Inhibin $\alpha$-subunit ($INHA$) gene and locus changes in paediatric adrenocortical tumours from TP53 R337H mutation heterozygote carriers


The R337H TP53 mutation is a low-penetration molecular defect that predisposes to adrenocortical tumour (ACT) formation in Brazilian and possibly other populations. Additional genetic defects may be responsible for the variable expression of ACTs in these cases. The inhibin $\alpha$-subunit gene ($INHA$) on 2q33-qter has been implicated in mouse adrenocortical tumourigenesis. We studied 46 pediatric patients with ACTs from Brazil for $INHA$ genetic alterations; 39 of these patients were heterozygous carriers of the R337H TP53 mutation. We first mapped the $INHA$ gene by radiation hybrid analysis and determined 10 linked microsatellite markers in an area flanked by D2S1371 and D2S206 on 2q33-qter. These markers were then used for loss of heterozygosity (LOH) studies in nine paired germline and tumour DNA samples. Mapping placed the $INHA$ gene in close proximity to D2S2848 (SHGC11864) with a log of odds (LOD) score of 5.84. LOH for at least one marker in the region was identified in 8/9 tumours (89%). Six patients were heterozygous for three $INHA$ mutations: one in exon 1, 127C>G, and two in exon 2, 3998G>A and 4088G>A, all leading to amino acid substitutions (P43A, G227R, and A257T, respectively). A257T is located in a conserved region, highly homologous to transforming growth factor-$\beta$; both G227R and A257T change polarity, and, in addition, G227R changes the pH. We conclude that these sequence alterations and the detected 2q allelic changes suggest that $INHA$ may be one of the contributing factors needed for ACT formation in pediatric patient carriers of the R337H TP53 mutation.

Adrenocortical tumours (ACTs) are rare, with an estimated incidence in the US of 0.3 per million per year. In southern Brazil, the incidence is 10-15 times greater, despite no evidence of occupational, endemic, or transmissible diseases that predispose to ACTs. Recently, a germline mutation of the TP53 gene (R337H) was described in 35 of 36 Brazilian children with ACTs. The same mutation was then identified by Latronic and co-workers in adult, as well as additional paediatric, Brazilian patients with ACTs. Furthermore, low-penetration mutations of the TP53 gene were recently identified in other populations. It appears likely that the tumourigenic effect of the low-penetration R337H mutation is related to pH-induced conformational changes in the p53 protein and/or other factors.

In the case of ACTs in the Brazilian population, it has long been hypothesised that additional germline or somatic mutations of other genes with an established tumourigenic role may act in synergy, or may even be required for adrenocortical tumourigenesis. The low penetrance of the R337H mutation could then be explained—only patients who also either carry or acquire additional defects will develop ACTs. Indeed, specific genetic changes may be present in these tumours, such as amplification of the 9q34 chromosomal region, which has not been reported in any other group of ACTs.

Additional factors that have been implicated in ACT formation and could be present in the tumours of this population include lack of imprinting and overexpression of the insulin-like growth factor II (IGF-II) gene, and possibly over-expression of angiotensin-II, endothelin-1 and adrenomedullin and urotensin II or under-expression of novH and CAMP early repressor.

Inhibin is a factor that has been associated with ACT formation at least in rodents. Inhibin is a glycoprotein secreted by the gonads, adrenals, pituitary gland, and pancreas. The complete protein consists of 364 amino acids, with the $\alpha$-subunit corresponding to the C-terminal 134 amino acids. Characterisation of inhibin c-DNA and genomic sequence led to the recognition of its homology with transforming growth factor-$\beta$ (TGF-$\beta$), and its possible role in tissue differentiation and development. The chromosomal localisation of the human inhibin subunit genes was determined by somatic hybrid analysis and reported in 1989. Alpha, $\beta$-A, and $\beta$-B subunits were mapped to human chromosome 2q33-qter, chromosome 7p15-p14, and chromosome 2qcen-q13, respectively.

The human inhibin $\alpha$-subunit gene ($INHA$) (GenBank accession: NT_005289) is encoded by two exons of 267 and 834 base pairs (bp), respectively. In a mouse model, the inhibin $\alpha$-subunit gene ($inh\alpha$) was demonstrated to be an important determinant of corticoid adrenal tumour development. Homozygous inh\textsuperscript{\textalpha} mice developed gonadal tumours at 4–5 weeks of age, and died at 12 weeks. Gonadectomy prior to gonadal tumour formation postponed the wasting syndrome, and allowed the development of ACTs at a later age (21 weeks); death followed at 33–36 weeks.

The results of studies of INHA expression in human ACTs have been less clear as regards its contribution to tumourigenesis. Immunoreactivity for INHA is present in the zona reticularis and in focal collections of cells in the zona fasciculata of the normal adrenal cortex, whereas zona
RESULTS

The INHA amplicon most closely mapped to the chromosone 2 marker D2S2848 (also known as SHGC11864), with a log-of-odds (LOD) score of 5.84 and an estimated distance of 19.5 cR10,000 (approximately 670 kb for chromosome 2). This LOD score of 5.84 and an estimated distance of 19.5 cR10,000 (approximately 670 kb for chromosome 2). The DNA analysis for TP53 mutations of these patients has been reported elsewhere. Out of the 46 patients, 39 were carriers of the R337H mutation and seven did not have any TP53 coding sequence changes (data not shown).

The 10 000 rad Stanford Human Genome Center (SHGC) G3 radiation hybrid (RH) panel from Research Genetics (Huntsville, AL) was used to determine the location of the inhibin gene with respect to polymorphic markers on chromosome 2. PCR was performed twice using a primer pair from an amplicon within the INHA gene. The first primer pair (INHA1-S: 5'-GGT GGC CCA CTC GAC C-3', INHA1-A: 5'-AGA CAT GGG CA-3') was used to amplify exon 1. Exon 1 was amplified with four primer pairs: INHA2-S: 5'-GCA GAT GCC AGC TGT GAG GAC AA-3' and INHA2-A: 5'-AGA CAT GGG CA-3'; INHA3-S: 5'-TCA CGG GGA GGC CCC GTC GTG-3' and INHA3-A: 5'-GGG GAC CAA GAG CC ACC ATC A-3'; INHA4-S: 5'-AGC CTC AAC TCC CCT GAT GTC CT-3' and INHA4-A: 5'-GAG GTG GAG CAC ACC ACC AT-3'; INHA5-S: 5'-TCA TTC TCT ACC ACT GTG ATG-3' and INHA5-A: 5'-ATA CAA GCA CAG TGC TGC GTG AG-3'. These primer pairs generated small-sized fragments of 281, 249, 257, 205 and 244 base pair (bp), respectively. PCR reactions were performed as follows: after an initial denaturation step at 94°C for 5 min and 35 cycles (94°C for 1 min, 60°C for 1 min, for INHA primer-pairs 1, 3, and 4); and 50°C for 1 min (for INHA primer pairs 2 and 5), followed by 72°C for 1 min, the samples were submitted to a prolonged extension cycle at 72°C for 7 min. PCR products were loaded on a 4–20% polyacrylamide minigel (Invitrogen-Novex, Carlsbad, CA, USA). Electrophoresis was performed in a minicell apparatus (MiniCell X-II, Invitrogen-Novex, Carlsbad, CA, USA) at 20 mA, with constant buffer temperature of 18°C. Running time was about 1.5–2.0 h, depending on the size of each fragment. In order to confirm the molecular size of the bands the samples were loaded in parallel to the D-15 molecular marker (Novex, San Diego, CA, USA). After electrophoresis, the gel was incubated for 30 min in TBE buffer containing ethidium bromide. Under UV light, the bands were detected and captured by digital photography (DG-120 digital camera, Kodak-Eastman, Rochester, NY, USA). Bidirectional sequencing using the same primers and fluorescent dideoxynucleotides was performed on an Applied Biosystems (Foster City, CA) model ABI 377 automated sequencer.

Abnormal mobility shifts were detected in the germline DNA of 22/46 cases. However, 16 patients had a 3850C mutation, and seven did not have a 3850C mutation. The PCR products were loaded on a 4–20% polyacrylamide minigel (Invitrogen-Novex, Carlsbad, CA, USA) at 20 mA, with constant buffer temperature of 18°C. Running time was about 1.5–2.0 h, depending on the size of each fragment. In order to confirm the molecular size of the bands the samples were loaded in parallel to the D-15 molecular marker (Novex, San Diego, CA, USA). After electrophoresis, the gel was incubated for 30 min in TBE buffer containing ethidium bromide. Under UV light, the bands were detected and captured by digital photography (DG-120 digital camera, Kodak-Eastman, Rochester, NY, USA). Bidirectional sequencing using the same primers and fluorescent dideoxynucleotides was performed on an Applied Biosystems (Foster City, CA) model ABI 377 automated sequencer.
3850C>T polymorphism abolishes one enzymatic site, resulting in four fragments of 10, 26, 80, and 141 bp.

The presence of the 3850C>T polymorphism was evaluated in our patients and 65 controls (blood donors, 50 males and 15 females, aged 18–45 years) with digestion by the Sau 96 I endonuclease for 4 h at 37˚C. After digestion, the samples were loaded on a 3.5% agarose gel and submitted to electrophoresis at 50 mA for approximately 3 h in TBE buffer with ethidium bromide. The bands were detected under UV light and photographed.

Three of our patients were homozygous for this polymorphism and 13 heterozygous. It seems to be an apparently common INHA polymorphism in the Brazilian population, since 19 of 65 normal controls carried the same sequence change, 17 in the heterozygous and two in the homozygous state (table 1). Statistical analyses of the distribution of the polymorphism versus that expected, according to the Hardy-Weinberg equilibrium, and between the patient and control populations were performed using the χ² test. There were no deviations from the expected frequencies, nor there were differences between patients and normal subjects (p>0.05 for both analyses).

In addition to the polymorphism, three previously undescibed INHA mutations, which were not present in normal controls, were also identified in the heterozygote state in six patients, five with the TP53 R337H mutation and one who did not carry this genetic defect (fig 2). One mutation was found in exon 1 of the INHA gene (127C>G) leading to an amino acid substitution (P43A) and two mutations were found in exon 2 (3998G>A and 4088G>A) which also led to amino acid changes (G227A and A257T, respectively). The P43A mutation was found in only one patient, while the G227A and A257T mutations were found in three and two, respectively. All these patients were from different families—each represented a single case of adrenocortical tumour in his/her kindred. In one patient with LOH in his tumour (patient 1), a mutation was identified. No tumour tissue was available from the other patients to test for INHA LOH.

Of the mutations, A257T is in the conserved INHA region of highest homology with transforming growth factor-β (TGF-β); both G227A and A257T may change the polarity of the protein, while G227A may also change its pH. The mutations are close to four conserved cystein residues that

**Figure 1** Location of the INHA gene on chromosome 2 (proximal to marker D2S2848); flanking markers were tested for loss of heterozygosity (LOH) in the nine paired blood–tumour DNA samples that were available. □ Indicates a normal result (retention of heterozygosity); ■ indicates LOH; non-checked squares were uninformative reactions (samples were homozygous in the blood DNA for the respective markers or produced microsatellite instability).

**Table 1** Characteristics of the adrenocortical tumours submitted to LOH analyses

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Side</th>
<th>Tumour function</th>
<th>Size (cm)</th>
<th>LOH (D2S markers)</th>
<th>MI (D2S markers)</th>
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<tr>
<td>1</td>
<td>F</td>
<td>0.5</td>
<td>L</td>
<td>Vi</td>
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<td>339; 408; 1363</td>
<td>1371; 126; 130</td>
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<tr>
<td>2</td>
<td>F</td>
<td>1.8</td>
<td>L</td>
<td>Vi</td>
<td>4.0±3.0</td>
<td>1363</td>
<td>1371; 163; 339; 130</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>2.0</td>
<td>R</td>
<td>Vi+Cu</td>
<td>4.2±3.5</td>
<td>130</td>
<td>1371; 163; 126</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>0.8</td>
<td>L</td>
<td>Vi</td>
<td>6.0</td>
<td>1363</td>
<td>1371; 173; 163; 130; 408; 351</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>2.8</td>
<td>L</td>
<td>Vi+Cu</td>
<td>5.0</td>
<td>206</td>
<td>1371; 173; 163</td>
</tr>
<tr>
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<td>M</td>
<td>2.7</td>
<td>R</td>
<td>Vi+Cu</td>
<td>8.0±6.0</td>
<td>339</td>
<td>130; 1363; 206</td>
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<tr>
<td>7</td>
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<td>R</td>
<td>Vi</td>
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<td>1371; 173</td>
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<tr>
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<td>F</td>
<td>2.2</td>
<td>L</td>
<td>Vi</td>
<td>6.6±5.8</td>
<td>206</td>
<td>1371; 173</td>
</tr>
</tbody>
</table>

Cu, Cushing syndrome; F, female; L, left; LOH, loss of heterozygosity; M, male; MI, microsatellite instability; R, right; Vi, virilisation.
INHA mutations among TP53 R337H carriers

DISCUSSION

In the present study, we examined paediatric ACTs from R337H carriers for LOH of the INHA locus and for germline mutations of the INHA gene. Most cases in which tumour tissue was available had undergone 2q allelic changes for at least one polymorphic marker in the immediate vicinity of the INHA gene. The LOH data prompted investigation of our paediatric patients for germline mutations of the INHA gene. Consistent with our original hypothesis, three missense mutations of the INHA gene were found in six of our patients and none of these sequence changes were present in the control population. All three mutations are predicted to affect the functional properties of the INHA protein because of corresponding predicted changes in its polarity and/or pH.

A recent review has discussed the body of evidence supporting the role of the INHA gene as a tumour suppressor gene in endocrine oncogenesis. However, up to now, there have been no reported INHA mutations in human neoplasms, including ACTs. In the present study we hypothesised that perhaps this was because INHA mutations act synergistically or as simple co-factors in the development of neoplasia, as we have suggested elsewhere. According to this hypothesis, molecular screening of random samples would not have a high likelihood of detecting INHA mutations. Rather, a specific sample, such as that of patients with the low-penetrance R337H TP53 mutation in the germline heterozygote state, would have to be analysed; in this patient population, other factors had long been postulated to play a role in ACT formation in certain patients and not in others.

The intrinsic mechanism by which inhibin may exert its possible tumour suppressive activity is not well understood but appears to be related to its structural and functional homology to TGF-β. Inhibin antagonises activin through a dominant-negative effect involving the binding of inhibin to the activin receptor type II. This action is further supported by INHA binding to betaglycan, a type III TGF-β receptor, which in turn mediates functional antagonism of activin signalling. The effects of inhibin on the TGF-β signalling pathway could be exerted by binding to betaglycan or inhibin-binding protein.

One possible consequence of the identified INHA mutations would be disruption of the αβ dimerisation, allowing for increased formation of ββ dimers in affected tissues. This apparent imbalance of inhibins (αβ) versus (ββ) activins at the tissue level has been proposed as a potential mechanism for tumour formation and progression. Additionally, the affinity and stability of the mutant inhibin when bound to its receptors could be altered.

The likely multiple interactions of INHA in tumourigenesis, and its cardinal role in reproduction and gonadal morphogenesis, suggest that if germline INHA mutations did contribute to human neoplastic transformation, they would be mostly missense mutations that would alter some of the functional properties of the molecule but would not completely abolish its activity. This hypothesis was supported by the evidence provided by the preliminary immunohistochemistry studies of INHA and other inhibin and activin subunits, at least in ACTs. Unfortunately, no additional tissue was available for immunohistochemistry in the present study.

In our patients and their families there was no increased incidence of other cancers. However, there is evidence suggesting that INHA allelic and sequence changes may be more widespread in endocrine tumourigenesis. First, chromosomal deletions, or even LOH, of genomic regions that harbour genes encoding for inhibin, activin, and their receptors and downstream effectors are frequently observed in tumours of endocrine and non-endocrine tissues, such as adenocarcinomas of the ovary and prostate. In ACTs, in particular, 2q losses have been detected in approximately 42% of tumours. Second, “low-penetrance” germline mutations

Figure 2 In addition to the polymorphism (3850C>T), three previously undescribed INHA mutations, which were not present in normal controls, were also identified in the heterozygote state in six patients. One mutation was found in exon 1 of the INHA gene (127C>G) leading to an amino acid substitution (P43A) and two mutations were found in exon 2 (3998G>A and 4088G>A) which also led to amino acid changes (G227R and A257T, respectively). Of these, A257T is close to the conserved INHA region of highest homology with transforming growth factor-β (TGF-β). G227A and A257T change the polarity of the protein, and G227A may also change its pH.

Figure 3 Schematic representation of the location of the identified inhibin α-subunit gene (INHA) alterations. The three mutations in codons 43, 227, and 257 and the polymorphism in codon 177 are indicated by black arrows. Circles represent the approximate location of cysteine residues and bold lines represent the regions of homology between the various inhibin subunits and TGF-β proteins. The 177 polymorphism and the three mutations are close to four conserved cysteine residues that participate in the formation of hydrogen bonds between the subunits.
of proven or suspected tumour suppressor genes (which have otherwise known lethal or highly deleterious “high penetrance” mutations) have been recently observed in a number of conditions. It is also possible that simple haplo-insufficiency for these mutations predisposes to tumour development, and that LOH is not necessary.

This is the first study to evaluate INHA molecular genetic involvement in human ACTs. Our findings, albeit preliminary, suggest that this gene may in fact be involved in human adrenocortical tumorigenesis, at least in the context of the R377H TP53 mutation. Our study also provides the genetic localization of, and linked markers for, the INHA gene, making future studies of this gene and its locus more accessible to investigators.

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Authors’ affiliations

CA Longui, O Monte, LEP Calliani, Pediatric Endocrinology, Santa Casa de São Paulo, School of Medicine, São Paulo, SP, Brazil

CA Longui, NM Rocha, BM Bello, Laboratory of Molecular Investigation, Santa Casa de São Paulo, School of Medicine, São Paulo, SP, Brazil

CP Lancelotti, Pathology Department, Santa Casa de São Paulo, School of Medicine, São Paulo, SP, Brazil

SHV Lemos-Marini, MT M Baptista, G Guerra-Junior, LSbragia-Neto, UNICAMP Pediatric Endocrinology Unit, State University of Campinas, Campinas, SP, Brazil

B Figueiredo, Pediatric Endocrinology, Federal University of Paraná, Curitiba, PR, Brazil

AC Latronico, BB Mendonca, FMUSP-SP Endocrinology Division, University of São Paulo, São Paulo, SP, Brazil

M Castro, AM Moreira, Endocrinology Unit, University of São Paulo, Ribeirão Preto, Brazil

R Liberatore Jr, Pediatric Endocrinology, School of Medicine, São José do Rio Preto, SP, Brazil

CA Watanabe, AM D Tardelli, AN Giri, Pediatric Endocrinology, Catholic University of Sorocaba, Sorocaba, SP, Brazil


polymorphic sequences maps at the Carney complex (CNC) and Doyne honeycomb retinal dystrophy (DHRD) loci. Genomics 1999;56:344–9.


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