Abstract. Aim: To characterize the differentially-activated mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K/Akt/mTOR) pathways in mutant (m) and wild-type (wt) GISTs and to investigate the role of insulin-like growth factor 1 receptor (IGF1R) expression. Materials and Methods: Ninety-nine paraffin-embedded gastrointestinal stromal tumors (GISTs) were selected. CD117, IGF1R, phospho-ERK1/2, phospho-Akt, p70S6, eukaryotic initiation factor 4E-binding protein-1 (4EBP1) and pS6 expression were investigated using immunohistochemical methods. KIT exons 9, 11, 13 and 17 and platelet derived growth factor receptor alpha (PDGFRA) exons 12 and 18 were amplified by PCR and sequenced. Results: Significant differences were found in the expression of phospho-ERK1/2 between mGISTs and wtGISTs. Complex evaluation of all PI3K/Akt/mTOR pathway markers revealed greater activation in mGISTs, particularly in PDGFRA-mutated GISTs. No significant correlation was observed between IGF1R expression and either mutational status or pathway activation. Conclusion: There appears to be no MAPK pathway activation in wtGISTs. Tumors harboring PDGFRA mutations tended to use the PI3K/Akt/mTOR signaling pathway. Most adult GISTs, irrespective of mutational status, displayed no IGFR1 expression; tumors positive for IGFR1 showed no preferential activation of the MAPK or AKT pathways.

Research into the signal transduction pathways involved in neoplastic cell transformation has enabled a more rational approach to the design of cancer therapies. The use of specific tyrosine kinase inhibitors such as imatinib mesylate (Gleevec, Novartis, USA) or sunitinib (Sutent, Pfizer, USA), in the treatment of gastrointestinal stromal tumors (GISTs) has revolutionized the study of these neoplasms. The results obtained with imatinib mesylate are particularly promising, in that this inhibitor achieves disease control in 70-85% of patients with advanced tumors; however, patient response is by no means uniform, and depends to a great extent on the mutational status of KIT and PDGFRA genes (1). Patients whose tumors lack mutations in either KIT or PDGFRA or who harbor imatinib mesylate resistant mutations, such as exon 17 mutations in KIT and exon 18 mutations in PDGFRA, have poorer response rates and shorter disease-free progression (2-5). The downstream signaling cascade activated includes the Ras-Raf-ERK/MAPK pathway, the phosphatidylinositol-3-kinase pathway (PI3K-AKT/mTOR) and the JAK-STAT kinase pathway (5, 6); research published to date indicates considerable variation in the degree of activation of these pathways (7-13).

Recently, insulin-like growth factor 1 receptor (IGF1R) has emerged as a player in a novel molecular signaling pathway, and attention has increasingly focused on its value as a potential therapeutic target in wtGISTs (14-18). IGF1R is a tyrosine kinase receptor that, after binding to ligands IGF1 or IGF2 stimulates the two main intracellular signaling pathways controlling proliferation rate and apoptosis: Ras-Raf-ERK/MAPK pathway, the phosphatidylinositol-3-kinase pathway (PI3K-Akt/mTOR) and the JAK-STAT kinase pathway (5, 6); research published to date indicates considerable variation in the degree of activation of these pathways (7-13).

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Key Words: Gastrointestinal stromal tumors (GISTs), Ras-Raf-ERK/MAPK Pathway, PI3K-Akt/mTOR pathway, insulin-like growth factor 1 receptor (IGF1R), CD117.
QIAamp DNA FFPE Tissue kit according to the manufacturer’s instructions was chosen for molecular analysis; DNA was isolated using a DNA isolation and molecular analysis.

For immunohistochemical analysis, representative samples were selected from a set of formalin-fixed, paraffin-embedded (FFPE) surgical specimens obtained from 99 GISTs. Samples were from patients undergoing surgery at the Hospital Universitario Virgen Macarena (Seville) and Hospital Torrecárdenas (Almería) over a 20-year period (1989-2009). Tumor sample collection was approved by the Hospital Ethical Committees. Hematoxylin–eosin-stained slides were reviewed and the pathological diagnosis was confirmed by tumor location, morphology, immunostaining for CD117 and sequencing results in the NCBI genebank.

The following clinical and pathological data were accessed: patient age and gender, tumor location (gastric, small and large bowel, extragastrointestinal), tumor size, histological type (spindle, epithelioid, mixed), mitotic index in 50HPF, stage at presentation (localized versus metastatic) and progression (patient alive and disease-free, alive with disease, dead due to disease, dead due to unrelated causes).

**Immunohistochemical analysis.** For immunohistochemical analysis, 5 μm serial sections were stained with a panel of antibodies (Table I) using the streptavidin-biotin-peroxidase complex technique. Negative (primary antibody replaced by normal horse serum) and positive controls (sections of a human breast cancer) were included in each slide run. All controls yielded satisfactory results.

Negative (primary antibody replaced by normal horse serum) and positive controls (sections of a human breast cancer) were included in each slide run. All controls yielded satisfactory results.

**DNA isolation and molecular analysis.** One selected paraffin block was chosen for molecular analysis; DNA was isolated using a QIAamp DNA FFPE Tissue kit according to the manufacturer’s instructions (Quiagen, Hilden, Germany). KIT exons 9, 11, 13 and 17 and PDGFRA exons 12 and 18 were amplified by polymerase chain reaction (PCR). The PCR reaction was carried out using a Taq PCR Master Mix (Quiagen, Hilden, Germany) including 0.1-1 μg of extracted DNA and 0.2 μM forward and reverse primers to a total volume of 30 μl.

Primers and experimental conditions are shown in Table II. PCR products were examined using a QuiAXcel DNA High Resolution Technology, USA.

**Table I. Antibodies used for immunohistochemical analysis.**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody (Cell Signaling Technology, USA)</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho-ERK1/2</td>
<td>Polyclonal</td>
<td>1:5000</td>
</tr>
<tr>
<td>Phospho-AKT</td>
<td>Monoclonal</td>
<td>1:50</td>
</tr>
<tr>
<td>pS6</td>
<td>Polyclonal</td>
<td>1:2000</td>
</tr>
<tr>
<td>4EBP1</td>
<td>Polyclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>P70S6Kinase</td>
<td>Monoclonal</td>
<td>1:1000</td>
</tr>
<tr>
<td>CD117</td>
<td>Polyclonal</td>
<td>1:20</td>
</tr>
<tr>
<td>IGFR1</td>
<td>Polyclonal</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

ERK1/2: Extracellular signal regulated kinases; AKT: seorine/threonine protein; pS6: S6 ribosomal protein; 4EBP: factor 4E binding protein; P70S6Kinase: ribosomal S6 protein kinase; CD117: KIT; IGFR1R: insulin-like growth factor 1 receptor.

**Patients and Methods**

**Patient selection and clinical features.** Representative samples were selected from a set of formalin-fixed, paraffin-embedded (FFPE) surgical specimens obtained from 99 GISTs. Samples were from patients undergoing surgery at the Hospital Universitario Virgen Macarena (Seville) and Hospital Torrecárdenas (Almería) over a 20-year period (1989-2009). Tumor sample collection was approved by the Hospital Ethical Committees. Hematoxylin–eosin-stained slides were reviewed and the pathological diagnosis was confirmed by tumor location, morphology, immunostaining for CD117 and sequencing results in the NCBI genebank.

**Results**

**Clinical features.** All tumors displayed clinical/pathological features consistent with GIST, and expressed CD117 and/or harbored KIT/PDGFR mutations. The median age was 64 years (range: 13-8, years), and samples were from 50 (51.5%) males and 47 (48.5%) females. Only two patients were under 20 years old. Forty-seven (48.5%) tumors originated in the stomach, 35 (36.1%) in the small bowel, 7 (7.2%) in the large bowel and 8 (8.2%) in extragastrointestinal locations in the mesentery and omentum. Mean tumor diameter was 7.65 cm (1-25 cm). Tumor diameter was less than 2 cm in 57 (60.6%)
cases, between 2 and 5 cm in 29 cases (30.9%), between 5 and 10 cm in 6 cases (6.4%) and over 10 cm in 2 cases (2.1%). Histologically, 77 (80.2%) neoplasms were of spindle type, 14 (14.6%) of epithelioid type and 5 (5.2%) showed mixed cytomorphology. Mitotic counts ranged from 1 to 60 in 50HPF; 49 samples (55%) displayed <2 mitotic counts; 15 (17%) between 2 and 5; and 25 (28%) >5. Follow-up was available in 67 cases, with a median duration of 42±32 months. Outcomes were as follows: 10 (15%) died of the disease, 2 (2.6%) died of unrelated causes, 36 (46.7%) were alive with no evidence of disease, whereas 29 (37.7%) were alive with the disease. Liver metastasis was found in 15 (22%) cases.

According to the NIH risk classification system (30), 4 (4.4%) patients had very low risk (tumor size <2 cm and mitotic count of <5/50 HPF). 25 (27.8%) were classed as low risk (2–5 cm and <5/50 HPF), 22 (24.5%) as intermediate risk (<5 cm and 6–10/50 HPF or 5–10 cm and <5/50 HPF) and 39 (43.3%) as high risk (>5 cm and >5/50 HPF or >10 cm regardless of mitotic activity or >10/50 HPF regardless of tumor size). Using Miettinen et al.'s classification (31), which includes location in the gastrointestinal tract, 66 (72%) of tumors were probably malignant, 4 (4%) probably benign and 22 (24%) were of uncertain malignant potential.

Immunohistochemical profile and mutational status. Immunohistochemical findings are shown in Table III. All cases displayed cytoplasmic staining for all proteins, except for 4EBP1, where staining was nuclear. Staining for CD117 exhibited a more variable cytoplasmic, membrane or dot-like (Golgi) pattern (Figure 1A).

Molecular analysis revealed KIT mutation in 68/99 (69%) cases, PDGFRα mutation in 11/99 (11%) and wtKIT/PDGFRα in 20/99 (20%). Among cases with KIT mutation, 64/68 (94%) had exon 11 mutations and 4/68 (6%) exon 9 mutations. There were no cases of exon 13 or exon 17 mutation. Among cases with PDGFRα mutation, 9/11 (82%) had mutations of exon 18 and 2/11 (18%) of exon 12. All CD117-negative cases had KIT (3, exon 11) or PDGFRα (2, exon 18; 1, exon 12) mutations.

Significant differences were found in phospho-ERK1/2 expression between mGISTs and wtGISTs (p=0.007) (Figure 1B, Table III). No significant differences were observed in phospho-Akt expression, although it was more frequent in the mGIST group (Figure 1C, Table III). Similarly, no significant differences in p70S6kinase (Figure 1D), 4EBP1 or pS6 (Figure 1E) expression were found between mGISTs and wtGISTs (Figure 1F) (Table III). Taking into account all the markers used to study the PI3K/Akt/mTOR signaling pathway, greater activation was observed in mGISTs-19/75 mGIST (25%) versus 3/20 wtGISTs (15%) - although the difference was not statistically significant (Table IV).

No significant differences in phospho-ERK, p70S6Kinase, pS6 or 4EBP1 expression were observed as a function of mutated gene type (KIT versus PDGFRα). However, PDGFRα-mutated tumors displayed greater phosphoAKT expression (p=0.026; Figure 2; Table V). Complex evaluation of all the PI3K/Akt/mTOR pathway markers revealed greater pathway activation in PDGFRα-mutated GISTs (p=0.019; Figure 2).

IGF1R expression (Figure 3) was observed in 22/97 (23%) cases; all except one of these stained positive by CD117. The CD117-negative sample was from a 73-year-old man with a 3 cm tumor located in the stomach. No significant correlation was observed between IGF1R expression and mutational status: 18/22 (82%) IGF1R-positive samples had a KIT mutation, 3/22 (14%) a PDGFRα mutation, and 1/22 (4%) was wtKIT/PDGFRα. No significant differences were found for the expression of phospho-ERK1/2 phospho-Akt, p70S6kinase, pS6 or 4EBP1 (Table VI). The two GISTs obtained from young patients (<30 years) were wtKIT/PDGFRα and stained negative by IGF1R.

Discussion

Activation pathways in GISTs have mostly been studied in tumor extracts from KIT mutant tumors, and findings with regard to the degree of activation of downstream pathways vary considerably. Strong KIT phosphorylation and evidence of activated downstream signaling pathways, including the MAP kinase pathway (RAF, MEK, ERK), the STAT pathway, and the PI3K/AKT pathway, have been reported (7-13, 32-36). Interestingly, phosphorylation of STAT5, a ubiquitous finding in hematological malignancies (35, 36), is infrequently observed in primary GISTs and GIST cell lines, while STAT3 is constitutively activated (7, 37). Some of these differences probably reflect the KIT mutation subtype or organ site (7), but may also be attributable to differences in the type of material studied (KIT-mutated cell lines versus paraffin tissue.
blocks with mutations in KIT, PDGFRA or wtKIT/PDGFRA (8-11), the number of cases reviewed, and the technical procedures used for phospho-protein identification (Western blot versus immunohistochemistry) (12, 13).

Here, significant differences were found in the level of MAPK and PI3K/AKT/mTOR pathway activation both between wtGISTs and mGISTs, and between the two different mutated genes (KIT vs. PDGFRA): no wtGIST displayed MAPK pathway activation, and tumors with PDGFRA mutations tended to use the PI3K/AKT/mTOR signaling pathway.

These findings differ markedly from those reported by Kang et al. (13), who found activation of the MAPK...
pathway regardless of mutational status in a limited number of cases studied by Western blot (20 mGIST versus 2 cases wtGIST); however, they are closer to the results described by Martinho et al. (38), who noted the absence of MAPK activation in 70% of the 26 wtGISTs studied by immunohistochemistry, and by Tarn et al. (15), who recorded higher levels of pAKT and mTOR in mGISTs.

It has been suggested that the PI3K/Akt/mTOR pathway may play an important role in the development of GISTs, especially those with imatinib resistance. However, few in vivo studies have examined the state of activation of this pathway; most research has focused on cell lines with KIT or PDGFRA mutations, rather than on wtGISTs (7, 8, 34-37). Here, positive staining for phospho-Akt was more common in GISTs with PDGFRA than with KIT mutations. Similar findings are reported by Duensing et al. (7), Bauer et al. (8), Sàpi et al. (12), Heinrich et al. (39), and Zhu et al. (40).

Recently, several authors have noted that IGF1R, a molecular receptor other than KIT and PDGFRA, is activated in wtGISTs. Nevertheless, published results on IGF1R expression are by no means uniform. Braconi et al. (14) evaluated the expression of IGF1R and its ligands IGF1 and IGF2 in 94 samples of paraffin-embedded GISTs, almost all from adults, and found strong cytoplasmic IGF1R expression in all cases, as well as

### Table IV. Staining results in wild type gastrointestinal stromal tumors (wtGISTs) and mutant GISTs (mGISTs).

<table>
<thead>
<tr>
<th></th>
<th>Phospho-ERK 1/2</th>
<th>Phospho-Akt</th>
<th>p70S6KKinase</th>
<th>pS6</th>
<th>4EBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
</tr>
<tr>
<td>wt GISTs</td>
<td>20/20 (100%)</td>
<td>0/20 (20%)</td>
<td>16/19 (84%)</td>
<td>3/19 (16%)</td>
<td>9/20 (45%)</td>
</tr>
<tr>
<td>mGISTs</td>
<td>50/75 (66.7%)</td>
<td>25/75 (33.3%)</td>
<td>54/76 (71%)</td>
<td>22/76 (29%)</td>
<td>32/76 (42%)</td>
</tr>
<tr>
<td></td>
<td>(17%)</td>
<td>(8%)</td>
<td>(20%)</td>
<td>(5%)</td>
<td>(10%)</td>
</tr>
</tbody>
</table>

ERK1/2: Extracellular signal regulated kinases; AKT: serine/threonine protein; pS6: S6 ribosomal protein; 4EBP: factor 4E binding protein; P70S6Kinase: ribosomal S6 protein kinase.

### Table V. Staining in KIT vs. PDGFRA mutated gastrointestinal stromal tumors (GISTs).

<table>
<thead>
<tr>
<th></th>
<th>Phospho-ERK 1/2</th>
<th>Phospho-AKT</th>
<th>p70S6kinase</th>
<th>pS6</th>
<th>4EBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
</tr>
<tr>
<td>KIT-mutated</td>
<td>43/65 (66%)</td>
<td>22/65 (34%)</td>
<td>50/66 (76%)</td>
<td>16/66 (24%)</td>
<td>29/66 (44%)</td>
</tr>
<tr>
<td>PDGFRA-mutated</td>
<td>7/10 (70%)</td>
<td>3/10 (30%)</td>
<td>4/10 (60%)</td>
<td>6/10 (10%)</td>
<td>3/10 (30%)</td>
</tr>
</tbody>
</table>

ERK1/2: Extracellular signal regulated kinases; AKT: serine/threonine protein; pS6: S6 ribosomal protein; 4EBP: factor 4E binding protein; P70S6Kinase: ribosomal S6 protein kinase.

### Table VI. Pathway protein expressions as a function of IGF1R staining.

<table>
<thead>
<tr>
<th></th>
<th>Phospho-ERK 1/2</th>
<th>Phospho-AKT</th>
<th>p70S6kinase</th>
<th>pS6</th>
<th>4EBP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
<td>−−</td>
</tr>
<tr>
<td>IGF1R positive</td>
<td>15/7 (21%)</td>
<td>15/71 (21%)</td>
<td>44/72 (61%)</td>
<td>27/72 (37.5%)</td>
<td>15/67 (21%)</td>
</tr>
<tr>
<td>IGF1R negative</td>
<td>9/22 (41%)</td>
<td>10/22 (45%)</td>
<td>10/22 (45%)</td>
<td>9/22 (41%)</td>
<td>7/22 (32%)</td>
</tr>
</tbody>
</table>

ERK1/2: Extracellular signal regulated kinases; AKT: serine/threonine protein; pS6: S6 ribosomal protein; 4EBP: factor 4E binding protein; P70S6Kinase: ribosomal S6 protein kinase.
moderate to strong expression of IGF1 and IGF2 in 73% and 49% cases, respectively; they reported no statistically significant association between IGF1R expression and mutational status. Tarn et al. (15), using Western blot analysis, high-density single nucleotide polymorphism arrays, genomic-based quantitative PCR assays, fluorescent in situ hybridization and immunohistochemistry in fresh and paraffin-embedded material from mutant and wtGISTs, found IGF1R expression and activation in all GISTs, especially in wtGISTs, including one pediatric wtGIST. They noted that this enhanced expression in the subset of wtGISTs was in part associated with IGF1R gene amplification. Agaram et al. (16) compared IGF1R gene expression profiles in GISTs from 17 patients under 30 years old with those of 8 adult wtGISTs, and found that pediatric GISTs showed a distinct transcriptional signature with overexpression of brain and acute leukemia (BAALC), pleomorphic adenoma gene 1 (PLAG1), IGF1R, fibroblast growth factor 4 (FGF4), and nel-like 1 (NELL1). Pantaleo et al. (17) studied gene expression profiling, genomic copy numbers and protein expression in 8 patients with gastric GIST, finding that IGF1R was up-regulated only in 2/8 patients; both cases were young patients (<30 years old) with wtKIT/PDGFRα tumors who had metastases at diagnosis; neither of them bore IGF1R amplification. More recently, Janeway et al. (18), using Western blot, high-density single nucleotide polymorphism arrays and fluorescent in situ hybridization found strong expression of IGF1R in 8/9 wtKIT/PDGFRα pediatric GISTs, without IGF1R amplification. Here, all except one of the samples staining positive for IGF1R expressed CD117 and displayed KIT or PDGFRα mutations. The results obtained here suggest that most adult GISTs, irrespective of mutational status, neither express IGF1R nor display preferential activation of MAPK or AKT pathways. The two patients under 30 years of age were wtKIT/PDGFRα, and neither expressed IGF1R. This would indicate that expression of IGF1R in GISTs varies greatly depending on patient age and mutational status; however, since few children were included in the present series, it is impossible to confirm the differences between adult and pediatric patients reported in the literature (14, 16, 18). Further research is required, with a larger number of young patients, to confirm potential differences between adult and pediatric GISTs.

**Conflict of Interest**

The Authors have no conflict of interest to declare.

**Acknowledgements**

This research was support by a grant from Novartis, Spain.


Received May 12, 2011
Revised July 1, 2011
Accepted July 2, 2011