of fluorescein isothiocyanate (FITC)-dCTP and FITC-dUTP (Dupont). Equal amounts (1 μg) of the labeled tumor and reference DNA were mixed with 20 μg of unlabeled human Cot-1 DNA (Gibco BRL, Gaithersburg, MD, USA) in 10-μL of hybridization buffer (50% formamide, 10% dextran sulfate, 2 × SSC [1 × SSC: 0.15 mol/L sodium chloride/0.015 mol/L sodium citrate, pH 7]) and hybridized onto normal metaphase slides. Before hybridization, the DNA was denatured for 5 min at 75°C, and the metaphase slides were treated in a 70%, 80%, and 100% ethanol series and denatured at 65°C for 2 min in a formamide solution (70% formamide/2 × SSC). Hybridization was performed in a humidified chamber at 37°C for 48 h, and washing was performed in 50% formamide/2 × SSC and twice in 2 × SSC. The slides were then mounted with an anti-fading medium containing a counterstain (Vector Laboratories, Burlingame, CA, USA).

**Digital image analysis**

Hybridization of antibodies was assayed using an Olympus fluorescence microscope and an ISIS digital analysis system (Meta-system GmbH, Altusheim, Germany), based on an integrated high-sensitivity monochrome CCD camera and automated CGH analysis software. Three color images with red, green and blue were acquired from 10-15 metaphase chromosomes. The chromosome regions were interpreted as over-represented (gained) when the green-to-red ratio exceeded 1.17 and as underrepresented (lost) when the ratio was less than 0.85.

**Statistical analysis**

The relationship among all clinical and pathological parameters (age, stage, lymphovascular invasion, lymph node involvement, degree of tumor differentiation, and calcitonin positivity/negativity) and the presence of disease recurrence or metastasis was evaluated by the χ² test and the statistical analysis was performed using the SPSS 10.0 software program.

**RESULTS**

**Clinical findings** (Table 1)

The general characteristics of the 29 patients showed an age distribution of 19-80 years (mean age: 39.9) and a predominance of females (F:M=18:11). The pre-operative serum calcitonin levels were above the normal baseline; the range was 31.7 to 20,900 ng/L. Two patients with hereditary MTCs were identified. One case was a MEN type II with a parathyroid adenoma, and the other was a familial MTC; an MTC was diagnosed in the mother and younger sister. The stages were as follows; I-4,

<table>
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<th>Parameters</th>
<th>Summary</th>
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<tr>
<td>Age</td>
<td>19-80 (mean 39.9) years</td>
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<tr>
<td>Sex</td>
<td>F:M=18:11</td>
</tr>
<tr>
<td>Preoperative calcitonin</td>
<td>31.7-20,900 ng/L</td>
</tr>
<tr>
<td>Familial setting</td>
<td>2 hereditary (MEN 2A, FMTC)</td>
</tr>
<tr>
<td>F/U (recur or metastasis)</td>
<td>3 mo-8 yrs (8/29, 27.59%)</td>
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</tbody>
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MEN, Multiple Endocrine Neoplasia; FMTC, Familial Medullary Thyroid Carcinoma.
II-6, III-8, IV a-9, and IV b-2. The finding of advanced stages (beyond stage III) was common (65.5%). The postoperative follow-up for serum calcitonin levels continued for three months to eight years. Disease recurrence or distant metastasis was found in eight cases.

Pathological findings (Table 2)

The histologic types were variable: solid (10 cases), trabecular (9), giant cell rich (3), paraganglioma-like (2), pseudopapillary (2), follicular (1), and microcystic (1). Among nine solid types, one case showed squamoid features (Fig. 1A) and two cases exhibited marked cytological atypia (Fig. 1B), suggesting morphologically poorly differentiated forms. Lymphovascular invasion was identified in six cases. Eight cases had multifocality, and three of these were bilateral. Five cases displayed hyperplasia of parafollicular C cells (Fig. 2).

### Table 2: Pathologic findings of 29 medullary thyroid carcinomas

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Summary</th>
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<tr>
<td>Pathologic stage</td>
<td>I (4), II (6), III (8), IV (11)</td>
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<tr>
<td>Histologic differentiation</td>
<td>Well (26), poorly (3)</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td>6/29 (20.7%)</td>
</tr>
<tr>
<td>Margin involvement</td>
<td>6/29 (20.7%)</td>
</tr>
<tr>
<td>C cell hyperplasia</td>
<td>5/29 (17.3%)</td>
</tr>
</tbody>
</table>

Fig. 1. Morphologically poorly differentiated tumor cells showing (A) squamoid, and (B) solid types with cytologic atypia.

Fig. 2. Parafollicular C cell hyperplasia in cases of multifocal masses: (A) more than 6 C cells in a follicle (HE), (B) calcitonin immunostaining.
Congo-red and immunohistochemical staining

Congo red-positive amyloid deposits were identified in 13 cases. All 29 cases were immunoreactive for chromogranin A, 26 cases were positive for CEA and 25 cases were positive for calcitonin.

**CGH results** (Table 3, Fig. 3)

Twenty-three cases revealed more than one DNA copy number change. The most common change was a 19q gain (65.5%). Gains of 22 (55.2%), 19p (51.7%), 16p (27.58%), 17q (17.24%), and losses of 4q (17.24%) and 3p (17.24%) were also found.

**Comparison of clinico-pathological factor**

Age, clinical stage, lymphovascular invasion, nodal status, histological differentiation, and a calcitonin negativity were not statistically correlated with disease recurrence or metastasis. Two of three morphologically poorly differentiated cases revealed losses of 4q and 9p24-pter, respectively.

**DISCUSSION**

In the present study of DNA copy number changes in thyroid medullary carcinomas, in a Korean population, a higher frequency (79.3%) was found than reported in previous studies. The predominant changes were gains of 19q, 22 and 19p. Hemmer et al. reported DNA copy number changes in five out of ten cases of MTC, predominantly genetic losses of chromosomes 3, 13 and 22. Frisk et al. observed that 60% out of 24 MTCs exhibited alterations in chromosomal balance and the most common aberrations were gains in chromosome 19q (29%), 19p (21%), 11c-q12 (12.5%), and 22q (12.5%), similar to our results. The higher frequencies observed in these two studies are most likely attributable to differences in cut-off points and sensitivity, racial differences between Western and Asian subjects, and the later stage of diseases common in this study. More advanced tumors generally have more genetic abnormalities.

Chromosome 19 has cancer-related mutations at 26 known genetic loci, eight of which are associated with zinc finger proteins. One of these is RREB-1, which is involved in the activation of the ras oncogene and played a role in raf signal transduction. RREB-1 is composed of 756 amino-acids and contains 4 zinc-finger domains, and is dependant on ras activation. Previous studies have demonstrated that the genes for two ligands (neutrin, persephin) of the RET receptor kinase are located on 19p, which is known to be related to neutrin mRNA overexpression in MTCs. Chromosome 22 is a very complex chromosome, composed of 1678 loci and containing multiple oncogenes and suppressor genes. PRKM1, which codes for mitogen-activated protein kinase (MAPK), is an important candidate.
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oncogene. Previous studies have shown that RET oncogene activation is related to the downstream pathway of MAPK.14,15 The MAPK inhibitor PD098059, attenuates DNA synthesis, which causes modest declines in cell count and DNA synthesis and minimal changes in apoptosis.16

The prognostic factors of MTCs are age, gender, extent (size, nodal metastasis, extrathyroidal extension), and preoperative calcitonin level.4 The present study revealed no significant relationship between the clinical and pathological parameters and disease recurrence or metastasis. The study has limitations including the following. This clinical data was obtained by retrospective chart review; only eight cases with clinical MTC had preoperative follow-up. The other cases were evaluated at the postoperative follow-up. And the sample size of the study was too small to demonstrate a statistical significance. Therefore, the ability to demonstrate a correlation with the serological markers studied was limited.

A group of 13 pathologists belonging to the French Calcitonin Tumor Study Group (GETC) examined the slides for histology and reviewed the medical records of 109 index cases with MTC diagnosed based on clinical features.17 Twenty-seven histological parameters were considered, including cellular heterogeneity, shape of the cells and characteristics of the cytoplasm. Five of the 27 parameters were significantly associated with a lower survival: presence of necrosis, squamous cell pattern, age more than 45 years, presence of oxyphil cells in the tumor, absence of cells with intermediate cytoplasm, and less than 50% of cells, in the tumor that were calcitonin immunoreactive. In this study, one case with squamous features and two with atypical features were associated with loss of 4q and 9p24-pter, respectively.

Patients with cell populations that are completely, or mostly, negative for calcitonin immunoreactivity have a worse prognosis than do calcitonin-positive individuals.17 Schmid et al. reported four cases among 142 MTCs that were calcitonin negative and classified as atypical MTCs.18 At the time, they were considered to have a different cyrogenetic background from the typical form, but the further studies did not support this conclusion. In the present study, four cases that were calcitonin and CEA negative were found and the CGH results were not different from the other cases (gain of 19q and 22). The interpretation of the results of the immunohistochemistry on array blocks is limited in cases with focal positive disease. If the positive area is not included within the three cores, it is recorded as a negative result. Therefore, a negative finding, may be a true or false negative. This is a limitation of the tissue array method. In conclusion, the results of this study showed that the DNA copy number changes associated with thyroid medullary carcinoma were more frequent (79.3%) than indicated by previous studies. The most frequent changes were gain of 19q, 22, and 19p. Further molecular studies using FISH for these genetic loci are needed. The presence of poor clinicopathologic parameters did not have a significant relationship to the large genetic aberrations identified.

REFERENCES


