High Rates of ras Codon 61 Mutation in Thyroid Tumors in an Iodide-deficient Area

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ABSTRACT

Using polymerase chain reaction and sequence-specific oligonucleotide hybridization, the frequency of three ras oncogene mutations (N-ras, Ha-ras, and K-ras) in thyroid tumors (25 adenomas, 16 follicular carcinomas, and 22 papillary carcinomas) was investigated in both iodide-deficient and iodide-sufficient areas. The ras oncogene mutation rate was significantly higher in the iodide-deficient area, being 85 versus 17% in the adenomas, and 50 versus 10% in the follicular carcinomas. No mutations were found in papillary carcinomas. The most common mutation site was Ha-ras codon 61 with Gln → Arg substitution. Two ras mutations at codon 61 (Gln → Lys in N-ras and Gln → Arg in Ha-ras) were found in a microfollicular adenoma specimen from Eastern Hungary. We conclude that dietary iodine may modulate ras oncogene mutations, and that in the iodide-deficient area, ras oncogene activation may play a more important role in the initiation and/or maintenance of follicular tumors. Additional factors are, however, necessary to initiate carcinogenesis.

INTRODUCTION

Thyroid epithelial tumors exhibit a broad spectrum of neoplastic pathology varying from well-differentiated benign tumors to highly malignant anaplastic carcinomas (1). They, therefore, provide an attractive model for studying the role of oncogene activation in different stages of tumorigenesis. Recently, it has been reported that all three ras oncogenes (Ha-ras, K-ras, and N-ras) were activated by point mutations in about 50% of follicular carcinomas and microadenomas, 17% of papillary carcinomas, but not in macrofollicular adenomas (2, 3). ras oncogene point mutation rates in thyroid tumors reported from elsewhere were less frequent (4, 5).

We considered the possibility that the differences may be related to the variation in dietary iodine intake. It is known that in iodide-deficient areas the rate of malignant transformation is higher than that of follicular relative to papillary carcinoma and the rate of undifferentiated cancer compared to that in iodide-sufficient areas (6). It is, however, not known whether dietary iodine intake could also influence the rates of ras oncogene mutations in thyroid tumors. In the present study, we investigated ras oncogene mutations of thyroid tumors from iodide-deficient and iodide-sufficient areas.

MATERIALS AND METHODS

Materials. Paraffin-embedded tissue blocks from a variety of thyroid tumors were randomly selected from the archives of Pathology Department, Health Sciences Centre, St. John's, Newfoundland, Canada, a high dietary iodine intake area (dietary iodide, 190-550 μg/day) and from those of the II Department of Surgery, Debrecen, Hungary, a low dietary iodine intake area (dietary iodide, 46-70 μg/day) with an 18% goiter rate. Synthetic Oligonucleotides and Polymerase Chain Reaction. The oligomers were purchased from Clontech Company (CA). A complete list of oligonucleotide sequences for primers and probes is available on request. Five-μm sections were cut from paraffin blocks and processed for polymerase chain reaction as described by Shibata et al. (7), with some modifications. Briefly, the sections were deparaffinized with 400 μl xylene and washed twice with absolute ethanol. The tissues were dehydrated and 50 μl of digestion buffer (100 mM Tris, 5 mM EDTA, pH 8.0) with 40 μg of protease K were added. The mixture was incubated at 37°C overnight. Proteinase K was inactivated by heating at 95°C for 10 min. Five μl of the supernatant were used for PCR in 50-μl volume with a Gene Amp kit (Perkin Elmer, Cetus, CA) according to the manufacturer's procedure. The primers were 20 bases long, each pair enclosing an amplified region of 100 base pairs. Samples were denatured at 94°C for 3 min and then cooled rapidly at 4°C. Forty cycles of amplification were used as follows: 30-s denaturation at 94°C, 30-s annealing at 56°C, and 30-s extension at 72°C. Five μl of the amplified products were run on 2% agarose gel to ascertain that specific amplification had occurred.

Southern Blot and Oligonucleotide Probe Hybridization. After an initial quantitation on the gel, 2-10 μl of each amplified product were run on 1.5% agarose gel and transferred to nylon membrane (Hybond-N; Amersham Canada). Replicate filters were prepared and DNA was fixed by UV illumination. Filters were prehybridized for 1 h at 50–56°C, depending on probe sequences in 5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 x Denhardt's solution (1 x Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% NaDodSO₄, and 10 mM sodium pyrophosphate. Hybridization was performed at the same condition for 2 h with [γ-32P]ATP end-labeled 20-mer oligonucleotide probes. Filters were washed twice in 2 x SSC, 0.1% NaDodSO₄, at 25°C for 20 min, followed by high stringency washes in 2 x SSC, 0.1% NaDodSO₄ for 10–20 min at 60–70°C. The filters were then subjected to autoradiography overnight at -70°C.

RESULTS

Since the activating mutations so far identified in naturally occurring tumors are at the codons 12, 13, and 61 (8), selective amplification of both regions was performed. Amplified DNA was analyzed with a set of oligomers each designed to be complementary to a different point mutation within these codons. This allows for the detection of all possible single base pair substitutions. We initially used the procedure recommended by Shibata et al. (7) to perform PCR amplification and found that amplification results were unpredictable. We were able to amplify DNA from only 40% of samples, even though fragments of tissue were clearly evident in the sample tubes. When, however, tissue fragments were treated overnight with proteinase K before PCR, DNA was amplified from more than 90% of samples. In our study, we used Southern blot instead of dot blot hybridization. We found that it could significantly increase the signal to noise ratio and overcome the difficulties of data analysis arising from occasional high background dot blot hybridization (data not shown).

The abbreviations used are: PCR, polymerase chain reaction; SSC, standard saline citrate; NaDodSO₄, sodium dodecyl sulfate.

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The rates of ras mutations were not significantly different ($\chi^2 = 2.886; P > 0.1$) between the adenoma and follicular carcinoma group.

<table>
<thead>
<tr>
<th>ras Oncogene</th>
<th>Mutant position</th>
<th>Mutation rate</th>
<th>Total mutation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ha-ras</td>
<td>Codon 61. Gln → Arg</td>
<td>11/13 (84.6)</td>
<td>84.6</td>
</tr>
<tr>
<td>N-ras</td>
<td>Codon 61. Gln → Lys</td>
<td>1/13 (7.7)</td>
<td>50.0</td>
</tr>
</tbody>
</table>

* K-ras mutations were not detected in the thyroid tumors.  
* Numbers in parentheses, percentage.

The histology of specimens from Debrecen was reviewed in St. John's before PCR amplification to exclude differences in diagnostic criteria.

Mutated ras oncogenes were identified in 11 of 13 thyroid adenomas (85%) and 3 of 6 follicular carcinomas (50%) obtained from Debrecen. Most of the adenomas (80%) were macrofollicular and the rest were mixtures of micro- and macrofollicular adenoma. No mutations in any of the three ras oncogenes were found in 12 papillary carcinomas. The most common mutation site was codon 61 with Gin → Arg substitution (Table 1; Fig. 1), accounting for 93% of the mutations. The ras protooncogene family includes three genes that have been designated Ha-ras-1, K-ras-2, and N-ras. These genes appear to be functionally involved in regulating cellular growth and cellular growth and differentiation (9). Mutations at codons 12, 13, or 61 of one of the three ras genes convert them into active oncogenes. Mutated ras genes are found in approximately 30% of human tumors and thus play an important role in the development of tumors (10). Certain mutations are found predominantly in human tumors: K-ras codon 12 in pancreatic carcinoma (11, 12) and adenocarcinoma of the lung (13, 14), and based on our present study and others (2), Gin → Arg substitution at codon 61 of Ha-ras in thyroid follicular tumors. Mutation at codon 61 is the most proficient in changing the conformation of ras proteins which is required to catalyze GTP hydrolysis (GTPase-inhibiting mutation) (15). The mutant proteins can bind to the GTPase-activating protein which mediates the signal transducing effect of ras protein on the cell (16, 17). By contrast to the wild type, however, oncogenic mutants are not converted to GDP-bound form during the interaction with GTPase-activating protein and stay in the active GTP conformation. As a result, constant growth-promoting signals are produced.

Dietary iodine intake in Eastern Hungary is suboptimal, whereas in North America it is more than sufficient (18-20). Our results show that ras genes are more frequently activated in follicular thyroid tumors in Eastern Hungary than in Newfoundland, and that they are activated at similar frequency in the early stages (adenoma) as in the late stages (follicular carcinoma) of tumorigenesis. In view of small sample numbers we combined our Hungarian data with those reported from Cardiff, United Kingdom (2) and compared them with the Newfoundland material. The ras oncogene mutation rate (11 of 21) was significantly greater than that from Newfoundland (1 of 10) ($\chi^2 = 14.03; P < 0.0001$). This exercise is justified by the borderline (mean, 90 μg/day) urinary iodide excretion in Wales, reflecting iodide intake below that recommended (150-300 μg/day) (18, 19), as well as the similarities in ras mutation rates. Iodide deficiency is known to produce thyroid hyperplasia, nodule formation, and ultimately malignancy in experimental animals (21). Some studies have also shown increased numbers of thyroid carcinomas in endemic goiter regions (22, 23). The excess of differentiated thyroid tumor is attributable to those with follicular histology (22, 24) in which ras mutations were found in this study. Our data suggested that in dietary iodine-deficient area ras oncogene activation may play a more important role in tumor initiation and/or maintenance. Additional factors are, however, necessary to initiate carcinogenesis.

Unlike follicular adenomas and carcinomas, we could not
Fig. 2. Paraffin sections of the thyroid adenoma harboring two ras mutants, showing (a) thick fibrous capsule (×10); (b) predominantly microfollicular structure with some follicles containing colloid (×40).

Table 2 ras oncogene mutation in thyroid tumors in Newfoundland, Canada

<table>
<thead>
<tr>
<th></th>
<th>Ha-ras</th>
<th>N-ras</th>
<th>Total mutation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant position</td>
<td>Mutation rate</td>
<td>Mutant position</td>
<td></td>
</tr>
<tr>
<td>Adenomas</td>
<td>Codon 61, Gln → Arg, 1/12 (8.3)</td>
<td>Codon 61, Gln → Lys, 1/12 (8.3)</td>
<td>16.6</td>
</tr>
<tr>
<td>Follicular carcinomas</td>
<td>Codon 61, Gln → Arg, 1/10 (10)</td>
<td>None (0/10)</td>
<td>10</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>None (0/10)</td>
<td>None (0/10)</td>
<td>0</td>
</tr>
</tbody>
</table>

* K-ras mutations were not detected in the thyroid tumors.
* Numbers in parentheses, percentage.

identify any ras oncogene mutations in 22 specimens with papillary carcinoma histology. Lemoine et al. (25) and Wright et al. (3) reported ras oncogene mutations in 20% of papillary carcinomas, while Fusco et al. (26) (also reporting from an area of relative iodide deficiency) failed to find any ras mutations in 20 papillary carcinomas. These results suggest that ras oncogene mutations may not be involved in the development of papillary carcinoma. Bongarzone et al. (27) reported high frequency (50%) of activation of two tyrosine kinase oncogenes (PTC and TRK) in papillary carcinomas. The activation of this class of oncogenes may be specifically involved in the pathogenesis of papillary thyroid cancer.
Parallels may be drawn between ras mutations and HLA-associated susceptibility to thyroid follicular carcinoma in the two populations under study. The susceptibility to benign thyroid adenoma was found to be associated with HLA-DR3, and follicular and papillary carcinomas with HLA-DR1 in an iodide-deficient area; in an iodide-sufficient area no such correlation was found (28–30). It seems that DR1 would be necessary for malignant transformation, although separate pathways may be involved in transformation to follicular versus papillary carcinomas (28–30). Clearly, iodide deficiency appears to be associated with high rates of ras mutations in thyroid nodular tissues. Additional genetic or environmental factors may be needed for malignant transformation to take place. It remains to be seen how HLA interacts with ras mutations in modifying the risk for follicular carcinoma.

REFERENCES

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