EGFR and gastrointestinal stromal tumor: an immunohistochemical and FISH study of 82 cases

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Gastrointestinal stromal tumor is the most common mesenchymal neoplasm of the gastrointestinal tract. Mutually exclusive *KIT* or *platelet-derived growth factor receptor- α* mutations are key events in gastrointestinal stromal tumor pathogenesis, and specific treatment targeting *KIT*/platelet-derived growth factor receptor- α activation is available. Epidermal growth factor receptor plays an important role in cancer biology and also constitutes a promising molecular target of therapy. Very few reports have been published in the literature about the relationship between gastrointestinal stromal tumor and epidermal growth factor receptor. The aim of this study was to investigate epidermal growth factor receptor immunohistochemical expression and *epidermal growth factor receptor* gene amplification in 82 consecutive gastrointestinal stromal tumor cases using tissue microarray technique. Hematoxylin- and eosin-stained sections and clinical information were reviewed, and expression of CD117 (*KIT*), CD34 and epidermal growth factor receptor was investigated by immunohistochemistry. *Epidermal growth factor receptor* gene copy number was determined using fluorescence *in situ* hybridization. Immunohistochemistry revealed that CD117 and CD34 were expressed in 96 and 57% of tumors, respectively. Variable epidermal growth factor receptor protein immunohistochemical overexpression was detected in 96% of gastrointestinal stromal tumor cases, but none of the 75 cases with represented tumor tissue cores and countable fluorescence signals exhibited *epidermal growth factor receptor* gene amplification by fluorescence *in situ* hybridization. These results show that there is no correlation between epidermal growth factor receptor protein overexpression by immunohistochemistry and *epidermal growth factor receptor* gene amplification by fluorescence *in situ* hybridization. Considering that the mechanisms of epidermal growth factor receptor protein overexpression are not well understood and the possibility that anti-epidermal growth factor receptor therapy may be beneficial for patients with gastrointestinal stromal tumor that overexpresses epidermal growth factor receptor, additional studies are encouraged.

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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal neoplasm of the gastrointestinal (GI) tract. GIST generally occurs in older individuals (over the age of 50) and it can arise anywhere along the GI tract or even outside of it (extra-GI GIST), as in mesentery, omentum, retroperitoneum and pelvis. GISTs show peculiar immunohistochemical (*KIT* or CD117 and CD34 positivity) and molecular genetic findings, the latter characterized by mutually exclusive *KIT* or *platelet-derived growth factor receptor- α* (*PDGFRA*) muta-

tions.^{1,2} The *KIT* and *PDGFRA* genes encode for similarly named transmembrane tyrosine kinase receptors that are involved in the development and maintenance of several cells. Activating (gain-of-function) mutations of *KIT* or *PDGFRA* permit the phosphorylation of the receptor tyrosine kinases, with activation of intracellular downstream effectors, resulting in increased cellular proliferation and decreased apoptosis, ultimately leading to neoplasia.^{3–6} The development of a *KIT* and *PDGFRA* tyrosine kinase inhibitor called STI571 or imatinib mesylate (Gleevec, Novartis Pharma, Basel, Switzerland)⁷ has brought new perspectives in GIST treatment by reducing *KIT* receptor tyrosine kinase activity.

The epidermal growth factor receptor (EGFR, HER-1/*ErbB*-1) is a tyrosine kinase receptor of the *ErbB* family also involved in intracellular signaling

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pathways that regulate key cellular processes such as proliferation, survival, adhesion, migration and differentiation. EGFR has been the subject of intense investigation in view of its involvement in some types of cancer such as non-small-cell lung carcinoma, being a potential molecular target of therapy with kinase inhibitors.^{8–10} However, very few reports have been published so far regarding the role of EGFR in GIST^{11–13} and further investigation was necessary to define if EGFR could be an additional molecular target in GIST treatment.

This study was designed to provide additional information about the relationship between GIST and EGFR, and to help define potential therapies for GIST by investigating EGFR protein expression and *EGFR* gene amplification.

Materials and methods

Case Material and Clinical Data

The study group included 82 consecutive cases of GIST received in consultation between June 2003 and May 2004 at *Consultoria em Patologia*, a reference consultation service in anatomic pathology located in Botucatu, Sao Paulo State, Brazil. Both GI tract and extra-GI tract GIST cases were included. All cases had morphological features of GIST. Clinical data including gender, age at diagnosis, tumor location and tumor size were obtained from the referring pathologists/oncologists and/or pathology reports.

Analysis of Histologic Parameters

Available hematoxylin- and eosin-stained slides were reviewed in each case. Cell morphology (spindle, epithelioid and mixed spindle/epithelioid) was recorded.

Tissue Microarray Construction

A tissue microarray block was constructed using a tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Each case was represented by three tumor cores of 0.6 mm that were taken from the original paraffin blocks. Serial sections of 3 μ m were cut from the tissue array block and used for immunohistochemical and fluorescence *in situ* hybridization (FISH) analyses. Proper positive controls cores were also included in the array block to provide adequacy of the antibodies used in the immunohistochemical study.

Immunohistochemistry

Polyclonal antibody against CD117 (c-kit, 1:200; DAKO, Carpinteria, CA, USA) and monoclonal antibodies against EGFR (clone 31G7, 1:50; Zymed,

San Francisco, CA, USA) and CD34 (clone My10, 1:400; Becton Dickinson Biosciences, Mountain View, CA, USA) were used for immunohistochemical analysis. Heat-induced (CD117 and CD34) and enzymatic (protease XXVI for EGFR) antigen retrieval was employed. Novolink polymer (Novocastra, Newcastle Upon Tyne, UK) was the detection system, and diaminobenzidine was the chromogen.

Immunostains for CD117 and CD34 were interpreted as positive or negative. EGFR was scored according to the intensity of the immunostaining as 0 (negative), 1 (positive, weak), 2 (positive, moderate) or 3 (positive, strong). EGFR was considered positive (1, 2 or 3) if staining was mainly membranous in at least 10% of the tumor cells.

Fluorescence *In Situ* Hybridization

FISH was used to estimate the copy number of the *EGFR* gene in each case using a 3- μ m-thick tissue section of the array block. Dual color EGFR FISH was performed with the SpectrumOrange LSI EGFR probe specific for the EGFR locus (7p12) and the SpectrumGreen CEP7 probe specific for chromosome 7 centromere (7p11.1–q11.1), according to the manufacturer's instructions (Vysis, Abbott AG, Baar, Switzerland) and as previously described.¹⁴ The quantification of *EGFR* gene copy number was performed using a Zeiss Axio Imager M1 fluorescence microscope (Carl Zeiss AG, Germany) with appropriate filters (Chroma Technology GmbH, Fuerstentfeldbruck, Germany) and the assistance of Isis FISH Imaging software (Metasystems, Altlusheim, Germany).

Results

GIST Clinical and Morphological Characterization

There were 48 males and 34 females and the mean age was 58 years (23–85 years). The tumors were located in the stomach (33%), small intestine (39%), large intestine (5%), mesentery (5%), pancreas/peripancreatic tissue (4%), liver (4%), abdominal cavity (4%) and retroperitoneum (1%). Five cases had unknown location. The tumors ranged in size from 2 to 31 cm (mean, 11 cm). The cell types were pure spindle (75%), pure epithelioid (10%) and mixed spindle/epithelioid (15%).

GIST Immunohistochemical Characterization

Immunohistochemistry revealed that CD117 was expressed in 79 out of 82 tumors (96%). CD34 expression was observed in 46 out of 81 GISTs (57%). Variable EGFR protein immunohistochemical overexpression was detected in 96% ($n=76$) of scorable GISTs ($n=79$). Only three (4%) tumors were scored 0. Ten tumors (12%) were scored 1,

Figure 1 EGFR protein expression in GIST. (a) GIST score 0—no EGFR expression. (b) GIST score 1—weak EGFR expression. (c) GIST score 2—moderate EGFR expression. (d) GIST score 3—strong EGFR expression.

21 (27%) were scored 2 and 45 (57%) were scored 3 (Figure 1).

EGFR Gene Copy Number Quantification

Seventy-five GISTs had tumor tissue cores represented in the section submitted to FISH and countable fluorescence signals. All 75 cases showed two orange (*EGFR* gene) and two green (chromosome 7 centromere) fluorescence signals, with a signal ratio *EGFR* gene/CEP 7 equal to 1, which means that all cases had no amplification of the *EGFR* gene (Figure 2). *EGFR* gene amplification is defined as a signal ratio *EGFR* gene/CEP 7 equal to or higher than 2.

Discussion

EGFR (HER-1) is a receptor tyrosine kinase that plays a key role in the regulation of essential normal cellular processes and in the pathophysiology of hyperproliferative diseases such as cancer. EGFR

Figure 2 Fluorescence *in situ* hybridization demonstrates the presence of two orange signals (*EGFR* gene) and two green signals (chromosome 7 centromere) per cell. No amplification of *EGFR* gene can be observed.

is known to be essential for mediation of both proliferative and survival signals to cells. Activation of the EGFR signaling pathway has been linked with increased cell proliferation, angiogenesis, metastasis and decreased apoptosis.

The literature has presented many reports that demonstrate the link between EGFR and some types of cancer,^{15–20} which are generally associated with poor clinical outcome. Small molecules that inhibit the tyrosine kinase domain of EGFR have become critical new weapons in the treatment of some tumors, including non-small-cell lung carcinomas,²¹ and they include gefitinib (Iressa, Astra Zeneca) and erlotinib (Tarceva, OSI Pharmaceuticals).

The role of EGFR has been studied in several types of human sarcomas,^{22–30} mainly in synovial sarcomas.^{14,23,29} On the other hand, very few reports about the relationship between GIST and EGFR have been published so far.^{11–13}

Cai *et al*¹¹ studied the expression of transforming growth factor- α (TGF- α) and two EGFR proteins (HER-1 and HER-2) in GISTs of the stomach and small intestine using standard immunostaining techniques in paraffin-embedded sections. Most GISTs expressed TGF- α , and a few expressed HER-1, suggesting that a TGF- α /EGFR autocrine loop was present in GIST and that TGF- α could promote proliferation of GIST tumor cells through its interaction with HER-1 in at least some GISTs.

Lanzafame *et al*¹² reported a GIST case occurring during pregnancy with immunohistochemical staining for EGFR, indicating that the expression of EGFR could provide pertinent biological information required to determine adequate therapeutic regimens. Interestingly, Yoo *et al*¹³ analyzed the *EGFR* gene in 60 GISTs for the detection of somatic mutations by using the polymerase chain reaction, the single-strand conformation polymorphism (SSCP) and DNA sequencing of exons 18, 19 and 21 encoding the kinase domain. The SSCP analysis revealed no evidence of *EGFR* mutations in those exons of the *EGFR* gene. The data indicated that the *EGFR* gene might not be mutated in human GISTs and suggested that therapies targeting the mutated *EGFR* gene products might not be useful in the treatment of GISTs.

On the other hand, Yang *et al*³¹ explored the effect of ZD6474 (Zactima, AstraZeneca, Macclesfield, UK), an orally available small-molecule inhibitor of vascular endothelial growth factor receptor-2 and EGFR tyrosine kinases, in GIST-T1 cells. ZD6474 induced growth arrest and apoptosis of GIST-T1 cells in association with blockade of KIT and its downstream effectors.

Considering some conflicting findings, additional investigation was mandatory. In the present study, we found that most GISTs had immunohistochemical overexpression of EGFR protein, but none of the cases studied by FISH had gene amplification, even though Tornillo *et al*³² found amplification for *EGFR* in 5.3% of GISTs. Results regarding EGFR over-

expression in the absence of gene amplification were also observed by Bode *et al*¹⁴ in synovial sarcomas. They studied 13 synovial sarcomas that exhibited strong diffuse or focal EGFR expression with no *EGFR* gene amplifications, but several point mutations were detected in exons 18–21 of two synovial sarcomas. They stated that the existence of mutations in the tyrosine kinase domain of the *EGFR* gene could make a small subset of synovial sarcomas suitable for treatment with EGFR inhibitors. Other authors^{33,34} observed similar findings in gliomas and suggested that the expression of EGFR protein could be enhanced by mechanisms other than simple alteration in *EGFR* gene copy number. Several mechanisms have been proposed to explain EGFR protein overexpression, including *EGFR* gene activating mutations, increased EGFR transcription or translation, decreased protein destruction and overexpression of receptor ligands.³⁵ Considering that the mechanisms of EGFR protein overexpression are not well understood and the possibility that anti-EGFR therapy may be beneficial for patients with GIST that overexpresses EGFR protein, further studies are necessary.

In summary, we found that EGFR protein was overexpressed in 96% of GISTs included in this study. Such result could make EGFR a possible molecular target of therapy, although *EGFR* gene amplification was not detected.

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Disclosure/conflict of interest

No conflict of interest to declare.

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