Screening of Three Exons of the RET Proto-oncogene in Turkish Patients with Papillary Thyroid Carcinoma

Papiller Tiroid Kanseri Türk Olgularda RET Proto-onkogenine Ait Üç Ekzonun Görüntülenmesi

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Anahatar Kellimeler: Adenokarsinom, papiller; polymerase chain reaction

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Papillary thyroid carcinoma (PTC) is the most frequently observed histotype of differentiated thyroid cancer, representing 75% to 85% of all thyroid cancer cases. Females are more likely to have thyroid
cancer at a ratio of three to one. Thyroid cancer can occur in any age group although it is most common after age 30, and its aggressiveness increases significantly in older patients. The RET (REarranged during Transfection), proto-oncogene localized to chromosome 10q11.2, comprises 21 exons, which encodes tyrosine kinase receptor expressed mainly in tumors of neural crest origin: medullary thyroid carcinoma, normal thyroid tissue, thyroid adenoma and papillary and follicular thyroid cell neoplasias derivatives and tumors of neural crest origin.2

RET proto-oncogene mutations are responsible for the genesis of PTC. A variable proportion of sporadic and radiation-associated PTCs have been linked to translocations involving the 3’ half of RET, which contains the tyrosine kinase (TK) and the 5’ end of several genes. Chimeric RET oncogenes are formed from the juxtaposition of the genomic region coding for the tyrosine kinase domain of RET with the 5’-promoter regions of a variety of unrelated genes. RET/PTC display a ligand independent constitutive tyrosine kinase activity and uniformly expressed in PTCs that are not derived from the neural crest, and with increased frequency in radiation-associated cancers after the Chernobyl nuclear accident. However, the frequency of RET rearrangements have also been reported in cancers after exposure to external radiation for benign and malignant conditions.2

The present study aimed to determine the frequency of mutation of the RET proto-oncogene in Turkish PTC patients and normal matched controls, and it verifies its correlation with RET proto-oncogene mutations and/or with the clinical features of PTC patients, and compare the frequency of the exon 10, 11 and 13 polymorphisms localized in the RET proto-oncogene involved in the pathogenesis of the PTC.

MATERIAL AND METHODS

PATIENTS

Thyroid samples were obtained from Ankara Dr. Abdurrahman Yurtsalan Oncology Education and Research Hospital, and Ankara Numune Education and Research Hospital between 2006 and 2008 years. Peripheral blood samples were collected from 82 PTC patients and 85 age-gender matched healthy people. All subjects signed consent forms. This study was conducted under approval of the Human Investigation Committee and the Ethics Committee of Gazi University.

A thorough review of the clinical data was carried out according to the case histories and none of the patients had a history of radiation exposure before surgery. The diagnosis and histological classification of the tumors were performed according to the standards of the World Health Organization (WHO).3

All patients received an ultrasound scan of the thyroid and neck (level I–VI), a chest X-ray, and thyroid function tests (including serum levels of free thyroxine, free triiodothyronine, thyrotropin, thyroid peroxidase antibody, thyroglobulin antibody and thyroglobulin) before surgery. Fine needle aspiration (FNA) was performed on all the thyroid cancer patients. Total thyroidectomy was performed routinely. In all, 24 patients with suspicious lymphadenopathy identified with ultrasound (US) and/or FNA was defined as clinical N1b (lateral neck lymph node involvement) and underwent level II–V neck dissection. Iodine-131(RAI) ablation and treatment was planned to all patients and administered to 19 patients prior to this study. All patients received life-long TSH-suppressive thyroid hormone replacement. After being evaluated by pathologists following surgery, 24 patients were identified as pathological N1b, respectively.

GENOMIC DNA ISOLATION

Genomic DNA was isolated from peripheral blood leukocytes using phenol-chloroform extraction, and was precipitated with ethanol and dissolved in Tris- Ethylenediaminetetraacetic acid (TE) buffer. Next, 25 ml of Red Blood Cell (RBC) lyses buffer was added to 9 ml of blood sample and shaken gently. The mixture was incubated on ice for 20 min and centrifugated at 4000 rpm for 20 min at 4 ºC. The supernatant was removed and 25 ml of RBC lyses buffer was added, then this process was repeated until all the red cells were removed. 20 µg/ml of proteinase K, 10% Sodium-dodecyl-sulfate (SDS)
(final concentration 0.5%) and 2.5 volume sodium chloride tris ethylenediaminetetraacetic acid (STE) were added and incubated overnight at 56 °C in a water bath. Afterwards, 1:1 phenol–chloroform–isoamyl alcohol (25:24:1) was added and shaken for 10 min. The mixture was then incubated on ice for 20 min and centrifuged at 4000 rpm for 20 min at 4 °C. The upper phase was transferred into a new tube, 1:10 volume of 2M sodium acetate (pH 5.2) and 95% ethanol (2 –fold the total volume) was added and shaken gently until the DNA was precipitated, which was then incubated overnight at -20 °C. DNA was centrifuged at 4000 rpm for 20 min at 4 °C and the supernatant was removed. DNA was washed in 500 µl of 70% ethanol and dissolved in 0.5-1 ml of TE buffer overnight in a 37 °C water bath. After amplification, PCR products were analyzed with 1.5% agarose tris-acetate-EDTA gel electrophoresis. The gel was stained with ethidium bromide and analyzed under UV light. A negative control was included in each amplification analysis and 373 bp, 561 bp, 346 bp amplification product bands were identified for exon 10, 11 and 13, respectively (Figure 1). The purified PCR products were directly sequenced using an automated system by Macrogen (Korea).

### POLYMERASE CHAIN REACTION AMPLIFICATION AND SEQUENCING

Template DNA (0.5-1.0 µg) was used in a Polymerase Chain Reaction (PCR) under sterile conditions. The primers for exons 10, 11 and 13 are given in Table 1. Polymerase Chain Reaction amplification was performed in a reaction volume of 50 µl that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 160 mM dNTP’s, 0.1 µM of each primer, 0.1 U of Taq polymerase, 60 ng of DNA and 2 mM MgCl2. Amplification was performed for initial denaturation at 95 °C for 10 min, followed by 40 cycles (denaturation at 95 °C for 30 s, annealing at the optimized temperature for 30 s, elongation at 72 °C for 1 min), and final elongation at 72 °C for 10 min in a Biometra thermocycler (Goettingen, Germany). After amplification, PCR products were analyzed with 1.5% agarose tris-acetate-EDTA gel electrophoresis. The gel was stained with ethidium bromide and analyzed under UV light. A negative control was included in each amplification analysis and 373 bp, 561 bp, 346 bp amplification product bands were identified for exon 10, 11 and 13, respectively (Figure 1). The purified PCR products were directly sequenced using an automated system by Macrogen (Korea).

### STATISTICAL ANALYSIS

The chi-square test, Fisher’s exact test, and the independent test were used to compare the characteristics of the RET mutations positive and negative groups. Multivariate analysis was performed with logistic regression analysis. A P value ≤ 0.05 was considered significant.

### RESULTS

#### “CLINICOPATHOLOGICAL CHARACTERISTICS”

Clinicopathological characteristics of the patients were summarized in Table 1. The mean ages were 41.6 years for the controls (58 Female / 27 Male). There is statistically significant difference (p=0.025) in distribution of the RET mutations among PTC patients and controls (patients: 43.9%, controls: 27.1%) (Table 2).

Papillary thyroid carcinoma was confirmed by pathologic examination in 82 patients (11 male and 71 female). Average age of the patients was 46 years (range: 16-79 years). Average tumor size was 2.03 (range: 0.2-6 cm). Tumors ≤1cm, 1-4cm and ≥4 cm were found in 17, 58 and seven of the patients, respectively (20.7%, 70.7%, and 8.6%). Multiple lesions were seen in seven patients. Classical papillary thyroid carcinoma was diagnosed in 52 patients (63.4%), whereas follicular and micropapillary variants were identified in 13, and 17 patients, respectively. Extension to the extrathyroidal soft tissues was noted in 18 patients (21.9%). Among the 24 patients that underwent lymph node dissection, 21 (87.5%) had cervical lymph node metastasis. Distant metastasis including two bone metastasis and three lung metastasis were observed in five patients. Recurrence developed in the lungs.
and cervical lymph nodes in two patients during a mean follow-up of 36 months. None of the patients died during the follow-up period.

**PREVALENCE OF RET MUTATION IN THYROID CANCER PATIENTS**

Clinical features, pathologic findings, and mutational analysis results for all the patients are shown in Table 1. In total, 82 PTC patients were screened for mutations in exon 10, 11, and 13 using PCR primers (Table 3, Figure 1) and sequence analysis. Mutation was seen in 13 of the patients and 26 patients had SNP (Table 4). In all, 12 different mutations were located in exon 11 and three different mutations were in exon 13. Molecular analysis revealed probable mutations in exon 11 of the *RET* gene at codons 630, 632, 633, 634, 635, 637, 659, 662, 697, 701, 702, and 703. Among the patients, 11 (13.41%) had mutation in exon 11. Additionally, mutations in exon 13 at codons 765, 770, and 795 were noted; three (3.65%) of the patients had mutation in exon 13. There following different amino acid substitutions were observed: Gly was replaced by Lys; Asn was replaced by Lys; Cys was replaced by Tyr; Lys was replaced by Ser; Cys was replaced by Ser; Ser was replaced by Arg; Ser was replaced by Cys; Arg was replaced by Glu; Cys was replaced by His; Asp was replaced by Thr; Glu was replaced by Ser; Leu was replaced by Trp; Cys was replaced by Trp; Arg was replaced by Ser; Arg was replaced by Ser. In all, 31 of the patients had SNP (37.80%), which was observed at codons 630 (6.09%), 691 (3.65%) and 702 (2.43%) in exon 11 and at codons 769 (26.82%) and 763 (1.21%) in exon 13 (Figure 2).

The most frequently observed SNP in the PTC patients involved codon 769 (exon 13) (n=22, 26.89%) in the tyrosine kinase domain.
A double mutation was observed in six patients and a triple mutation was observed in only one PTC patient with lung metastasis.

In control group consist of 85 people, 58 of whose are female and 27 of whose are male. Mutations were observed in 23 people, 17 of them are female and 6 of them are male, of 85 control group. Three mutations for male control group were both in exon 11 and in exon 13. Among female control group, six mutations were in exon 11, eight mutations were exon 13 and three people had mutations in both exons.

**CORRELATION WITH CLINICOPATHOLOGICAL FEATURES AND CONTROLS**

The demographics and other clinicopathological features of the papillary carcinomas with specific mutations are summarized (Table 1). Tumors with PTC mutation were associated with patient age (P<0.05). There was gender preponderance in tumors with PTC mutation groups and overall in all papillary carcinomas (Table 4). There was no correlation between PTC mutation and non-mutation groups in tumor size and capsular invasion (P > 0.05). Significant correlation was not found in patients with lymph node metastases and PTC mutation groups (p>0.05).

**DISCUSSION**

Thyroid tumors are the most frequently seen malignancies of the endocrine system. Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy. Prevalent mutations in papillary thyroid carcinomas are point mutations of BRAF, RAS and RET/PTC rearrangement. Several recent studies on the molecular characterization of PTC have been published. RET encodes a receptor tyrosine kinases expressed primarily on neural crest-derived and urogenital cells. It is required for maturation of several cell lineages of the peripheral nervous system, kidney morphogenesis, and

<table>
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<tr>
<th>E</th>
<th>PCR Amplification</th>
<th>PCR Product</th>
<th>A T</th>
<th>MgCl₂</th>
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<tr>
<td>10</td>
<td>10F:5’GGGCCCTATGCTTGCGACACCA3’</td>
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<td>11R:5’CAGCTGGCCAGCCCTCAGAG3’</td>
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<tr>
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**TABLE 3:** Primers and PCR conditions for amplification of exon 10, 11 and 13.

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<tr>
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**TABLE 4:** The relation of prognostic factors and RET mutation.
spermatogenesis. RET is mutated by different mechanisms in different types of thyroid carcinoma. RET rearrangement was discovered in papillary thyroid carcinoma and it is an important pathogenic event in this cancer. Activating germline mutations of RET causes multiple endocrine neoplasia type 2 (MEN 2), an inherited cancer syndrome characterized by medullary thyroid carcinoma (MTC), pheochromocytoma (Pheo), and parathyroid adenomas.

Gain-of-function alterations within the RET proto-oncogene are responsible for the development of medullary, as well as PTC, making it a candidate for the design of targeted therapies. Identification of the mutations in the RET proto-oncogene may aid the clinical diagnosis of person with thyroid carcinoma syndromes. In the present study, mutations were observed in 13 (15.85%) of all thyroid carcinoma patients. There were also mutations in exon 11 than in exon 13. The affective region was codon 630-703 in exon 11, and codon 765 and 795 in exon 13.

Specific mutations in different codons might influence the phenotypic expression. The mutation in codon 634 substitution of cysteine for arginine is significantly predictive of the development of pheochromocytoma and parathyroid disease. A germline mutation in RET was observed in 4% of apparently sporadic MTC patients, in 100% of patients with MTC and pheochromocytoma, or MTC and clinical features of multiple endocrine neoplasia type 2B, and in 100% of probands of clinically established kindreds. The most affected codon was 634 (58%) followed by codon 804 (16%).

Data from other studies show that the most frequent mutations are in exon 11 at codon 634 (47.8%), mostly the replacement Cys634Arg. Differences in the frequency of specific RET mutations in thyroid carcinoma phenotypes have been reported in series from different countries, suggesting that the occurrence of these mutations may be influenced by genetic background. Nonetheless, in the present study five of the 82 PTC patients had Cys630Ser (TGC→AGC, 6.09%) which is the most common mutation at codon 630 in exon 11. In addition, Cys630Ser mutation is associated with MTC; however, our genetic testing diagnosed it in five patients with PTC. Therefore, the relationship between Cys630Ser variation and development of PTC should be explored in greater detail.

Furthermore, several polymorphisms in the coding region of the RET proto-oncogene have been described. The most frequent polymorphisms were reported by Mulligan et al., Ceccherini et al., and Sáez et al., and include those at codons 45, 125, 432, 691, 769, 836, and 904. All of the investigated polymorphisms were silent mutations,

FIGURE 2: Sequence analysis of RET gene in PTC patient. The circular indicate the position of mutation. A T to G transition at codon 769 in exon 13 was exchanged from CTT to CTG, resulting in a silent mutation.
except codon 691 polymorphism, which resulted in the glycine being replaced by serine amino acid. The codon 691 polymorphism was noted in two (GGT/AGT) of the 82 PTC patients.

Erdogan et al. studied RET proto-oncogene mutations in Turkish families with multiple endocrine neoplasia. They reported RET mutations of these diseases in Turkish families were similar to those reported from other populations. Gursoy et al. identified several single nucleotide polymorphisms (SNP) of the RET gene in medullary thyroid carcinoma (MTC) patients. Erdogan et al. studied exon 10, 11, 13, 14, 15 and 16 of the ret gene in fifty-six MTC patients. They found mutations at codon 634 in exon 11, one at codon 618 in exon 10. In present study we had the same mutation in exon 11, but we observed no mutations in exon 10. In the exon 13 region of the RET proto-oncogene we observed that these possible mutations were expressed as an alteration in the form of TCC (serine) replacement with TGC (sistein) at codon 765, CGA (arginine) replacement with CAA (glutamic acid) at codon 770. The other mutation in exon 13 was at codon 770, Arg was replaced with Gln. The large number of PTC patients with SNP at codon 769 in exon 13 (n=22) was an interesting finding that should be explored further.

Acknowledgements

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REFERENCES


