Serpin Peptidase Inhibitor Clade A Member 1 as a Potential Marker for Malignancy in Insulinomas

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Abstract

Purpose: The biological behavior of insulinomas cannot be predicted based on histopathologic criteria in which the diagnosis of malignancy is confirmed by the presence of metastases. In this study, microarray and quantitative real-time reverse transcription-PCR were applied to identify differentially expressed genes between malignant and nonmalignant insulinomas to search for useful biomarkers to recognize the metastatic potential of insulinomas.

Experimental Design: CodeLink human bioarrays were used to analyze differences in ~20,000 genes between six well-differentiated endocrine tumors of benign behavior compared with one well-differentiated endocrine carcinoma (WDEC) and three metastases of endocrine carcinomas (MEC). Quantitative real-time reverse transcription-PCR was used to validate differential expressions of five genes in a series of 35 sporadic insulinomas. Serpin peptidase inhibitor clade A member 1 (SERPINA1; α-1-antitrypsin) expression, identified as up-regulated in malignant insulinomas, was also evaluated by immunohistochemistry.

Results: Analysis of microarray data resulted in 230 differentially expressed genes. Gene Ontology analysis identified serine-type endopeptidase activity and serine-type endopeptidase inhibitor activity as pathways presenting significant differential expression. Protease serine 2 and complement factor B (from serine-type endopeptidase activity pathway) were respectively confirmed as up-regulated in well-differentiated endocrine tumors of benign behavior (WDET) and in WDEC/MEC. Angiotensinogen and SERPINA1 (from serine-type endopeptidase inhibitor activity pathway) were confirmed as up-regulated in WDEC/MEC. SERPINA1 was shown to be expressed in 85.7% of malignant versus 14.3% of nonmalignant insulinomas by immunohistochemistry.

Conclusions: Our data are consistent to the possibility that SERPINA1 is a marker of malignancy in insulinomas. Given the widespread availability of antibody anti-α-1-antitrypsin in pathology services, SERPINA1 expression evaluation might be of clinical utility in recognizing patients more likely to develop an aggressive presentation.

Although insulinomas are rare endocrine tumors with an estimated annual incidence of four cases per million population (1, 2), they are the most frequent functional islet of Langerhans cell tumors (3). In contrast to the other types of pancreatic endocrine tumors, the majority of insulinomas are benign at the time of diagnosis (4). The average percentage of malignancy is 8.4% and its diagnosis can only be made in the presence of gross local invasion and/or distant metastases (5, 6). However, even the presence of metastasis cannot predict the clinical course, and there are patients with metastatic disease who present good clinical evolution. Insulinomas with malignant behavior contain a higher number of genetic alterations than benign tumors, but only scarce information is available on molecular mechanisms underlying insulinoma carcinogenesis. Accumulation of several genetic alterations, including c-myc and transforming growth factor-α/epidermal growth factor receptor overexpression, c-K-ras point mutations, and p53 protein overexpression, may contribute to tumor development and/or progression from a benign to a malignant phenotype (7, 8).

The difficulty in predicting the biological behavior of insulinomas based on histopathologic criteria alone has long been recognized (4). Jonkers et al. (9) have shown that chromosomal instability is a powerful indicator for the development of metastatic disease in patients with sporadic
insulinoma, and Vezzosi et al. (10) have suggested that the presence of telomerase activity within a primary endocrine tumor might indicate a malignant behavior. In the present study, microarray and quantitative real-time reverse transcription-PCR (RT-qPCR) analyses were applied in an attempt to identify differentially expressed genes between malignant and nonmalignant insulinomas with the aim of searching for useful biomarkers to recognize the metastatic potential of insulinomas.

**Materials and Methods**

**Patients and tissue specimens.** Tissue collection was carried out in compliance to the Institution's Ethics Committee and in accordance to The Declaration of Helsinki, with informed and free consent being required of each subject or subject's guardian. From 1999 to 2005, tumor tissues were obtained from patients diagnosed with sporadic insulinomas based on the clinical features of Whipple's triad of symptoms with endogenous hyperinsulinemia. During laparotomy, tumor fragments were collected in sterile containers and immediately frozen in liquid nitrogen. As shown in Table 1, 35 insulinoma samples collected from 33 patients were included in the study as follows: 5 liver metastases of insulinomas and 30 primary tumors, 2 of them corresponding to the primary insulinoma of hepatic metastases.

Tumor fragments were resected by a single experienced surgeon, and the fragments for RNA extraction were carefully removed from the central portion (by a single person) to avoid contamination with normal pancreatic tissue. Most tumors were encapsulated and samples evaluated in the microarray analysis presented an expansive growth pattern, which reduces the possibility of acinar cell presence.

**Histologic and immunohistochemical analyses.** Sections of all tumor tissues were histologically analyzed by H&E staining. Histologic examination included mitotic index (number of mitosis per 10 high-power fields) and presence of perineural and vascular invasion. Immunohistochemical staining was done in paraffin-embedded blocks by avidin-biotin peroxidase complex method using antisera anti-chromogranin A (BioGenex Laboratories), synaptophysin, insulin, proliferation-related Ki-67 antigen, and serpin peptidase inhibitor clade A member 1 (SERPINA1; α-1-antitrypsin; DakoCyto- mation Denmark A/S). Mitotic index was graded from 0 to 3, perineural and vascular invasion was graded as 1 (no) or 2 (yes), proliferation rate (Ki-67) was graded as 0 (<2%) or 1 (>2%), and tumor sizes were graded as 1 (<10 mm), 2 (10 to 20 mm), 3 (20 to ≤40 mm), or 4 (>40 mm). Based on these histopathologic features, insulinomas were classified according to the 2000 WHO International Classification of

### Table 1. Clinical, histopathologic, and immunohistochemical features of tumor specimens studied

<table>
<thead>
<tr>
<th>No. patient</th>
<th>Tumor type</th>
<th>Outcome</th>
<th>Follow-up (mo)</th>
<th>Tumor size (mm)</th>
<th>IMH hormones</th>
<th>IMH Ki-67 (% cells)</th>
<th>IMH SERPINA1 (% cells)</th>
<th>Mitotic index (/10 HPF)</th>
<th>WHO classification</th>
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<tr>
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</tr>
<tr>
<td>32</td>
<td>M</td>
<td>Recurrence</td>
<td>15</td>
<td></td>
<td>Ins —</td>
<td>&gt;1</td>
<td>—</td>
<td>MEC</td>
<td></td>
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<tr>
<td>33</td>
<td>PT</td>
<td>Deceased (sepsis)</td>
<td>62</td>
<td>20</td>
<td>Gluc &gt;2</td>
<td>&gt;1</td>
<td>1</td>
<td>WDEC</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>M</td>
<td>Deceased (sepsis)</td>
<td>62</td>
<td>10</td>
<td>Gluc —</td>
<td>&gt;1</td>
<td>—</td>
<td>MEC</td>
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</table>

Abbreviations: PT, primary tumor; M, metastasis; IMH, immunohistochemistry; Ins, insulin; Gluc, glucagon; SST, somatostatin; NA, not available; HPF, high-power field.

*1 Laparotomic biopsies.

*1 Multiple nodules (largest nodule, 20 mm in diameter).
Endocrine Tumors as follows: well-differentiated endocrine tumors of benign behavior (WDET-BB), confined to the pancreas, nonanorgiav- nusive, no perineural invasion, <2 cm in diameter, less than two mitoses per 10 high-power fields, and <2% Ki-67–positive cells; well- differentiated endocrine tumors of uncertain behavior (WDET-UB), confined to the pancreas and one or more of the following features: ≥2 cm in diameter, 2 to 10 mitoses per 10 high-power fields, >2% Ki-67–positive cells, angioinvasive, and perineural invasion; well- differentiated endocrine carcinoma (WDEC), in the presence of gross local invasion and/or metastases; and metastasis of endocrine carcinoma (MEC, ref. 6). A single pathologist has evaluated all samples.

**Study design.** As a screening strategy to determine which genes were differentially expressed in malignant (WDEC and MEC) compared with nonmalignant insulinosomas (WDET-BB and WDET-UB), we did gene expression profiling by microarray analysis of six tumors classified as WDET-BB compared with four malignant tumors, one classified as WDEC and three as MEC. The criteria for discovering differentially expressed genes by microarray analysis are comprehensively described in Microarray Data Analysis section. To validate microarray results, five differentially expressed genes were selected and their expressions were relatively quantified by RT-qPCR in a larger set of insulinosoma samples as follows: WDET-BB (n = 17), WDET-UB (n = 11), WDEC (n = 2), and MEC (n = 5). SERPINA1 (α-1-antitrypsin), identified as up-regulated in malignant insulinosomas (WDEC/MEC), was also evaluated by immunohistochemistry in all studied tumor tissues.

**Microarray.** Gene expression profiling was done using CodeLink UniSet Human 20K 1 Bioarray (GE Healthcare Biosciences). The Minimum Information about a Microarray Experiment checklist containing all information is included as Supplementary Data 1. Furthermore, a detailed description of the microarray experiment, according to the Minimum Information about a Microarray Experiment criteria, is also available online at Gene Expression Omnibus (accession number GPL2891).6

Total RNA from tumor tissues was prepared using Trizol reagent (Invitrogen) according to standard protocols provided by the manufacturer. RNA samples were purified with RNeasy Mini kit (Qiagen GmbH), and total RNA integrity was assessed by denaturing agarose gel electrophoresis. Concentrations were quantified by spectrophotometry (GeneQuant Pro DNA/RNA Calculator, GE Healthcare Biosciences), and 2 µg were used from each studied subject. Target synthesis and hybridizations were done with the CodeLink Expression Assay kit (GE Healthcare Biosciences) according to the manufacturer’s instructions.

**Microarray data analysis.** The ~20,000-spot intensities on the microarray image were quantified in CodeLink Expression Array Software (GE Healthcare Biosciences), filtering out genes with missing spots, and the expression values from duplicated were averaged. Intensity of each spot was divided by the intensity median of the array to provide a scaled and comparable number across multiple arrays. The expression values of these genes were normalized individually across all arrays. Expression ratio was calculated by log2, between six WDET-BB and four WDEC/MEC. Unpaired Student’s t test was used for two-group statistical comparisons. Differentially expressed genes were selected according three basic criteria: presence of at least 40% of “C” (good) flag in analyzed arrays, differential expression either up-regulated or down-regulated by at least 2-fold in the average WDET-BB compared with WDEC/MEC expression data, and statistically significant log2; ratio at probability levels of P < 0.05. Correction for multiple testing was then done using the method of Reiner et al. (11) to derive a false discovery rate estimate from the raw P values. A false discovery rate of 5% was set as a cutoff for statistical significance. Supervised hierarchical clustering analysis was done among differentially expressed genes and different tumor tissue analyzed. All statistical analysis was done using IMP Release 5.1.1 software (SAS Institute, Inc.).

**Gene Ontology analysis.** Microarray gene expression data were analyzed by GeneSifter program.7 Gene ontology routine included in the program was chosen for further interpretation of the data about molecular function-related genes according to Gene Ontology (GO) Consortium categories (12).8 All the pathways in the GeneSifter database were examined to determine whether a significant number of altered genes in GO term were affected. We examined z-scores that express the frequency of genes fulfilling an increase ≥2 in each GO term and compared with null hypothesis expected frequency for that GO term based on the total number of genes examined on the array. The z-score was derived by dividing the difference between the observed number of genes meeting the criterion in a specific GO term and the expected number of genes based on the total number of genes in the array meeting the criterion. This value was then divided by the SD of the observed number of genes under a hypergeometric distribution. Positive z-scores indicate GO terms with a greater number of genes meeting the criterion than is expected by chance. Negative z-scores indicate GO terms with fewer genes meeting the criterion than expected by chance. A z-score near zero indicates that the number of genes meeting the criterion approximates the expected number. A z-score of ≥2 is considered a statistically significant association (approximately equivalent to a P ≤ 0.05) between the differentially regulated genes and their corresponding GO terms (13, 14). A false discovery rate of 5% was also set as a cutoff for statistical significance.

**Quantitative real-time reverse transcription-PCR.** Five genes identified by microarray analysis were also evaluated by relatively RT-qPCR to validate transcriptional responses in a large tumor series: protease serine 2 (PRSS2), chymotrypsinogen B1 (CTRB1), SERPINA1, angiostensi- nogen (AGT), and complement factor B (CFB). Relative expression of each gene was measured in reference to the housekeeping gene proteasome 26S ATPase subunit 6 (PSMC6) and normalized with a reference sample from a unique insulinoma patient.

For stability comparison of some candidate housekeeping gene presented in CodeLink array, the NormFinder software (15) was applied (freely available online).9 It is a Microsoft Excel add-in that calculates the stability values of individual candidate reference genes for normalization. Stability value is based on combined estimate of intragroup and intergroup expression variations of the genes studied. The PSMC6, used as a reference gene in a previous study (16), presented one of the lowest stability value compared with well-known house-keeping genes.

Total RNA was extracted from 35 insulinosomas as mentioned previously in microarray analysis. Reactions were conducted on a Rotor- Gene RG-3000 (Corbett Research) using SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). RT-qPCR was done in a final volume reaction of 25 µL with 5.0 µL RNA sample (20 ng/µL). 0.5 µL SuperScript III RT/Platinum Taq Mix, 12.5 µL × SYBR Green Reaction Mix (containing 3 mmol/L MgSO4), and 0.2 sense/antisense primer. Negative samples were run for each RT-qPCR assay consisting of no RNA in the reverse transcription reaction and no cDNA in the PCR. To confirm accuracy and reproducibility of RT-qPCR, the intra-assay coefficients of variation for each gene calculated with five replicates of a unique sample within one run varied from 3.6% to 8.3% and the interassay coefficients of variation for each gene calculated with the very same sample repeated in duplicate in each assay varied from 6.1% to 9.8%. Reactions were carried out under the following cycling conditions: 5 min at 50°C and 5 min at 95°C followed by 35 three- temperature cycles (20 s at 95°C, 30 s at 55°C, and 30 s at 72°C). Fluorescence changes were monitored after each cycle, and melting curve analysis was done at the end of cycles to verify PCR product identity (72°C ramping to 99°C at 0.2°C/s with continuous fluores- cence readings). Specificity of amplicons was also ensured by agarose

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6 http://www.ncbi.nlm.nih.gov/geo/
7 http://www.genesifter.net/web/
8 http://www.geneontology.org/GO.doc.html
9 http://www.mdl.dk.
gel electrophoresis to visualize a unique product fragment with appropriate size. Gene-specific primer pairs were located on two adjacent exons to achieve a high level of specificity and to avoid detection of genomic DNA. Primers were designed to have similar guanosine-cytosine content and melting temperatures by using Primer3 Program\textsuperscript{10} (17): PRSS2 (NM_002770), 5’-CCCTACCAGGTTCCTGGAA-3’ (sense) and 5’-AGTCGCGCTTTGATTTGG-3’ (antisense; 204-bp product); SERPINA1 (NM_000295), 5’-CACCCAGATATCATCACCA-3’ (sense) and 5’-TCAGTCCCTTTTCGTCGAT-3’ (antisense; 237-bp product); AGT (NM_000029), 5’-GAAGTCTGGATGTTGCTGCA-3’ (sense) and 5’-GCTGTTGTCCACCCAGAAC-3’ (antisense; 189-bp product); CFB (NM_001710), 5’-TAATGCAGACTGGGATCGGTA-3’ (sense) and 5’-ATCTGCCTTCATCCGGTGTGAA-3’ (antisense; 239-bp product); CTRB1 (NM_001906), 5’-AGGGATCCACCGACGC-3’ (sense) and 5’-TGGAGGTGGAGCAGGTGT-3’ (antisense; 151-bp product); and PSMC6 (NM_002806), 5’-GCTGCGTCCAGGAA-3’ (sense) and 5’-TGCGAACATACCTGCTTCAG-3’ (antisense; 196-bp product).

To evaluate the amplification efficiency of each target and housekeeping genes, standard curves were constructed from a reference sample of RNA using duplicate serial dilutions with six different RNA concentrations (0.2, 0.8, 3.1, 12.5, 50, and 200 ng/\(\mu\)L). The mathematical model described by Pfaffl (18) was used to evaluate the relative expression ratio for all genes compared with PSMC6 and efficiency values for each set of primers, additionally normalized to the amount of RNA reference. Data were analyzed by an unpaired, two-tailed Student’s \(t\) test with statistical significance attributed to \(P < 0.05\).

**Statistical analysis of RT-qPCR.** Statistical tests were evaluated by JMP Version 5.1 statistical computer program (SAS Institute). Because assumptions for a parametric test were not valid (\(P < 0.05\), Kolmogorov-Smirnov), all data were evaluated by Kruskal-Wallis ANOVA complemented by Scheffe test as a multiple comparison method. Statistical power was calculated for each comparison and considered adequate when >80%. The Spearman test was used to assess the statistical significance of correlations between mRNA expression and histopathologic data that could imply any malignant feature, such as mitotic index, perineural and vascular invasion, proliferation rate (Ki-67), and tumor size. These variables were graded as described in the Histologic and Immunohistochemical Analyses section. Data are expressed in mean ± SE. Statistical significance was fixed at probability levels of <0.05.

**Results**

**Clinical and histopathologic findings.** The cohort observed in our series was similar to other large series of patients (1) with hypoglycemia syndrome. Disease duration ranged widely from 1 to 168 months with a median of 24 months. The frequency of malignancy was 15%. Clinical and biochemical data are summarized as Supplementary Table S1.

Table 1 describes outcome data and follow-up for all patients and relevant histologic and immunohistochemical features from 35 tumor specimens analyzed in 33 patients. Among the patients with WDET-BB (median follow-up, 42; range, 12-89 months) and WDET-UB (median follow-up, 43; range, 22-74 months), no recurrence of disease was observed at any time during follow-up. Mean largest diameter of the tumors was 19.7 ± 20.5 mm (mean ± SD) ranging from 6 to 120 mm. There was no evidence of perineural invasion and necrosis in any samples. Few tumors (8.6%) presented mitotic index more than two mitoses per 10 high-power fields. None of the tumors analyzed presented necrosis in the histopathologic

\textsuperscript{10}http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

Fig. 1. Expression profiles of 10 samples representing 6 WDET-BB and 4 WDEC/MEC analyzed by supervised hierarchical clustering using 230 differentially expressed genes. Row, single gene; column, tissue sample.
differentially expressed resulted on the dendrogram (Fig. 1),

<table>
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NOTE: Genes changed refers to the number of distinct genes that met the criteria considered for the expression data set analysis. The table shows molecular function GO IDs and GO terms, number of perturbed genes (up-regulated and down-regulated in relation to WDET-BB), and z-scores for up-regulated and down-regulated genes. Serine-type endopeptidase activity and serine-type endopeptidase inhibitor activity pathways presented a z-score >2 for genes up-regulated in WDET-BB. Z-score is the standard statistical test under the hypergeometric distribution used to check if the genes in the GO term would show a difference in the ratio of genes meeting the criteria considered for the expression data set analysis compared with the background percentage in the entire GO. A positive z-score indicates that there are more genes meeting the criteria in the GO term than would be expected by random chance. A negative z-score indicates that the there are fewer genes meeting the criteria than would be expected by random chance.

examination. Immunohistochemical study for Ki-67 exhibited an important immunoreaction (>2% of positive cells) in 14.3% of all analyzed tumor tissues. Ki-67 immunostaining and mitotic index were not applicable for analysis in metastatic tumors. Immunoperoxidase staining for chromogranin A and synaptophysin was positive in all tumor tissues analyzed. Hormonal immunohistochemical analysis showed positive insulin staining in all samples tissues, except in the primary tumor from sample 33, despite unequivocal clinical hypoglycemia. Immunoreaction for other hormones was observed in nine tissue samples: six positive somatostatin staining (two up-regulated in WDET-BB and four WDET-UB) and three positive glucagon immunoreaction (one WDET-UB and two WDEC).

**Identification of genes differentially expressed by microarray.** Six WDET-BB (patients 1, 2, 3, 4, 5, and 6) and four WDEC/MEC (patients 7PT, 7M, 8, and 9) were analyzed for gene expression using CodeLink UniSet Human 20K I Bioarray containing ~20,000 known human genes. After data normalization, 13,060 genes presented reliable measurements of at least 40% of G flag value as assigned by the CodeLink software. Student’s t test analysis of the individual gene expression ratios (log2 ratio between WDET-BB and WDEC/MEC) resulted in 230 considered differentially expressed, defined as those meeting criteria mentioned above. From them, 120 genes were overexpressed in WDET-BB and 110 in WDEC/MEC. Supervised hierarchical clustering done on the log2 values for the 230 genes differentially expressed resulted on the dendrogram (Fig. 1), depicting the relationship between gene expression and tissue types, according to the histologic classification. A complete listing of transcriptional changes from all differentially expressed genes is provided as Supplementary Table S2.

**Biological pathway significance.** GO analysis used to map differentially expressed genes to candidate biological terms identified two pathways with significant z-score (positive and >2) correlated to molecular function GO terms, namely chaperone regulator activity (GO ID: 30188; z-score, 4.08) and enzyme regulator activity (GO ID: 30234; z-score, 2.42), respectively, more disturbed in WDET-BB and WDEC/MEC. Although significant change has been observed in chaperone regulator activity, this pathway was not chosen because only six genes were represented on the array from which two were differentially expressed. According to previous reports, GO terms presenting fewer than five genes changed have not been considered for validation (15, 16). On the other hand, in the enzyme regulator activity (GO ID: 30234) pathway, 42 from 535 genes were altered. A group of genes with related functions represented by the final branch of this GO term is the serine-type endopeptidase inhibitor activity pathway (GO ID: 4867; z-score, 2.75) containing eight genes differentially expressed (two up-regulated in WDET-BB and six up-regulated in WDEC/MEC). Another closely related pathway, serine-type endopeptidase activity (GO ID: 4252), also presented a relevant z-score value, although its initial molecular function GO term (catalytic activity, GO ID: 3824) did not had a z-score >2.0. Table 2
depicts all GO terms from molecular function and the final branches of serine-type endopeptidase activity and serine-type endopeptidase inhibitor activity pathways.

**Gene expression by RT-qPCR.** Among the up-regulated genes in WDET-BB (Supplementary Table S2) belonging to serine-type endopeptidase activity pathway, we selected for RT-qPCR validation the two most differentially expressed genes, **CTRB1** and **PRSS2**, and with 7.9- and 5.2-fold changes, respectively. Similarly, along with the up-regulated genes in WDEC/MEC (Supplementary Table S2) from serine-type endopeptidase inhibitor activity pathway, the genes **AGT** (6.1-fold change) and **SERPINA1** (6.6-fold change) were chosen. **CFB** gene, belonging to serine-type endopeptidase activity pathway and found to be up-regulated in WDEC/MEC (3.9-fold change), was also selected for validation because its expression had been previously identified in other human cancer cells (19–21).

Transcriptional changes of **PRSS2**, **CTRB1**, **SERPINA1**, **AGT**, and **CFB** were examined by RT-qPCR using a set of independent RNA samples prepared from 35 tumor tissues. Changes in mRNA levels (>2-fold) of four of five genes were concordant with those observed in microarray analysis. As shown in Fig. 2A, mRNA expression of **PRSS2** presented increased expression in the group of WDET (BB and UB; 0.87 ± 0.40) compared with malignant tumors (WDEC and MEC; 0.001 ± 0.001). The opposite regulation was observed in mRNA content of **SERPINA1** (Fig. 2B), **AGT** (Fig. 2C), and **CFB** (Fig. 2D), with increased expression in malignant insulinomas (WDEC and MEC; 76.00 ± 39.25, 193.46 ± 127.86, and 20.43 ± 16.34, respectively) than in WDET (BB and UB; 0.47 ± 0.14, 2.66 ± 1.08, and 0.75 ± 0.27, respectively). Comparison between WDET-BB and WDET-UB for all studied genes did not present any statistical difference. No statistically significant difference was observed in **CTRB1** expression between the different histologic groups [WDET-BB and WDET-UB (1.64 ± 0.83) and WDEC and MEC (0.02 ± 0.01)] as depicted in Fig. 2E.

Tumors identified as plurihormonal by immunohistochemical staining showed the same average mRNA expression as other tumor types in all genes analyzed.

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**Fig. 2.** mRNA expression values of **PRSS2** (**A**), **SERPINA1** (**B**), **AGT** (**C**), **CFB** (**D**), and **CTRB1** (**E**) in sample tumors classified according to WHO classification (WDET-BB, WDET-UB, WDEC, and MEC). Box diagram comparing relative mRNA expression levels of genes in WDET-BB/WDET-UB compared with WDEC/MEC. The horizontal line within the box plot represents the median value, the box plot limits refer to 25th to 75th percentiles, and the box plot bars include the 10th to 90th percentiles for mRNA levels.
No statistical significant correlation was found between mRNA expression of studied genes and histopathologic variables by Spearman rank test (data not shown).

**Immunohistochemical analysis of SERPINA1.** Six of 7 (85.7%) samples of malignant insulinomas (including WDEC and MEC) showed positive immunohistochemical staining for SERPINA1, whereas only 4 of 28 (14.3%) WDET samples presented positive immunostaining for SERPINA1 (Fig. 3). From the positive cases, three corresponded to WDET-BB (patients 11, 12, and 19) and one to WDET-UB (patient 24).

**Discussion**

In the present study, we compared global gene expression profiles between WDET-BB and WDEC/MEC using microarray analysis in an attempt to identify molecular markers for an aggressive biological behavior in insulinomas. Even recognizing the limitation of WHO classification in predicting malignancy, this categorization was used because it constitutes a standardized pathologic evaluation that has been considered useful for clinical purposes. Despite the small number of tumors initially evaluated, the microarray findings seem to be representative based on the concordant results of four (PRSS2, CFB, SERPINA1, and AGT) of five selected genes validated by RT-qPCR on 35 fresh-frozen tissue specimens.

From ~20,000 transcripts analyzed, 230 genes showed >2-fold differential expression between tumor classes. Our objective to search for markers that could be validated by RT-qPCR was based on stringent selection criteria and probably neglected several subtle modifications in gene expression that will be considered for future studies. The supervised hierarchical clustering of these 230 genes resulted into two distinct tumor groups (WDET-BB and WDEC/MEC), represented by nodes 1 and 2 (Fig. 1).

Two genes belonging to serine-type endopeptidase activity pathway, CTRB1 and PRSS2, were selected for validation by RT-qPCR based on the magnitude of their expression ratio differences as well as on our interest in studying PRSS2, which was previously identified as one differentially expressed gene between benign and malignant insulinomas following suppressive subtractive hybridization (22). Among the genes identified as up-regulated in WDEC/MEC, one gene from serine-type endopeptidase activity pathway, CFB, and two genes belonging to serine-type endopeptidase inhibitor activity pathway, SERPINA1 (also known as \(\alpha1\)-antitrypsin) and AGT (also known as SERPINA8), were chosen for RT-qPCR validation. PRSS2, CFB, SERPINA1, and AGT had their differential expressions confirmed between WDET-BB and WDEC/MEC in 35 tumor samples.

Trypsinogen-2 is the precursor of PRSS2, which is produced in pancreatic acini (23) and also by tumors (named tumor-associated trypsinogen 2; ref. 24, 25), whose expression has been shown to correlate with the malignant phenotype of some cancer cells through activation of latent matrix metalloproteinases, which may stimulate tumor invasion and metastasis (24, 26, 27).

PRSS2 expression in insulinomas might reflect either the presence of acinar cells in the tumor or production of trypsinogen-2 by the tumoral cells. Although in the current study immunohistochemistry for specific acinar cell markers had not been done, a carefully histologic analysis did not show morphologic features of acinar cell differentiation, characterizing the tumors as purely endocrine. Yantiss et al. (28) have recently studied pure pancreatic endocrine tumors, including insulinomas, for the presence and degree of acinar differentiation by immunohistochemical analysis. Overall, 66% of tumors stained positively for at least one acinar cell marker, 37% for trypsin, and 25% for chymotrypsin. The authors did not find any correlation between the expression of acinar cell markers and clinical outcome and suggest that cytochemical evidence of concomitant acinar and endocrine differentiation strongly suggests the existence of a common progenitor stem cell. Based on this premise, we speculate that, about insulinomas, a higher degree of differentiation would explain the higher expression of the acinar cell marker PRSS2 in well-differentiated tumors. Alternatively, we cannot rule out that lower expression of this gene in WDEC/MEC is associated with promoter hypermethylation as already reported by Yamashita et al. (29).
in esophageal squamous cell carcinomas and gastric adenocarcinomas.

CFB, thezymogenic of the catalytic site of the C3/C5 convertase of the alternative pathway of complement (30), had its up-regulation in WDEC/MEC validated by RT-qPCR. CFB expression has already been reported in the exocrine pancreas, where it is regulated by interleukin-1β, tumor necrosis factor-α, and IFN-γ and probably synthesized by ductal epithelial cells (19), as well as in human cancer cells, including gastric (20) and astrocytoma cell lines (31). Doustjali et al. (21) have recently shown immunoreactivity for CFB in malignant breast lesions, whereas benign lesions negatively stained for this protein. Whether expression of CFB is a direct determinant of tumor aggressiveness or it is a consequence of host defense mechanisms as the tumor becomes more invasive remains to be elucidated.

Another gene identified as up-regulated in WDEC/MEC was AGT, previously described in human insulinomas, where the RNA abundance was shown to be higher than in normal pancreas (32). AGT, the product of this gene, is cleaved by renin to generate angiotensin I, which is subsequently converted into angiotensin II by angiotensin 1–converting enzyme. AGT shares structural homologies with the serine protease inhibitor (serpin) family of proteins, but it has no inhibitory activity. Célèrier et al. have shown that AGT, as well as the fragment resulting from its cleavage (desangiotensin I AGT), presents antiangiogenic activity. Because angiogenic effect is associated to angiotensin II, they suggested that the final effect on angiogenesis depends on local conditions, such as the clearance rate of angiotensin II and the presence or absence of renin (33). Considering that renin, as well as angiotensin I–converting enzyme RNA expression, was detected in all tumor sample evaluated by microarray (data not shown), it is tempting to speculate that up-regulation of AGT in WDEC/MEC is associated with a final proangiogenic effect, which contributes to tumor growth.

The expression of SERPINA1, which codes for α-1-antitrypsin, a major plasma serpin that has a broad inhibitory spectrum against serine proteases, has been related to the invasive potential of gastric (34), lung (35), and colorectal adenocarcinomas (36). Our finding of SERPINA1 mRNA up-regulation in WDEC/MEC agrees with data published by Hansel et al. (37) reporting SERPINA1 overexpression in metastatic versus nonmetastatic tumors in a study that evaluated global gene expression profiles from well-differentiated pancreatic endocrine neoplasms. Although SERPINA1 expression had not been validated in this series, which differs from ours in that is not composed only of insulinomas, its identification as up-regulated in metastatic tumors also suggests that SERPINA1 plays a role in pancreatic endocrine neoplasia progression. The participation of this protein in tumor invasiveness seems to be related to its degradation by matrix metalloproteinases, resulting in the production of a COOH-terminal fragment that increases tumor growth in vivo, possibly due to its modulatory effects on natural killer cell activity against the tumor cells (38).

Immunohistochemical analysis identified SERPINA1 expression in 85.7% of malignant versus 14.3% of nonmalignant insulinomas. Interestingly, the MEC (sample 8) presenting negative immunostaining for SERPINA1 was a hepatic metastasis that determined hypoglycemia 12 years after the resection of the primary tumor, characterizeg an indolent clinical course in spite of being a metastatic tumor. The four patients whose tumors classified as WDET-BB or WDET-UB presented positive SERPINA1 immunostaining being closely followed to assess their long-term evolution.

To our knowledge, this is the first study on a series comprising only insulinomas approached by microarray analysis that provided the identification of genes associated with tumoral malignancy. Considering the widespread availability of antibody anti-α-1-antitrypsin in pathology services, the confirmation of SERPINA1 overexpression in larger series of WDEC might be of clinical utility in identifying patients more likely to develop aggressive disease.

References


Serpine Peptidase Inhibitor Clade A Member 1 as a Potential Marker for Malignancy in Insulinomas

Sandra Valéria de Sá, Maria Lúcia Corrêa-Giannella, Márcio Carlos Machado, et al.


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