Gene expression in thyroid and thyroid cancer
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Absence of activating mutations in ras and gsp oncogenes in a cohort of nine patients with sporadic pediatric thyroid tumors

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Abstract

BACKGROUND: Characterization of the genetic background of pediatric thyroid carcinomas could aid in distinguishing between differently staged tumors with respect to treatment and prognosis. Two known genetic factors associated with thyroid carcinoma, the proto-oncogenes gsp and ras were investigated.

PROCEDURE: DNA was extracted from paraffin sections from both tumor and normal thyroid tissue of 9 patients (ages 9-16 years). Of these patients 8 were diagnosed with papillary carcinoma and one with follicular adenoma. The coding exons of gsp and the three known ras genes (H, K, and N-ras) were screened for mutations using SSCP-analysis.

RESULTS: There were no mutations present in the ras and gsp proto-oncogenes hot spots, however LOH of H-ras (chromosome location 11p15.5) was found in tumor tissue from one patient and a homozygous mutation in exon 12 of gsp causing a Pro→Ser conversion was present in thyroid tumor tissue from another patient. Two silent polymorphisms were detected, H-ras exon1, 86T→C and gsp exon 5, 81T→C.

CONCLUSIONS: Our results indicate that the ras/gsp mutations found are probably late events in the tumorigenesis representing general oncogenic stress. In conclusion it seems that ras/gsp activation is not a factor in the mechanism causing sporadic thyroid carcinoma in children.
Introduction

Thyroid cancer accounts for 1.3% of all childhood carcinoma and can be divided into four major groups; papillary, follicular, medullar and anaplastic tumors. In children as well as in adults the papillary type is the most frequent (75%), medullar tumors (15%) can be part of the MEN2 syndrome and follicular and anaplastic carcinomas occur at a low frequency (1-5%) [1]. Prognosis is generally good, a relative 5-year survival rate of 100% for thyroid carcinoma in children from 0-14 years was reported [1]. Treatment generally involves total thyroidectomy and/or ablative radio-iodine therapy. The treatment of thyroid carcinoma results in the need for life-long substitution of thyroid hormone. Since thyroid hormone balance is indispensable for proper physical and mental development of children it is however preferable to keep endogenous thyroid hormone production and normal endocrine regulation pathways intact. Identification of genetic factors in pediatric thyroid carcinoma could eventually aid in pinpointing the stage of disregulation and distinguish between invasive and non-invasive thyroid carcinomas with respect to treatment and prognosis.

Activating mutations in G-protein genes ras and gsp have been identified in a variety of human neoplasms including thyroid [2, 3]. The mechanism behind the activation of the ras and gsp gene-products is well documented. Single base substitutions causing amino-acid changes in the guanine nucleotide binding site (ras codons 12,13; gsp codon 201), or in the effector domain containing the intrinsic GTPase activity (ras codon 61; gsp codon 227) have been shown to cause overstimulation by keeping the protein in a constitutively active state [4, 5, 6]. Studies on thyroid carcinoma show activation of ras and gsp in 18-62% of papillar y carcinoma and an even higher percentage in follicular carcinoma [7, 8]. As for radiation-exposed Chernobyl children with thyroid carcinoma no mutations in ras/gsp hot spots were reported [9, 10]. Starting with the assumption that the etiology of differently originated thyroid carcinoma are not necessarily identical, screening of coding exons of the ras genes H-ras, K-ras and N-ras and of gsp using SSCP-analysis (Single Strand Conformation Polymorphism) was performed thyroid tumors from children not exposed to radiation to look for mutations which could give some insight into the genetic background of these tumors.

Material & Methods

Thyroid carcinoma tissue specimens

From 13 patients (9-18 years of age) diagnosed with thyroid carcinoma in the Netherlands paraffin-embedded tissue was recovered. Tissue samples were pathologically revised according to WHO classifications. Tumor tissue samples with a tumorcell content of at least 90% were selected for DNA studies. Isolation of high-quality genomic DNA proved possible from 9 patients. From several patients more than 1 tumor tissue sample and/or tissue samples from lymph node metastasis were present, in a few cases normal thyroid tissue was also available. Information about screened patients is summarized in table 1 (page 41).

DNA Extraction

DNA was extracted from 50μm slices of paraffin-embedded thyroid tissue. After deparaffinization with xylol, tissue was treated with proteinase K at 65°C overnight followed by phenol-chloroform extraction and sodium acetate ethanol precipitation. DNA from nine samples was of sufficient amount and quality to be able to perform PCR-amplification.
PCR Amplification
For ras/gsp exons 100ng of genomic DNA was amplified in 25μl standard PCR-reactions for 30 cycles at 95°C for 1 min., 55°C for 1 min. and 72°C for 1 min. using AmpliTaq (Perkin Elmer, Foster City, USA).

Primer sequences used to amplify exons from flanking intron sequences were designed as follows:

Gsp primers: 2f-aaaatgcctccctctacacgaga; 2r-tctgttcctcttacttggtg; 3f-tcctccaccagtgctgtctcagcgc; 3r-cagctgctgttctgacttctgttgttggttgp; 4f-tggcttttggttacagagccattga; 4r-gtgacacacaggacgccacgcgaaa; 5f-ctccagccagtgctgttccctgaccg; 5r-ctggcccatgtgcagggctgtcactcatgtt; 6f-gattagttcaagctgttccctgaccg; 6r-ttgtctgtttatgttctgtgatgg; 7f-tggcttttggttacagagccattga; 8f-ggttattccagagggactggggtgaa; 9f-gacattcacccagctccctctgaaat; 9r-aagcggctttctgaacacgcaaag; 10f-ctgggtgttttgaagagagccg; 11f-caggaggcccttggtcacttgg; 11r-agaacacccgcaatgaacagc; 12f-tgctagacccagctctgtgatgg; 12r-agaggaggacagagaggaacct; 13f-catacaggatggtttctcctgtgatgg; 13r-taaggcttttataatatatgtgtggggttc; 14f-ggtgaaacctgtttgttgga; 14r-atacacagaggaagcttcg.

In subsequent amplifications 1μl of amplified material was used in a 10μl radioactive PCR using 33nM (α^32P)dATP under identical conditions as used for the initial PCR.

SSCP (Single Strand Conformation Polymorphism)
10μl of radiolabeled PCR-product was diluted 5 times in SSCP-dilution buffer (0.1% SDS, 10mM Tris-HCI, 1mM EDTA) and again 7 times in SSCP-sample buffer (10mM Tris-HCI, 1mM EDTA, 95% formamide). 5μl sample was loaded on a SSCP-gel (8% acrylamide; 0 and 10% glycerol) and electroforized overnight at room-temperature at 7W constant power [11]. Gel was exposed to Fuji medical film. To determine the frequency of the H-ras polymorphism 40 normal alleles (20 normal genomic DNAs) were screened using RFLP (restriction fragment length polymorphism) The point mutation in exon 1 (86T→C) generates a DraIII-restriction site.

DNA sequencing
DNA samples showing SSCP aberrations were sequenced to determine the nature of the mutations. Reactions were performed using the Dyedeoxyterminator Cycle Sequencing kit (Applied Biosystems/Perkin Elmer, Foster City, USA) according to protocol. Samples were analysed using the ABI Prism 377 DNA sequencer and Sequence Analysis 3.0 software.

Results
From 13 patients paraffin-embedded thyroid tumor material was recovered of which genomic DNA was isolated. In 4 cases DNA was severely degraded and PCR-amplification could not be performed. In 8 cases of papillary carcinoma and 1 case of follicular adenoma ras proto-oncogenes (H-, K-, N-ras) and gsp proto-oncogene were screened for mutations using SSCP. As a control DNA from normal thyroid tissues (frozen and paraffin-embedded) were used in all experiments. Besides screening hot-spot point-mutations
responsible for activation of RAS and Gsα proteins other coding exons of ras and gsp genes were also screened. Exon 1 of gsp could not be amplified due to GC-rich (>90%) domains flanking the exon. Exons 3 and 4 of N-ras were not screened because no intronic sequence data was available to design exon-flanking PCR-primers. No mutations were found in already described hot spots of ras; codons 12/13 (exon 1), codon 61 (exon 2) and gsp; codons 210 (exon 8), codon 226 (exon 9).

Figure 1 shows an example of a typical SSCP pattern of an amplified K-ras exon 1 fragment. Next to the absence of an aberrant pattern in the patient samples as compared to normal controls the positive control samples show a distinct shifted pattern. Control +1 (SW480 colon carcinoma cell-line, codon 12 (GGT→GTT) homozygous), +2 (SW1398 colon carcinoma cell-line, codon 12 (GGT→TGT) heterozygous), +3 (A549 lung carcinoma cell-line, codon 12 (GGT→AGT) homozygous).

In three cases mutations were found. DNA from patient I shows a homozygous (C→T) transition in K-ras exon 4b, 27 bases downstream of TAA stop codon in the 3'UTR (result not shown). Using the H-ras polymorphism for which patient VIII with a follicular variant of papillary carcinoma was heterozygous we detected LOH (loss of heterozygosity) from region H-ras exon 1 (chromosome location 11p15.5) in one out of two tumor DNAs from this patient.
Figure 2A shows the LOH in lane 3 of an SSCP analysis of H-ras exon 1 where a band representing an allele is missing in contrast to lanes 1 and 2, control and an additional tumor sample from the same patient, which have both alleles still present. A coding mutation was found in tumor DNA from patient VI. An homozygous (CCC→TCC) conversion in exon 12 was present resulting in an amino acid substitution (Pro328Ser).

Figure 2A. SSCP-gel showing LOH of H-ras 11p15.5. lane 1: Control thyroid tissue shows two different alleles because of 86T→C polymorphism in exon 1; lane 2: tumor sample #1 of patient VIII where both alleles are present; lane 3: tumor sample #2 of patient VIII, only 1 allele present, LOH.

Figure 2B. SSCP-gel showing a homozygous point-mutation in gsp exon 12. lane 1: tumor DNA sample #1 of patient VI; lane 2: metastasis DNA sample of patient VI; lane 3: tumor DNA sample #2 of patient VI; lane 4: control thyroid DNA. Lanes 1, 2 and 4 show wild-type SSCP pattern, lane 3 shows homozygous band-shift.

Figure 2B shows the SSCP band shift in both bands corresponding to two alleles from exon 12 in lane 3 indicating a homozygous mutation in this DNA sample. Other DNA samples from patient VI shown in lanes 2 and 4 of figure 2B, one from tumor tissue and another from a metastasis did however not show this mutation as is also seen in the control DNA (lane 1). The results from all 9 patients are summarized in table 1.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age of Diagnosis (yrs.)</th>
<th>Type of Tumor*</th>
<th>Mutation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>F</td>
<td>16</td>
<td>papillary</td>
<td>C→T homozygous silent point mutation in exon 4b of K-ras 27bp downstream of stop codon in the 3’-UTR.</td>
</tr>
<tr>
<td>II</td>
<td>M</td>
<td>9</td>
<td>papillary</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>M</td>
<td>13</td>
<td>papillary</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>M</td>
<td>11</td>
<td>papillary</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>M</td>
<td>10</td>
<td>papillary</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>F</td>
<td>15</td>
<td>papillary</td>
<td>CCC→TCC (Pro328Ser) homozygous coding point mutation in exon 12 of gsp</td>
</tr>
<tr>
<td>VII</td>
<td>F</td>
<td>13</td>
<td>follicular adenoma</td>
<td>-</td>
</tr>
<tr>
<td>VIII</td>
<td>F</td>
<td>13</td>
<td>papillary, (follicular variant)</td>
<td>LOH† of H-ras exon 1 (11p15.5)</td>
</tr>
<tr>
<td>IX</td>
<td>F</td>
<td>15</td>
<td>papillary</td>
<td>-</td>
</tr>
</tbody>
</table>

a Pathologically revised according to WHO classifications.

b Mutations were found using SSCP screening followed by sequencing of coding exons from H-ras, K-ras, N-ras and gsp proto-oncogenes.

c LOH - loss of heterozygosity scored using H-ras exon 1 polymorphism (figure 2A).

Discussion

No hot-spot mutations were found in either ras or gsp oncogenes. Three other mutations were found from which the point mutation in the 3’-region of the K-ras gene was silent and therefore not expected to be involved in tumorigenesis. LOH of H-ras was found in one patient. LOH of 11p15.5 is associated with several other childhood tumors, for example rhabdomyosarcoma [13] and Wilms’ tumors [14], in which tumorigenesis is presumably related to loss of a still unknown tumor-suppressor gene located at 11p15.5, but in the case of patient VIII, since LOH is present in only one out of two tumor samples, it is probably a late event in tumor progression and a result of the oncogenic stress, as in elevated proliferation, in which a particular subset of tumor cells behave. Since thyroid tumors are almost always clonal, a tumor inducing mutation should be present in all tumor cells. Similarly, patient VI showed a homozygous coding point mutation in exon 12 of gsp but only in out of three tumor samples. Because of this we can rule out the possibility of this mutation being tumor related and conclude that this mutation also resulted from the oncogenic stress of the tumor cell population.

Overall these results suggest that in sporadic pediatric thyroid carcinoma the G-protein associated oncogenes ras/gsp do not play a role in the pathogenesis. This was also shown in Chernobyl children with thyroid papillary carcinoma [9, 10]. Rather than to suggest a difference in the mechanism of carcinogenesis in radiation-exposed and non-exposed children with thyroid carcinoma these results and data from studies on adult thyroid carcinoma show a difference between adult and pediatric thyroid tumorigenesis.
especially in the papillary cases. In adult thyroid carcinoma ras and gsp activating mutations are frequently found in follicular as well as papillary tumors and show a distinct radiation-dependable pattern [15, 16]. Up till now no ras/gsp activating mutations have been described in pediatric thyroid carcinoma. These results may suggest an additional genetic factor in the carcinogenesis of pediatric thyroid cancer next to the activation by ras/gsp mutation described in adult thyroid tumors.

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