

Ki-67 expression score correlates to survival rate in gastrointestinal stromal tumors (GIST)

Escore de expressão de Ki-67 correlaciona-se com taxa de sobrevida em tumores estromais gastrointestinais (GIST)

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ABSTRACT

PURPOSE: To evaluate the immunohistochemical expression of p16, Ki-67, p53 and Bcl-2 proteins in gastrointestinal stromal tumors (GIST); to assess the possible association between these variables and clinical and histopathological factors of cancer; and to check for prognostic value of these variables (survival and recurrence).

METHODS: A sample of 55 patients treated surgically for GIST in three hospitals was studied. The surgically excised tumors were confirmed as GIST by KIT, vimentin, desmin S100 protein, CD117, 1A4 and CD34 assessment in paraffin blocks.

RESULTS: Only 9 (16%) cases of GIST were positive for p53, p16 was positive among 43.6%; 80% of GISTs showed staining for Bcl-2. The proliferative index (expressed as the proportion of positive cells) assessed by immunohistochemical expression of Ki-67 was high in 49% of cases. Elevated Ki-67 scores were associated to high histological grade ($p=0.0026$) and mitosis index, MI ($p=0.0001$). High Ki-67 index was associated to death. Expression of p53, p16 and Bcl-2 did not correlate to morphological or clinical variables.

CONCLUSIONS: Ki-67 immunohistochemical evaluation should be included in preoperative evaluation of GIST biopsies or surgical specimens as a prognostic tool for clinical staging; and all other proteins studied (Bcl-2, p53 and p16) did not play a role in GIST metabolic or carcinogenic process, remaining without prognostic value.

Key words: Gastrointestinal Stromal Tumors. Ki-67 Antigen. Immunohistochemistry. Survival Rate.

RESUMO

OBJETIVO: Avaliar a expressão imunoistoquímica de p16, Ki-67, p53 e Bcl-2 proteínas em tumores gastrointestinais estromais (GIST); determinar a possível associação entre essas variáveis e fatores clínicos e histopatológicos de câncer, e para verificar o valor prognóstico destas variáveis (sobrevivência e recorrência).

MÉTODOS: Uma amostra de 55 pacientes tratados cirurgicamente para GIST em três hospitais foi estudada. Os tumores extirpados cirurgicamente foram confirmados como GIST por KIT, vimentina, proteína desmina S100, CD117, 1A4 e avaliação de CD34 em blocos de parafina.

RESULTADOS: Apenas nove (16%) casos de GIST foram positivos para p53, p16 foi positiva em 43,6%, 80% dos GIST apresentaram coloração para Bcl-2. O índice proliferativo (expresso como a proporção de células positivas), avaliado pela expressão imunoistoquímica de Ki-67, foi elevado em 49% dos casos. Escores de Ki-67 elevados foram associados com alto grau histológico ($p=0,0026$) e índice de mitose, MI ($p=0,0001$). Alto índice de Ki-67 foi associado à morte. Expressão da p53, p16 e Bcl-2 não se correlacionou com as variáveis morfológicas ou clínicas.

CONCLUSÕES: A avaliação imunoistoquímica de Ki-67 deve ser incluída na avaliação pré-operatória de biópsias ou peças cirúrgicas de GIST como uma ferramenta prognóstica para o estadiamento clínico, e todas as outras proteínas estudadas (Bcl-2, p53 e p16) não desempenharam um papel no processo metabólico ou carcinogênico em GIST, mantendo-se sem valor prognóstico.

Descritores: Tumores do Estroma Gastrointestinal. Antígeno Ki-67. Imunoistoquímica. Taxa de sobrevida.

Introduction

Gastrointestinal stromal tumors (GIST) are considered a rare entity, encompassing for 2.2% of gastric neoplasias, 13.9% in small intestine and only 0.1% of colon tumors. However, in the United States 3000 to 5000 new GIST cases are diagnosed yearly¹. Before 1986 this entity was encompassed among gastrointestinal mesenchymal tumors. With the identification of KIT protein expression in these lesions, a novel malignant tumor referred as GIST was identified^{2,3}.

In the 2001 consensus, immunopositivity was defined as a key element for the diagnosis of GIST, and the classification of malignancy is based on tumor size and mitotic count³⁻⁵. However, lack of uniform diagnostic criteria led to controversial epidemiological and survival data. Some tumors previously considered GIST could be diagnosed as other particular GI sarcomas (as leiomyosarcomas or schwannomas)^{3,6}. Differential diagnosis among GIST and other muscular or neural tumors require therefore immunohistochemical assessment with standard profile including S100 protein, CD34, 1a4, desmin, and CD117 (c-KIT) antibody, determinant of GIST diagnosis³.

Only 5% of GISTs are sensitive to classic chemotherapy⁷ and radiotherapy is ineffective in this particular entity. Surgical resection was the main therapeutic approach to GIST until mesylate imatinib (Glivec), a molecular target drug that blocks KIT signalization (STI-571)⁸, was available for adjuvant therapy in advanced or irressectable GIST cases^{7,9}.

However, it is still difficult to determine which cases will metastasize or present an aggressive behavior based uniquely in morphological variables. Several potential prognostic variables were assessed in GIST, such as DNA ploidy, protein expression (S100, CD34, VEGF, S-VEGF) and genetic alterations (deletion of exon 11) without reaching statistical significance and, more important, with lack of technical reproducibility. Recently, we evaluated the role of p53 and Ki-67 immunohistochemically with preliminary favorable results¹⁰.

The objectives of this study are: 1) To evaluate the immunohistochemical expression of p16, Ki-67, p53 and Bcl-2 proteins in 55 cases of GIST; 2) to assess the possible association between these variables and clinical and histopathological factors of breast cancer; and 3) to check for prognostic value of these variables, relating them to survival and recurrence.

Methods

A total of 55 samples of surgically excised GIST, diagnosed between February of 1992 and September of 2006, were retrospectively identified from the archives of the Department of Surgical Pathology of three university Hospitals of Sao Paulo, Brazil, and were included in this study. Ethics Committees of all the three institutions approved this study.

Inclusion criteria were primary GIST tumors previously detected by immunohistochemical profile including KIT, vimentin, desmin, S100 protein, CD117, 1A4 and CD34 immunoreactivity assessment (as suggested by Fletcher *et al.*³); paraffin blocks and clinical data available for further analysis and tumors with no preoperative chemo or radiotherapy. GIST tumors had been graded according to the standard criteria³, and to morphological categories, presence of necrosis, mitotic index (50 high power camps, hpc), tumor width and histological type and grade. These variables were morphologically assessed in hematoxylin-eosin (HE) slides by two pathologists (AFL and RAN). Clinical, survival and recurrence rates were calculated based on the follow up of all patients until October 2006.

Immunohistochemical assessment

Representative tumor paraffin-embedded tissue blocks were cut into 5 micra slides. Immunohistochemistry analysis was performed on each tumor slide block for p53, p16, Bcl-2 e Ki-67, with standard streptavidin ABC methodology. Two minutes of pressure cooking at pH 6.0 was used for antigen retrieval with p53 clone (1:x) (BioGenex Inc., San Ramon, CA, USA). Immunohistochemical assay with the tumor markers was performed using the antibodies and conditions shown in Table 1.

TABLE 1 - Immunohistochemical markers used to assess cell proliferation and apoptosis in gastrointestinal stroma tumor.

Antibody	Clone	Provider	Recovery	Detection	Dilution
Bcl-2	124	DAKO	Microwave	AntimouseABC	1:400
P53	DO-7	DAKO	Pressure pan	Ervision-mouse	1:2000
P16	16P07	Neomarkers	Microwave	Ervision-mouse	1:100
Ki67	MIB-1	DAKO	Pressure pan	Ervision-mouse	1:300

For p53, p16, and Ki-67, nuclear expression was assessed by identification of nuclear brown staining of neoplastic cells for further analysis at the nuclear markers¹¹⁻¹⁴. A semiquantitative method for nuclear immunohistochemical grading was used¹⁵.

Bcl-2 showed a cytoplasmatic diffuse staining. A lesion

was designated as positive if at least 10% of true neoplastic tumor cells expressed the antibody studied¹⁵⁻¹⁷. In some tumors virtually every cell expresses the marker and others may show a focal or variable percentage of positive cells.

Statistical analysis

Full clinical follow-up data, including presence of metastatic disease, were available for all GIST cases. Associations between p53, p16, Bcl-2 e Ki-67, immunoreactivity and clinic pathological parameters – including tumor size (as defined by TMN [tumor-node-metastasis] staging, 7th edition), tumor grade (as morphological criteria) local recurrence, and distant metastasis – were evaluated by Fisher’s exact test or chi-squared 2 test as appropriate. Mean age at diagnosis and positivity for p53, p16, Bcl-2 e Ki-67 were compared using t test and Kolmogorov-Smirnov test. Survival analyses were conducted for overall survival (OS), disease-free survival (DFS), survival to metastases at specific sites, and time from first recurrence to death. DFS was defined as time to any type of recurrence, distant metastasis, or death from any cause. Survival curves were calculated using the Kaplan-Meier method. Tests of differences in survival between groups were performed using the log-rank test and were expressed as hazard ratios (HRs), which were estimated using Cox regression. Cox regression analysis was also used to evaluate any independent effect of prognostic factors on DFS, OS, and survival from recurrence. Factors found to be significant (at a p value of less than 0.05) in the survival analysis, together with for p53, p16, Bcl-2 e Ki-67 as the factors of principal interest, were included in the regression analyses. All tests were two-tailed, and 95% confidence intervals were presented where appropriate. All analyses were carried out using software SAS 9.1 (Statistical Analysis System, Cary, NC, USA).

Results

Clinical and morphological data

Among the 55 studied patients, 13 died from the disease (23.6%) and 42 were alive (76.4%) at the last follow up assessment. Among the survivors, 34 had no sign of disease (80.9%) and 8 were currently presenting recurrence (14.5%). All clinical epidemiological and morphological data are disposed in Table 2.

TABLE 2 - Clinical and demographic data on gastrointestinal stromal tumor (GIST) patients.

Characteristics	GIST (n=55)	%	
Median age (range)			
Gender	Male	27	49
	Female	28	51
Age	Median	56	
	Maximum	87	
Histological type	Fusiform	32	58
	Epitelioid	15	27
	Mixed	8	15
Site	Stomach	22	40
	Small bowel	20	36
	Retroperitonium	5	9
	Colon	4	7
	Others	4	7
Histological grade	High	33	60
	Moderate	13	24
	Low	9	16
Tumor size	Up to 5 cm	6	11
	5 to 10 cm	22	40
	More than 10 cm	27	49

The stomach was the most frequent affected organ (22 cases, or 40%). However, atypical locations as appendix, mesenterium, rectum and pelvis were represented with one case each (disposed as “others” in Table 2). Median tumor width was 12.78 cm (0.7 a 32 cm) with predominance of fusiform differentiation (32 or 58.1%).

More than 20% of the cases were composed of high-grade tumors (Table 2). Tumor necrosis was present in 31 cases (56.4%). High mitotic index (MI) (more than 10 mitosis per high power fields) was observed in 21 GIST cases (38%), while 8 (12%) presented intermediary MI (5 to 9 mitosis per 50 hpf) and

27 (49%) only a low MI (up to 4 mitosis in 50 cpf).

When all morphological and clinical variables were statistically analyzed altogether, necrosis was associated to tumor width ($p=0.0032$), to MI ($p=0.0001$) and also to histological grade ($p=0.0013$). Presence of necrosis was marginally associated to evolution to death and the also to the epithelioid variant, but it did not reach statistical value ($p=0.0599$ and $p=0.0562$, respectively).

Besides necrosis, MI was associated to evolution, to death ($p=0.0153$) and to the “stayed alive” ($p=0.0099$) conditions; and correlated to tumor width ($p=0.0009$) and histological grade ($p=0.0001$). Finally, histological grade was also associated to tumor width ($p=0.0001$) and evolution, since high histological grade patients’ survival was significantly shorter when compared to lower-grade patients ($p=0.0163$).

p53, p16, Ki-67 and Bcl-2 expression

Preliminary diagnostic immunohistochemical profile of all tumors included analysis of desmin (seven positive cases, 12.7%), S100 protein (17 stained cases, 30.1%), vimentin (almost all cases stained strongly, 49 or 89.1%), CD34 (reactivity present in 35 cases or 63.6%), and 1A4 expression (present in 22 cases, 40%). These data were not significantly correlated to any morphological or clinical variables.

Only nine (16%) cases of GIST were positive for p53, and the proportion of neoplastic positive cells were largely variable among the positive cases.

p16, however, was positive among 25 reactive cases (43.6%); with positivity in the majority of the transformed cells. Interestingly, more than 75% of GIST analyzed also showed staining for Bcl-2 (44 cases or 80%).

According to the proliferative index, tumor cells were negative for Ki-67 in 27 cases (49%) and positive in the remaining 28.

Among the biological markers, Ki-67score reached significant correlation with other important prognostic variables. Higher Ki-67 scores were associated to high histological grade ($p=0.0026$ with Fisher test, $p=0.007$ with Kruskal-Wallis test) and MI ($p=0.0001$). Tumors with high Ki-67 indexes presented necrosis ($p=0.0257$) and p53 positive results ($p=0.0018$) more frequently. Moreover, a high Ki-67 index was associated to death: 13 of 24 Ki-67-positive GIST patients died during the study and association to evolution was confirmed with Fisher and Kruskal-Wallis tests ($p=0.049$ and $p=0.01$, respectively). Figure 1 shows the survival curves of positive and negative Ki-67 cases.

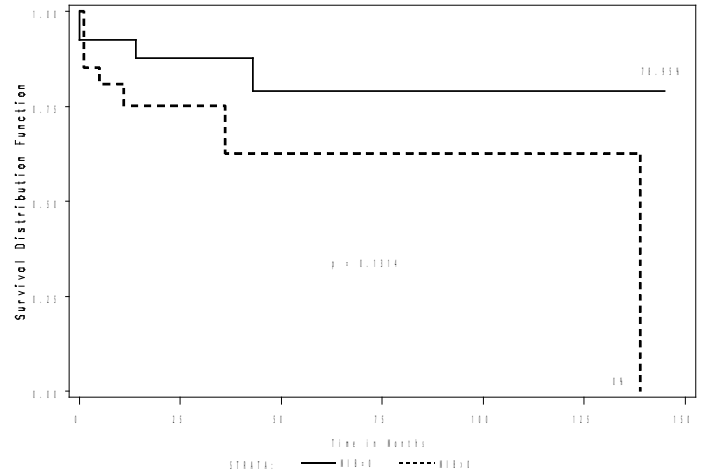


FIGURE 1 - Survival curve of patients with GIST tumors and positive or negative for Ki-67 (Mib).

Expression of p53, p16 and Bcl-2 did not show any association and did not correlate to any morphological or clinical variables (Table 3).

TABLE 3 - Statistical significance (p value) of the correlations between the variables.

Variables	p16	Bcl-2	Ki-67	Degree	Mitosis	Necrosis	Size	Evolution
p53	0.3402	1.000	0.0018	0.5614	0.1446	0.8648	0.8524	0.5617
p16	-	0.5698	0.8460	0.3985	0.8913	0.2433	0.4944	0.2055
Bcl-2	-	-	1.000	0.9302	0.2528	0.2766	0.5185	0.1454
Ki-67	-	-	-	0.0026	< 0.001	0.0257	0.0991	0.0161
Histological grade	-	-	-	-	< 0.001	0.0013	< 0.001	0.1417
Mitosis	-	-	-	-	-	< 0.001	< 0.001	0.0202
Necrosis	-	-	-	-	-	-	0.0032	0.0872
Size	-	-	-	-	-	-	-	0.7314

Discussion

The current prognostic classification in GIST is based in tumor width and mitotic index as proposed by Fletcher *et al.*³ However, the classical mitotic index evaluation requires 50 high power camps and usually small samples are insufficient

to complete the analysis. Moreover, there are irregularities. For instance, fixation may shrink tumor size and mitoses may “go over” during fixation¹⁸.

Recently, diagnostic approach to GIST started to include ultrasound-guided fine needle aspiration or core biopsy. Preliminary results from our group¹⁰ and also from Ando *et al.*¹⁹ suggested that Ki-67 expression is a powerful prognostic tool for easy assess in smaller samples. The new Food and Drug Administration (FDA)-approved indication of imatinib mesylate in the adjuvant therapy turns biopsy specimens assessment necessary. Currently, there is no consensus in literature about the most suitable methodology for proliferation assessment and Ki-67 expression evaluation in GIST. Besides the variation of reading and assessment methodologies, limit values for positive and negative results remain controversial²⁰⁻²⁴.

We used a previously described method for Ki-67 assessment (Allred method for estrogen receptor). By using a high score for intensity¹³, since it is assumed that it would be equivalent in all samples and not variable, adapted this method to proliferation assessment. Distribution remained with punctuation from 0 to 5, leading to an index from 4 to 8. The final result is expressed in crescent categories, very reproducible. One advantage is that it constitutes an easily understandable method among distinct observers, as in ER expression, and practical for clinical application. Another important feature of this approach is that it can be performed in biopsy specimens, therefore allowing the pathologist to have an idea of proliferation status before surgery. The strong correlation to other important prognostic variables, such as histological grade ($p=0.0026$), mitotic index ($p=0.001$) and presence of necrosis ($p=0.0257$) endorsed the potential clinical role of this Ki-67 index. Finally, the association of Ki-67 index to evolution ($p=0.0161$) was evidenced, demonstrating that higher Ki-67 index is associated to poor survival rate.

The evaluation of cell cycle-related proteins in neoplasms by immunohistochemistry has been included in prognostic staging in central nervous system^{25,26} and other tumors^{21,27-30}. We verified if these same proteins could also play a prognostic role in GIST^{20,31}.

Bcl-2 protein is highly expressed in many neoplasms and also in GIST. Our results confirmed that GISTs are Bcl-2 expressing tumors. Classically, chemotherapy agents hit cells in an apoptosis pathway. In carcinomas and lymphomas, a higher contingent of apoptotic cells may represent a better response to chemotherapy^{30,32}. Unlike many chemotherapeutic agents, imatinib mesylate, used in GIST is not based on a proliferating contingent of cells, but rather a blocking tyrosine kinase receptors and the further induction of cellular proliferation³³. Therefore,

Bcl-2 would not be useful to predict response to imatinib. Indeed, most of the studies could not associate the highly expressed Bcl-2 to other prognostic factors or to drug response in GIST^{22-24,34}. We confirmed that Bcl-2 assessment could not improve staging or prognosis assessment in GIST. The reason why Bcl-2 is so up regulated in GIST is not clear yet. One could speculate that uncontrolled proliferation in ultimately stable mesenchymal cells could trigger cell mechanisms to balance increasing population or cell transformation in GIST affects Bcl-2 gene regulation with protein stabilization and accumulation. Molecular assessment would be necessary to clarify this issue.

Gene mutations in TP53 tumor-suppressor gene are one of the most common genetic alterations in human solid tumors. Loss of p53 protein function may increase the risk for carcinogenesis^{35,36}. There are dozens of publications on p53 expression in GIST with a large methodological variation among them. However, the series of patients are usually smaller than ours and the results are very heterogeneous, ranging from 0 to 100% of positive neoplastic cells. In five larger studies, comprehending more than 60 patients, p53 staining was present in less than 50% of neoplastic cells and prognostic value remained controversial^{35,36}.

We found nine p53 positive GIST cases in 55 analyzed (16%), and p53 staining correlated to Ki-67 index, but not to any other clinical or morphological variable. The smaller contingent of p53 positive cells could mean that p53 alterations are not adamant to GIST early steps in carcinogenesis and may occur later in this process^{37,38}. Also p53 deletion is not frequent and missense mutation seems confined to less than 50% of neoplastic cells³⁹.

The assessment of p16 protein in GIST is still incipient and three previous studies⁴⁰⁻⁴² with positivity ranging from 34% and 62% assessed p16 by PCR, RNA and tissue microarray (TMA). We found 25 immunohistochemically positive cases (45.4%). Metilation of p16 gene was previously demonstrated by House *et al.*⁴² with may partially explain the diversion of results.

Ki-67 protein is one of several factors involved in cell proliferation that may be assessed easily by immunohistochemistry. Ki-67 index reflects the proportion of cycling cells in a given population. In GIST, Ki-67 expression has been previously associated to morphological variables and also survival, but results are hardly comparable due to methodological different approach^{19,20,23,31}.

Conclusions

The Ki-67 immunohistochemical evaluation should be included in preoperative evaluation of GIST biopsies or surgical

specimens as a prognostic tool for clinical staging. All other proteins studied (Bcl-2, p53 and p16) did not play a role in GIST metabolic or carcinogenic process, remaining without prognostic value.

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