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Serum c-kit Protein Detection as a Reliable Biomarker for the Diagnosis of Gastrointestinal Stromal Tumors: A Case-Control Study

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Abstract

Background: In the last decade, the diagnostic approach of Gastrointestinal Stromal Tumors (GIST) has drastically changed with the utilization of tissue-specific biomarkers, such as c-Kit. However, a serum biomarker aiding in differential GIST diagnosis prior to surgery would provide added benefit in the daily clinical practice. Thus, the aim of the study was to investigate whether detection of c-Kit protein in the serum could be used as a reliable diagnostic biomarker.

Methods: Twenty-seven patients with histologically confirmed GIST (16 men; age 41-80 years), 27 healthy controls (14 men; age 45-76 years) and 24 patients with submucosal tumors other than GIST (10 men; age 25-85 years) were enrolled in the study. Detection of serum c-Kit protein in blood samples of all patients prior to any treatment-related intervention and of healthy participants was accomplished using flow cytometry. The sensitivity and specificity of the assay were estimated.

Results: Of the 27 patients with GIST, 25 were serum c-Kit positive. In the control group of 27 healthy participants, all except one were serum c-Kit negative, while among the 24 patients with tumors either than GIST, 22 were serum c-Kit negative and 2 were positive. Based on these results, the sensitivity of the assay was 92.6%, while the specificity was 96.3% when compared with the healthy volunteers and 91.7% in comparison with the non-GIST group.

Conclusion: c-Kit protein detection with flow cytometry could represent a reliable sensitive and specific serum bioassay for differential GIST diagnosis.

Keywords: Gastrointestinal stromal tumors; c-Kit; Soluble c-Kit; Biomarker; Diagnosis; Flow cytometry

Abbreviations:

ELISA: Enzyme-Linked Immunosorbent Assay; GI: Gastrointestinal; GIST: Gastrointestinal Stromal Tumors; NPV: Negative Predictive Value; SCF: Stem Cell Factor; PDGFRA: Platelet-Derived Growth Factor A; PPV: Positive Predictive Value

Introduction

Gastrointestinal Stromal Tumors (GISTs) are the most common mesenchymal tumors of the Gastrointestinal (GI) tract. They account for 1-3% of all GI neoplasms and are characterized by features that overlap with those of other mesenchymal tumors, such as leiomyomas and leiomyosarcomas. About 70% occur in the stomach, 20-35% in the small intestine and less than 5% in the esophagus [1].

The majority of GISTs express the c-Kit tyrosine kinase receptor (also known as CD117), the product of the KIT proto-oncogene2.c-Kit binds to Stem Cell Factor (SCF), forming a dimer that activates its intrinsic tyrosine kinase activity, leading to phosphorylation and subsequent activation of signal transduction molecules that propagate the signal in the cell. Signaling through c-Kit has been shown to be involved in cell survival, proliferation, and differentiation [2]. Approximately 85-90% of GISTs are associated with KIT gene mutations that result in a constitutively activated ligand-independent form of KIT [1-3]. Mutations in KIT exon 11 are the most common, and may be frame deletions, missense mutations, or internal tandem duplications, while KIT exon 9 mutations (mainly duplications) are the next most common KIT mutations identified in GIST tumors. Mutations in KIT exons 13 and 17 are rare (accounting for approximately 1% each) [2,4]. A small proportion of GISTs (5-7%) are associated with mutations of the Platelet-Derived Growth Factor Receptor A (PDGFRA), the gene encoding PDGFR-α [5].

Based on the pathophysiological association between KIT receptor tyrosine kinase tissue expression and GIST tumors, the detection of

soluble c-Kit protein in the plasma has been explored as an attractive surrogate biomarker for response to therapy. Preliminary reports have suggested that a decline in plasma c-Kit levels in GIST patients might correlate with response to imatinib [6,7] and sunitinib [8]. These initial findings were further supported by the study of De Primo et al. [9], demonstrating that plasma circulating c-Kit levels detected using a prototype quantitative sandwich Enzyme-Linked Immunosorbent Assay (ELISA) could serve as a surrogate marker for clinical outcome in GIST patients. Specifically, this study showed that the median time to progression was 34.3 weeks in sunitinib-treated patients whose serum c-Kit levels at the end of the second cycle were reduced compared to baseline, while patients whose serum c-Kit levels were increased compared to baseline had a median time to progression of 16 weeks.

Thus, it would be of interest to investigate whether serum levels of c-Kit would be useful not only as a marker of disease response to treatment, but also as a reliable biomarker that could contribute to the differential diagnosis of GISTs from other GI submucosal tumors.

Therefore, in the present study we evaluated the hypothesis that serum c-Kit may be used as a biomarker of GIST differential diagnosis. c-Kit serum levels were determined in patients with GIST by flow cytometry and were compared with results obtained from patients with submucosal tumors other than GIST and to results from healthy controls. Flow cytometry was chosen due to the fact that in studies using ELISA as a method to detect serum c-Kit levels, serum c-Kit protein fragments were detectable not only in the serum of patients with GISTs, but also in the serum of patients with pathological conditions other than GIST as well as in healthy individuals [10].

Subjects and methods

Study participants

A total of 27 patients with histologically confirmed GISTs diagnosed between February 2008 and December 2011 at the Gastroenterology Department of Army Veterans Hospital or at the 'Thriasio' General Hospital of Elefsina were recruited for this prospective, case-control study, and their clinical data were analyzed. In addition, 27 healthy subjects were recruited to serve as a control group while 24 patients with submucosal tumors other than GIST were recruited to serve as a comparator group (Figure 1). The first subject was recruited into the study on 13-Feb-2008. The last-subject-last-visit was 31-Dec-2011.

For enrollment into the study participants had to fulfill the following inclusion criteria: age >18 years; absence of diagnosis of other epithelial tumor; and no prior antitumor medication. For enrollment into the GIST group, GIST diagnosis had to be histologically-confirmed.

Serum c-Kit levels were determined in all 27 patients with GIST before any treatment procedure was performed and their results were compared with those of the 27 healthy participants, who were used as a control group and those of the 24 patients with other than GIST submucosal tumors (8 leiomyomas, 6 lipomas, 3 carcinoid tumors, 4 ectopic pancreatic tissue masses, 1 schwannoma, 1 pancreatic pseudocyst and 1 neurofibroma), who were endoscopically diagnosed during the same period in the study center and served as a comparison group.

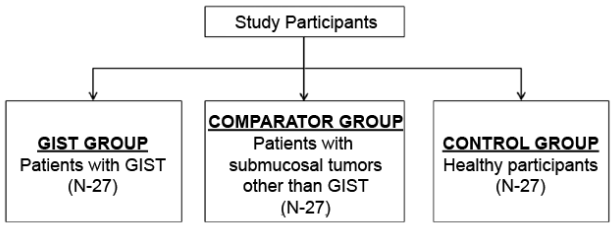

Figure 1: Study participant groups. The distribution of study participants in the 3 study groups is presented. Control group denotes healthy patients (n=27), comparator group those with submucosal tumors other than GIST (n=24), and GIST group patients with GIST (n=27)

This study was performed in accordance with the International Conference on Harmonisation Good Clinical Practice Guidelines, the Declaration of Helsinki and all applicable laws and regulations. The study was approved by the University of Athens Medical School and Army Veterans Hospital Ethics Committees. All participants gave written informed consent prior to enrollment into the study. No participant withdrew from the study prior to completion of study-related requirements.

Clinicopathological assessment

Blood samples were collected prior to biopsy collection from subjects who were suspected to have GIST based on clinical, endoscopic or other imaging assessments. Blood samples were stored until histological diagnosis of GIST.

Tumors were histologically evaluated by 2 pathologists, blinded to the procedures of the study. Patient demographic characteristics including age and gender, and clinicopathological factors including tumor size, location, histological type, tumor TNM staging, mutational status and risk stratification were recorded.

Blood samples and c-Kit measurement

Blood samples were collected from all patients with GIST and non-GIST submucosal tumors as well as from healthy controls (5ml from each participant) in EDTA containing tubes (K2EDTA), after informed consent was obtained. After centrifugation at 1000 rpm for 15 min, the supernatant obtained was collected with a Pasteur pipette in eppendorf tubes (approximately 2ml of serum), was treated with a proteinase inhibitor and was subsequently stored frozen at 30°C until use.

Measurement of c-Kit serum levels was accomplished by flow cytometry performed at a central laboratory, using the MILLIPLEX MAP Total c-Kit and Phospho c-Kit MAPmates pair in conjunction with the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit (Merck Millipore Co, Billerica, MA, USA), to detect the presence of both total c-Kit and Phospho c-Kit in serum samples of study participants using the Luminex® 100 IS System (Luminex Corp). We aimed to report the abundance and expression levels of the target protein, as well as phosphorylation levels (direct measurement of kinases activated within the cell, in order to confirm the proliferation

process). Cut-off levels for both positive and negative values were calculated according to the manufacturer's instructions.

Statistical analysis

Continuous variables are summarized as mean±standard deviation (SD) and categorical variables are displayed as frequency tables (N, %). Data were statistically analyzed using t-test as appropriate. The correlations between the parameters and serum c-Kit levels were also assessed statistically using the chi-square test and the Fisher's exact test. All statistical tests were performed at a 0.05 significance level.

Results

Characteristics of study participants

Participant characteristics according to gender and age are presented in Table 1. For the majority of GIST patients (66.7%). All tumors were associated with Kit mutations; 19 (70.4%) had an exon 11 KIT mutation while the rest (29.6%) had an exon 9 KIT mutation. The characteristics of the GIST patient population according to location, tumor size, histological type, mutational status, mitotic rate, resectability and presence of metastasis at diagnosis is presented in Table 2.

	GIST Group	Comparator Group	Control Group
N	27	24	27
Male/Female	16/11	10/14	14/13
Age, range	41-80	25-85	45-76
Age, mean	63	51	62

Table 1: The characteristics of the different populations (control group, comparator group and GIST group) participating in this study are depicted according to gender and age. Control group denotes healthy patients, comparator group those with submucosal tumors other than GIST, and GIST group patients with GIST

The Fletcher risk table, also known as the NIH risk table, was used to classify GIST patients into categories based on their risk of recurrence or metastasis [11]. Based on the mitotic rate and tumor size, 7.4% (n=2) of the patients were classified as 'very low risk', 40.7% (n=11) as 'low risk', 33.3% (n=9) as intermediate risk and the remaining 18.5% (n=5) as high risk. The results for total and phospho c-Kit determination were in complete agreement with each other.

In the GIST group there was no significant association of serum total c-Kit positivity with clinicopathological characteristics, such as patient's age, gender, tumor location and size, histological type or metastatic status (Table 3).

	N (%)
Gender	
Male	16 (59.3)
Female	11 (40.7)
Age (years)	
<55	9 (33.3)
≥55	18 (66.7)
GIST location	
Stomach	18 (66.7)
Duodenum	4 (14.8)
Jejunum	3 (11.1)
Ileum	1 (3.7)
Colon	1 (3.7)
Tumor size	
≤2 cm	2 (7.4)
>2 cm	25 (92.6)
Histological type	
Spindle cells	19 (70.4)
Epitheloid cells	8 (29.6)
Mutational status	
Exon 11 KIT	19 (70.4)
Exon 9 KIT	8 (29.6)
Mitotic rate	
<5/50 high power fields with tumor size <5 cm	13 (48.1)
6-10/50 high power fields with tumor size <5 cm	9 (33.3)
>5/50 high power fields	5 (18.5)
Resectability	
Resectable	23 (85.2)
Unresectable	4 (14.8)
Presence of metastasis at diagnosis	4 (14.8)

Table 2: The characteristics of the GIST patient population are presented according to gender, age group, GIST tumor location, tumor size, histological type, mutational status, mitotic rate, tumor resectability and presence of metastasis

VARIABLES	SERUM c-KIT		p-value
	NEGATIVE	POSITIVE	
Age			
<55 years	2	0	NS
≥55 years	0	25	
Gender			
Male	2	14	NS
Female	0	11	
Location			
Stomach	2	16	NS

Small intestine	0	8	
Colon	0	1	
Tumor size			
≤2 cm	2	0	NS
>2 cm	0	25	
Histological type			
Spindle cells	1	18	NS
Epithelioid cells	1	7	
TNM stage			
I/II/III	2	21	NS
IV	0	4	

Table 3: Association between the preoperative levels of serum c-Kit and clinicopathological factors of the patients with GISTs Associations between the preoperative levels of serum c-Kit and clinicopathological factors of the patients with GISTs were examined with the t-test. Clinicopathological variables include age, gender, location, tumor size, histological type and TNM stage. NS, Not significant ($p<0.05$)

Preoperative values of serum total c-Kit (as well as phospho c-Kit) were positive in 25 of the 27 patients with GIST. Specifically, all 25 patients with tumor size > 2 cm (T2 TNM staging) were serum total and phospho c-Kit positive, while both patients with a tumor size ≤2 cm were serum total and c-Kit negative (as well as phospho c-kit negative). Furthermore, there seems to be a correlation between serum total c-Kit levels and TNM stage, as in the two patients with TNM stage I and II, serum total and c-Kit values were negative, whereas total c-Kit levels were positive in all 25 patients with TNM stage III and IV. There was a strong correlation between serum total c-Kit levels and immunohistochemical staining of the surgical specimen. More specifically, in 25 out of 27 histologically confirmed patients with GIST, immunohistochemical staining revealed total c-Kit positive results in correlation with positive serum total c-Kit (Figures 2A and 2B)

On the other hand, immunohistochemical c-Kit staining as well as soluble total c-Kit was negative for 2 of the 27 cases. The immunohistochemical c-Kit staining results for the 2 patients with negative serum c-Kit were: c-Kit (-), SMA (+), S-100 (+), CD34 (+), Ki-67 (+15%), actin specific (-), desmin (-), myosin (-) for the first case and c-Kit (-), SMA (-), S-100 (+), CD34 (+), Ki-67 (+10%), actin specific (-), desmin (-) , myosin (-) for the second case with negative serum total c-Kit.

Of 27 patients enrolled in the healthy control group, all except one participant was serum total c-Kit negative. Comparison of these results with those of the GIST group, yields a sensitivity of 92.6% and a specificity of 96.3% for the assay, whereas the positive (PPV) and negative (NPV) predictive value of the method used was 96.3% and 92.6 % respectively.

In the group of 24 patients with other than GIST submucosal tumors, 2 out of 24 patients (both with carcinoid tumors), were serum total c-Kit positive (Fisher's exact test: $p<0.0001$). The sensitivity and specificity of the test, comparing the GIST group with the other than GIST submucosal tumor comparator group, was found to be 92.6 % and 91.7 % respectively, while the PPV and NPV predictive value was estimated to be 91.7 % and 92.6 %, respectively.

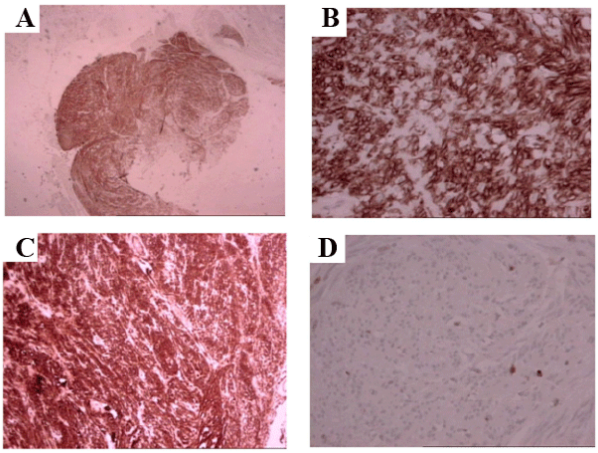

Figure 2: Immunohistochemical staining of GIST gastric mucosa specimens. Immunohistochemical staining of GIST gastric mucosa specimens with alkaline phosphatase - anti-alkaline phosphatase (APAAP).CD117 positive expression in tumor cells at (A) magnification (x20) and (B) x400 (same specimen). (C) Positive staining for CD 34 (x100). (D) Ki-67 (x400) staining

Discussion

GISTs share common features with other submucosal tumors, such as leiomyomas, leiomyosarcomas and schwannomas. The diagnostic difficulty in submucosal tumors lies in the fact that preoperative conventional imaging techniques (i.e., sonography, endoscopy, and computed tomography), cannot always provide enough information to differentiate between these types of tumors. Hence, for the differential diagnosis a histological sample obtained prior to surgery is needed. c-Kit immunohistochemical positivity along with the histological morphology is used to distinguish GIST tumors from other submucosal tumors [12,13]. However, a serum biomarker aiding in differential GIST diagnosis prior to surgery would provide added benefit in the daily clinical practice.

Identification of biomarkers that aid in the diagnosis as well as the assessment of clinical activity have been the focus of many recent oncology studies due to their potential to provide information on clinical outcomes in a more timely manner and with employment of fewer patients than many traditional endpoints. Numerous studies have provided evidence on the pathophysiologic and etiologic association between aberrant Kit expression and activity in GIST cases, thus providing the rationale for investigating serum c-Kit as a surrogate marker, not only for the clinical outcome of GIST patients under specific therapy as previously reported [6,9], but also for the differential diagnosis of GISTs from other submucosal tumors of the GI tract.

In the present study, we aimed to investigate whether total c-Kit protein serum level detection with the use of flow cytometry could serve as a highly sensitive and specific diagnostic biomarker of GIST, which would enhance the diagnostic procedures providing a quick,

non-invasive, easy to perform test in everyday clinical practice. Moreover, we aimed to study and evaluate the role of serum c-Kit protein detection in the differential diagnosis of GIST from other submucosal tumors, such as leiomyomas, leiomyosarcomas, lipomas, schwannomas, etc.

Measuring serum total c-Kit levels in patients with histologically confirmed GISTs and comparing the results with those of healthy controllers and of patients with other than GIST submucosal tumors, we found that circulating total c-Kit levels seem to function as a surrogate biomarker which could contribute to the differential diagnosis of submucosal tumors of the GI tract. Specifically, when comparing results obtained from the group of GIST patients with those of the control group the sensitivity and specificity of the performed procedure were 92.6% and 96.3%, respectively. Moreover, when the results of the serum total c-Kit levels were compared between the GIST group and the comparator group of other than GISTs submucosal tumors, the sensitivity and specificity of the performed procedure were calculated to be 92.6% and 91.7%, respectively.

Serum c-Kit has been suggested to originate from proteolytic cleavage of the extracellular domain of KIT on KIT expressing cells. Among cells that normally express KIT are mast cells, melanocytes, gastrointestinal tract pacemaker cells (Cajal cells), hematopoietic stem cells, cutaneous basal cells, Leydig cells, spermatogonia, spermatids and breast epithelial cells [14-17]. Abnormal concentration of c-Kit has been reported in various diseases. Specifically, elevated concentrations of circulating c-Kit in the serum are often detected in patients with chronic myeloid leukemia, but c-Kit may also be detectable in the serum of healthy individuals [18]. Initial studies have revealed the presence of elevated levels of a c-KIT in the serum of patients with acute myeloid leukemia which decreased to normal levels after effective chemotherapy [19]. A recent study has correlated circulating c-kit levels with the outcome of metastatic breast cancer [20]. In regards to patients with GISTs tumors, previous reports have suggested that a decrease in c-Kit plasma levels might correlate with response to imatinib [6,7] and sunitinib [9] therapy. Moreover, Bono et al. [7], showed that patients with GISTs had elevated pretreatment serum c-Kit levels, as compared with healthy controls.

The results of the present study are quite promising, as we found that the frequency of positive total c-Kit in the serum was higher among GIST patients than in healthy controls or patients with non-GIST submucosal tumors. Among the patients with GIST, no significant correlation of circulating total c-Kit levels with patient's age and gender, tumor location and histological type was found. However, serum levels of this protein (both total and phosphorylated form) might be correlated with tumor size, as we found elevated levels of total and phospho c-Kit in all patients with tumor size > 2 cm, as opposed to patients with tumors ≤ 2 cm, for whom total and phospho c-Kit levels were registered below cut-off levels. The validity of this association merits further investigation in a larger study. Nevertheless, these observations, in conjunction with previous reports that response to therapy and subsequent reduction in tumor volume is related to lower c-Kit serum levels [6-9], suggest that a significant proportion of soluble c-Kit fragment might originate from tumor cells.

In the present study, the detection of both circulating serum total and phospho c-Kit was achieved by using a flow cytometry method rather than ELISA used in previous studies. Detection ofc-Kit in serum with flow cytometry has been previously used to confirm diagnosis in patients with chronic lymphocytic leukemia and has specific benefits over existing methods [18,19,21].

Theoretical advantages of flow cytometry over ELISA have been described by the initiator of the technique and the kit manufacturer. Based on a recent customer study, the benefits of xMAP technology over ELISA include among others decreased turnaround time; substantial savings in reagent costs; reduced sample volume; and same workflow as ELISA. Furthermore, with flow cytometry performed with the MILLIPLEX xMAP technology, using the Luminex IS100 System, biological tests or bioassays are performed on the surface of color-coded microspheres rather than on the surface of a plate of the bottom of a well, such as with ELISA. The analytes to be measured within a sample are captured on the surface of these microspheres, which are suspendable in liquid, thus allowing assays of several analytes to be performed simultaneously in a process described as 'multiplexing'. As an example pertaining to the potential application of serum samples of patients with GIST, the presence of c-Kit in the sample may be examined at the same time as the presence of PDGFR-α. The notion of a 'suspension array' as opposed to a flat, solid array (ELISA), allows true representative quality control. Furthermore, multiplexing versus single plexing (ELISA), allows the use of truly internal controls (positive and negative) within the same sample [22].

Conventional ELISA technology is designed to conduct bioassays quickly, performing only one test per sample. However, this approach cannot efficiently study the entire process, but only one or a few of the individual components. The xMAP technology is more accurate and efficient than ELISA. In addition, xMAP technology contributes to substantial cost savings due to lower reagent usage and lower labor costs than traditional testing methods, such as ELISA [21,22]. Importantly this technique is also considered advantageous over ELISA due to its potential to analyze additional biomarkers of GIST, e.g. PDGFR-α, which might aid in the differential diagnosis of GIST, simultaneously with c-Kit.

It is noted that the cut-off levels of the c-Kit assay performed using flow cytometry have not been optimized for clinical testing purposes. 'Positive' or 'negative' results are user-defined and depend on the results of the positive and negative internal controls of each kit used. Moreover, the cut-off levels can differ from one kit to another and this is due to several factors, such as different kit lot numbers, incubation times, etc. As such, the samples are defined as positive or negative compared to the internal controls (positive and negative) of each kit, which determine the cut-off levels.

The present study has some potential limitations such as the well known effects of several diseases on soluble c-Kit levels. Besides the gastrointestinal tract pacemaker cells (Cajal cells), c-Kit receptor is broadly distributed within hematopoietic cells and is also found in other tissues. As a result, some studies have revealed the presence of elevated levels of soluble c-Kit in patients with acute and chronic myelocytic leukemia, melanoma, breast cancer, small-cell lung cancer, and seminoma. Moreover, asthmatic subjects can overexpress soluble c-Kit. The results of a recent study support that the c-Kit level was higher in the group of non-severe asthmatics compared with healthy controls. It was observed that treatment with systemic glucocorticoids or high doses of inhaled glucocorticoids could be responsible for the decrease of serum c-Kit. Furthermore, soluble c-Kit shed from mast cells and other KIT-bearing hematopoietic cells might be expected to increase in case of disordered hematopoiesis. As a result, high levels of c-Kit can be detected in patients with mastocytosis and anaphylaxis [23]. Elevations in soluble c-Kit were found to correlate with the extent

of bone marrow involvement. Being aware of all the above, and counting on histological contribution in confirming GIST diagnosis, we could minimize the potential limitations.

Notably, the results of the study may also be limited by the observation of patients with histologically confirmed GIST, but with negative soluble total c-Kit. Nevertheless, it is noted that patients with negative serum c-Kit were also negative by immunohistochemical staining and that they represent a relatively low percentage of the patient population (about 7%). In addition, as mentioned previously, the cut-off levels of the total and phospho c-Kit assay have not been optimized. However, cut-off point and assay optimization were not objectives of the present analysis, as it would be impossible in a small initial study, such as the present one, to define the cut-off levels of the c-Kit assay. Moreover, the generalizability of the study results is limited by the small number of patients and control subjects enrolled and the fact that the sample size was not statistically validated. As such, further large, multi-centered, well-organized, randomized studies and clinical trials are required, in order to establish the use of the present assay in everyday clinical practice. Nevertheless, this study was designed to explore the potential of serum total c-Kit as a biomarker and the results provide initial evidence of its potential diagnostic utility in GIST patients.

Conclusions

The results of this small prospective case-control study suggest that detection of totalc-Kit in the serum was more specific for patients with GISTs rather than for healthy controls and patients with non-GIST submucosal tumors. Specifically, the assay had a sensitivity of 92.6%, whereas the specificity was 96.3% and 91.7%, respectively, when compared with the healthy volunteers and with the non-GIST group to differentially diagnose patients with GIST. Due to its ease of detection, circulating total c-Kit levels may function as a reliable surrogate biomarker for clinical purposes. Further studies are warranted, in order to confirm the results and refine the methodology. Moreover, studies are required to evaluate circulating c-Kit (total or phospho) levels as a biomarker in other tumors that have a pathology linked to KIT overexpression, such as small cell lung cancer, breast cancer, germ cell tumors and melanoma.

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