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Tumor Deletion Mapping of Chromosomal Region 13q14 in 43 Growth Hormone Secreting Pituitary Adenomas

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Previous studies have reported allelic loss in chromosomal region 13q14 in pituitary tumors. However, the role of *RB1* in this region has not been clarified. We performed a tumor deletion map of chromosomal region 13q14 with pituitary adenomas and matched blood samples of 43 patients with acromegaly. Twenty-one patients had non-invasive tumors, 19 had invasive tumors, and in 3 this information was not available. Results showed loss of heterozygosity in at least one microsatelite marker of region 13q14 in 12% (5 of 43) of the somatotropinomas. Retention of marker *D13S1325*, telomeric to *RB1*, suggests that the putative tumor suppressor gene is located centromeric to this region, which includes *RB1* locus. The participation of *RB1* was excluded in four of the five cases because retinoblastoma protein was shown to be positive in these tumors in our previous study. Allelic loss occurred in similar frequency in invasive and noninvasive adenomas. In summary, we confirmed the participation of chromosomal region 13q14 in a subset of GH-secreting adenomas with no regard to tumor grade. *RB1* was not implicated, suggesting the participation of another tumor suppressor gene in this region during the first steps of somatotropinoma development.

Key Words: Somatotropinoma; *RB1*; tumorigenesis; chromosome 13q14; retinoblastoma.

Introduction

Genetic markers related to pituitary tumors are gradually being described but only few abnormalities have been clearly associated with the initiation and/or progression of GH-secreting pituitary adenomas (somatotropinomas). Oncogene *gsp* is the most frequent and specific mutation found in somatotropinomas. It is detected in up to 40% of GH-secreting adenomas *(1–6)*, and it is equally frequent in invasive and noninvasive tumors *(7)*. Other genetic abnormalities such as loss of heterozygosity of locus 11q13 *(8–18)*, low expression of p27 *(19–21)*, and overexpression of *PTTG (22,23)* are found in lower frequency. Retinoblastoma protein (RB), a product of *RB1* located at chromosomal region 13q14, is an important regulator of cell cycle. Loss of RB function leads to progression from G1 to S phase, favoring replication of the cell *(24)*. Mice heterozygous for *Rb1* inactivation develop tumors of the intermediate pituitary lobe with near complete penetrance *(25,26)* and less frequently anterior lobe pituitary adenomas *(27)*. However, the role of this tumor suppressor gene (TSG) in the pathogenesis of human pituitary tumors, including somatotropinomas, is controversial. Simpson et al. *(28)* found that RB was negative in 27% (9 of 33) of somatotropinomas, and we have reported that RB was underexpressed in 20% (10 of 49) of GH-secreting tumors *(29)*. In both studies expression of RB was not different in invasive and noninvasive tumors suggesting that low levels of this protein may represent an early event in the pathogenesis of this tumor subtype. Conversely, Honda et al. *(30)* did not find any abnormality in gene expression or sequencing of *RB1* in a group of pituitary adenomas, including 12 GH-secreting adenomas. Evidence for participation of another TSG in chromosomal region 13q14 different from *RB1* in progression toward aggressive tumors has been suggested by two studies *(31,32)*. Pei et al. *(31)* described that all 13 malignant and highly invasive pituitary tumors, but only 4 of 17 benign pituitary adenomas, had loss of heterozygosity (LOH) in polymorphic microssatellite markers surrounding *RB1* at the long arm of chromosome 13. Immunohistochemistry analysis, however, revealed presence of RB in tumors with LOH at the *RB1* locus. Bates et al. *(32)* found that the chromosomal region 13q14 presented one of the highest rates of LOH among pituitary adenomas (16%, 14 of 85), especially in high-grade tumors. These allelic losses were more frequently associated with a microssatelite marker centromeric to *RB1*.

Hence, the role of chromosomal region 13q14 in pituitary tumors has not been clarified. In the present study

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we assessed the participation of this chromosomal region in somatotropinomas by performing a tumor deletion map (TDM) using pituitary adenomas of Brazilian patients with acromegaly.

Results

Tumor Deletion Mapping

Tumor deletion mapping of 43 cases is summarized in Fig. 1. Twelve percent (5 of 43) had LOH for at least one of the evaluated microsatellites (Fig. 2). In 3 of the 5 cases, allelic loss included *RB1* intragenic marker *D13S153*, and in two cases (15 and 23), this marker could not be assessed because it was not informative. In case 23 the presence of LOH in markers centromeric and telomeric to *RB1* suggests that allelic loss involves the entire chromosomal region 13q14. Notably, retention of heterozygosity of *D13S1325* in cases 4 and 8 suggests that the area of chromosomal loss is centromeric to this microsatellite marker.

Frequency of LOH in Chromosomal Region 13q14 in Noninvasive and Invasive Adenomas

Presence or absence of LOH in markers of region 13q14 in invasive and noninvasive adenomas is shown in Table 1. Although the fraction of invasive tumors in the group with LOH in 13q14 markers (60%, 3 of 5) was slightly larger than in the group without LOH (46%, 16 of 35), this difference was not statistically significant ($p = 0.471$).

Discussion

Previous studies suggested the participation of a TSG located at chromosomal region 13q14 in the pathogenesis of pituitary tumors *(28,31,32)*. Our results support this observation in 12% of GH-secreting adenomas. Result of TDM indicates that the area of chromosomal loss is centromeric to microsatellite marker *D13S1325* and includes *RB1* locus. We did not find correlation between allelic loss at the studied chromosomal region and tumor biological behavior.

Four of the five cases that showed LOH in region 13q14 (cases 4, 8, 15, and 23) had previously been evaluated for RB expression, and in all these cases this protein was present in at least 30% of the adenomatous cells *(29)*. Therefore, the participation of a TSG different from *RB1* within the same chromosomal region should be sought in these cases. Several TSGs have been identified in region 13q12-21 and are related to some neoplasms. Analysis of our TDM suggests an overlapping region of allelic loss centromeric to marker *D13S1325*; therefore, TSGs that should be initially considered are the ones in this region (Table 2). *BRCA2* is an important target for development of early onset breast carcinoma *(33)*. Its participation in pathogenesis of pituitary adenomas has previously been studied, but the results do not support a significant role for this gene. Simpson et al. *(28)* evaluated chromosome 13q in nonfunctioning and GH-secreting adenomas and found that both tumor subtypes had 10% of LOH in the marker related to *BRCA2*. However, the area of allelic loss also included *RB1* intragenic marker in some of these tumors. *CDX2* gene expression is restricted to intestine epithelia in adults; therefore, the chances of its participation in development of pituitary tumors are very small. *LATS2* gene plays an inhibitory role in cell cycle progression but its role in the pathogenesis of pituitary tumors has not been assessed. The function of the other TSGs in this chromosomal region awaits clarification.

In addition, chromosomal region 13q14 is one of the loci that contain micro-RNAs (miRs) genes. miRs are small noncoding RNAs that most likely function as antisense regulators of other RNAs *(34,35)*. *miR-15a* and *miR-16-1* genes are located at region 13q14, and their expression was shown to be decreased in 10 somatotropinomas and 10 prolactinomas compared to normal pituitary tissue *(36)*. Moreover, levels of miR-15a and miR-16-1 inversely correlated with tumor diameter, suggesting that *miR-15a* and *miR-16-1* may be involved in regulation of pituitary tumor growth. The mechanism through which miRs interfere with tumor growth is not fully understood. Allelic loss at chromosome 13q14 of somatotropinomas in our series could potentially result in decreased expression of *miR-15a* and *miR-16-1*.

We did not find correlation between allelic loss at chromosomal region 13q14 and tumor biological behavior. A possible explanation for this finding is that a TSG in this region may play a role in the first steps of pituitary tumor development. In opposition, previous studies have found LOH in chromosomal region 13q14 to be more frequent in aggressive non-functioning *(28)* and mixed pituitary tumor subtypes *(31,32)*, favoring the participation of allelic loss on tumor progression. Interestingly, this pattern does not seem to occur in somatotropinomas; Simpson et al. *(28)* and we found similar frequency of LOH in chromosomal region 13q14 in noninvasive and invasive GH-secreting pituitary tumors. Therefore, allelic loss in region 13q14 may participate at different stages of pituitary tumor development in different adenoma subtypes. This could be due to presence of more than one TSG in this region.

In conclusion, this study supports the role of a TSG in region 13q14 different from *RB1* in the first steps of the pathogenesis of a subset of GH-secreting pituitary adenomas.

Materials and Methods

Subjects

Forty-three sporadic somatotropinomas with matched blood samples were obtained from acromegalic patients. Thirty-eight of these cases have previously been evaluated for tumor RB expression *(29)*. The ethical research committee of our institution approved this protocol and informed consent was obtained from all subjects. Diagnosis of acromegaly was made when serum GH was not suppressed below 1 μg/L during oral glucose tolerance test and/or when serum

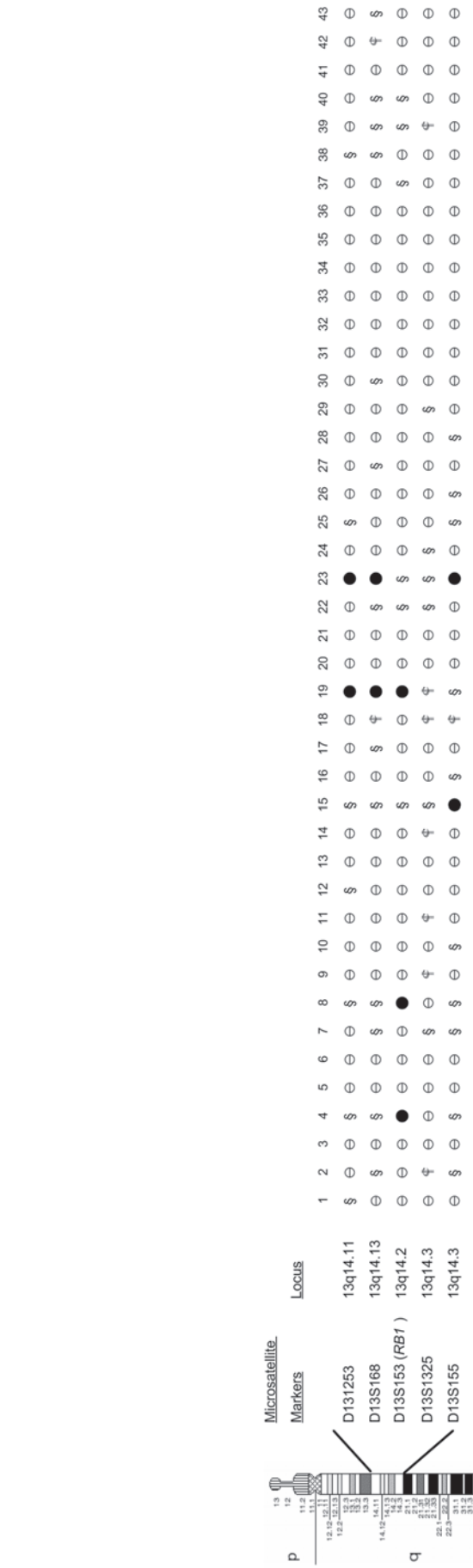

Fig. 1. Tumor deletion map of chromossomal region 13q14.

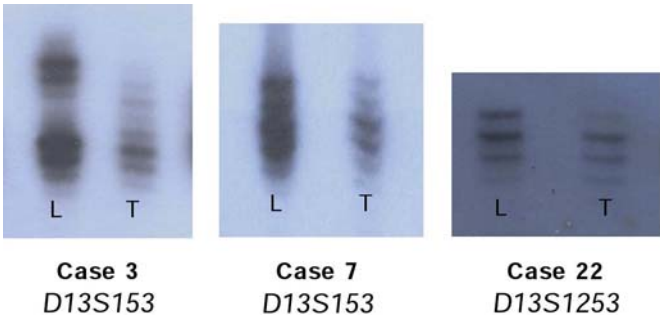

Fig. 2. Shown are images of gels containing PCR-amplified DNA from tumors (T) and leukocytes (L) of cases that presented LOH in microssatelite markers in chromosomal region 13q14.

Table 1
Distribution of Cases According to Tumor Behavior and Tumor Deletion Map Result

TDM* of region 13q14	Noninvasive tumors	Invasive tumors
LOH** (% of total number of cases with LOH)	2 (40%)	3 (60%)
Retention of heterozygosity (% of total number of cases without LOH)	19 (54%)	16 (46%)

*TDM, tumor deletion map.
**LOH, loss of heterozygosity.

Table 2
Candidate Tumor Suppressor Genes for GH-Secreting Adenomas

Tumor suppressor gene	Locus	Described role	Function	Reference
LATS2	13q11-q12	Lung carcinoma	Negative regulator of cell cycle	*40*
CDX2	13q12.3	Colorectal cancer	Homeobox gene; in adults, it is expressed only in intestinal epithelium	*41,42*
BRCA2	13q12.3	Early onset breast carcinoma	DNA repair	*33*
RFP2	13q14	B-CLL*	Unknown	*43*
DICE1	1314.12-q14.2	Lung carcinoma	Putative RNA helicase	*44*

*B-CLL: B-Cell lymphocytic leukemia.

IGF-I was above normal range for age and sex *(37)*. Diagnosis of GH-secreting pituitary adenoma was performed by standard hematoxylin–eosin and immunohistochemical staining. Tumors were evaluated through computed tomography (CT) and/or magnetic resonance imaging (MRI) prior to surgery and graded according to criteria based on a modified Hardy's classification *(38)*. Briefly, tumors were classified as noninvasive when the sella turcica was intact and when extrasellar extension, if present, was limited to the suprasellar cistern up to the III ventricle. Tumors were classified as invasive if the sella turcica was partially or totally destroyed and/or if the tumor extended intracranialy or invaded lateral cavernous sinus. Based on these criteria, 21 patients had noninvasive and 19 had invasive tumors (Fig. 3). In three cases (14, 31 and 35) tumor grade could not be ascertained because we did not have access to preoperatory CT or MRI. There were no carcinomas in this series.

Tissue Collection and DNA Extraction

Pituitary adenoma tissue was obtained from frozen samples or from paraffin-embedded tissue blocks. Peripheral blood samples were collected from all patients. DNA was extracted according to manufacture's instructions as follows: frozen tumor DNA was obtained with DNAzol (Life Technologies, San Diego, CA), paraffin-embedded tumor DNA with DNeasy tissue kit (Qiagen, Hilden, Germany), and leukocyte DNA with GFX Genomic Blood DNA Purification Kit (Pharmacia Amersham, Buckinghamshire, UK).

Tumor Deletion Mapping

Oligonucleotide sequences (Life Technologies) specific to highly polymorphic microsatellite markers spanning chromosomal region at 13q14 were selected based on UniSTS *(39)*. Marker *D13S153* lies in intron 2 of *RB1*; *D13S1253* and *D13S168* are centromeric, and *D13S1325* and *D13S155* are telomeric to *RB1*. Tumor and leukocyte DNA were amplified as follows: PCR reaction mixture (20 μL) contained 1X PCR buffer (Life Technologies), 1–1.5 m*M* $MgCl_2$ (Life Technologies), 0.2 m*M* deoxy (d)-NTPs (Life Technologies), 200 p*M* primers (Life Technologies), and 2.5 U Taq Polimerase (Life Technologies) and was submitted to 39 cycles consisting on denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 10 min. Leukocyte and tumor DNA products were electrophoresed in 6% polyacrylamide urea gel. Visualization of PCR products was performed either by autoradiography or by silver staining. For autoradiography, reverse primer of each oligonucleotide pair was end labeled with [γ-^{32}P]ATP using the 5' DNA Terminus Labeling System kit (Life Technologies). For silver stain, gel was treated in silver nitrate 1 g/L in 0.15% formaldehyde for 20 min, sodium carbonate 15 g/L in 0.15% formamide for 5 min

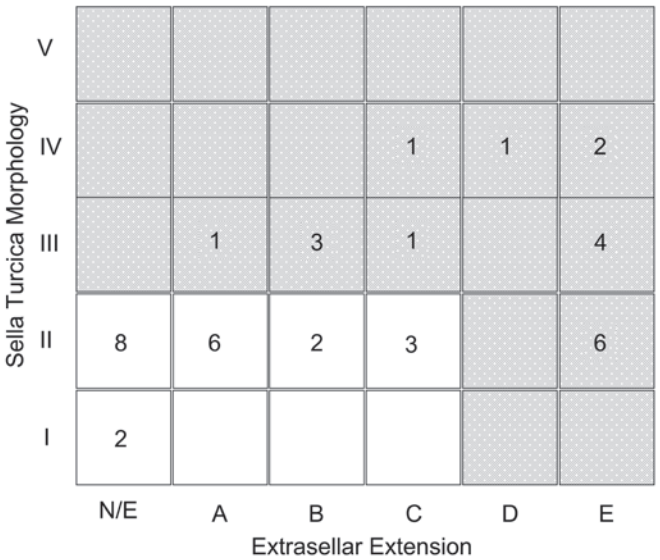

Fig. 3. Tumor grade according to Hardy's modified classification. Sella turcica morphology: I, intact, normal contour; II, intact, enlarged; III, partially destroyed; IV, totally destroyed; V, distant spread via cerebrospinal fluid or blood. Extrasellar extension: N/E, no extrasellar extension; A, suprasellar cistern; B, recesses of III ventricle; C, whole anterior III ventricle; D, intracranial; E, lateral cavernous sinus. The numbers indicate the number of adenomas with different combinations of sella turcica morphology and extrasellar extension. The non-shadowed area correspond to the noninvasive tumors (total = 21) and the shadowed area corresponds to the invasive tumors (total = 19). This figure does not include the three cases that could not be classified because the pituitary preoperatory imaging was not available.

and sodium carbonate 15 g/L in 0.075% formamide until stained. For informative cases, absence or significant reduction in signal of one allele in tumor sample relative to corresponding allele in the leukocyte sample was scored as loss of heterozygosity (LOH). Two observers with no information about tumor grade independently scored the results. A LOH result was recorded only if reduction in intensity was clear and concordant among the observers and was confirmed by repeating the experiment two or three times.

Statistical Analysis

Chi-square test was used to compare allelic loss in TDM and tumor grade, using SPSS program version 11.0 for Windows (SPSS Inc.). Statistical significance was established at the level of $p < 0.05$.

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