Image analysis of tumor infiltrating lymphocytes components of EGIST microenvironment

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Abstract

Extra gastrointestinal stromal tumor (EGIST) are sarcomas originating from Cajal like cells. They are prone to events triggering inflammation.

Aimes and Objectives: To describe into a retrospective study, the intratumoral lymphocytes in a series of EGIST and correlate their density with the proliferation index and morphology.

Methods: Using antibodies against CD5, CD20, CD45, we demonstrated lymphoid cells and structures by using immunohistochemical methods. Slides were analyzed using QuPath for tumor-infiltrating lymphocyte (TIL) density. Tertiary lymphoid structures (TLS) were reported per sample. Statistical analysis was done using the Kruskal-Wallis corrected test and the Pearson coefficient.

Results: We selected 22 cases with an average age of 51 years. Histologically, the batch was composed of fusiform, epithelioid, and mixed EGIST (63%; 14% and 23%). Numerical analysis of immunohistochemical stains showed an average Ki67 of 19%. The average TIL density of T and B cells was 1.6*103 cells/mm2 and 263 cells/mm2. The TLS have an average of 2.41 households/sample. The correlation between T and B cell density and between TIL with Ki67 shows $p > 0.05$ in our EGIST series. The correlation between EGIST histological variants with TIL type density resumed a $p>0.05$.

Conclusion: TIL cell densities are independent histological parameters for EGIST. The TIL arrangement is inhomogeneous in EGISTs; T cells predominate.

Keywords: Extra gastrointestinal stromal tumor, tumor-infiltrating lymphocyte; digital image analysis

INTRODUCTION

Extra gastrointestinal stromal tumor (EGIST) are sarcomas located outside the digestive tube and originating from cells that show differentiation toward Cajal like cells [1]. Down regulation of immune system has been associated with development and progression of various malignancies. Intra-tumoral lymphocytes as part of the microenvironment is a histoprognostic factor as well as a therapeutic target in a large range of neoplasia. Sarcoma's microenvironment represents a frequent subject in numerous studies focusing on diverse components. The microenvironment represents a part of a general response of host organism to invading tumors. It is expected that even if the response is a generally a common one regardless of tumor type, it is modulated by tumoral intrinsic factors. This can lead to various amount of intra-tumoral lymphocytes as the host response must be triggered by different stimulating factors. Thus, the benefits of appreciating TIL into a specific type of neoplasia can be exploited only if a specific type of neoplasia is investigated descriptively from point of view of active actors in this process. Intra-tumoral leucocytes are represented by myeloid series, lymphocytes and histiocytes. Their proportion is expected to vary among neoplasm types as well as their subtypes. From the classical immunological point of view, main actors in tumors progression and suppression are theT cell. Accumulating evidence is supporting that the B cell lineage as well that histiocytes [2], [3]. Immune checkpoint regulating drugs acting on CTLA-4 and anti-PD1 / PD-L1 have been introduced in clinical practice, treating many cancers, including lung, breast, colon, and bladder cancers. The response to these drugs is only partial, estimated to around 30%. Few studies have investigated these actors in EGIST, both as presence and as possible correlation between their morphology, proliferative index, and TIL density. EGIST and their digestive tube counterpart are well known tumors that are susceptible to events that can't trigger host inflammatory responses.

Aim and objectives

We aim to describe in a prospective study, the intra-tumoral leucocytes in a small series of EGIST and corelate their density with the proliferation index and morphology. The text included in the sections or subsections must begin one line after the section or subsection title.

MATERIAL AND METHODS

Archives and databases of "Victor Babes" National Institute of Pathology (INCDVB) and Bucharest University Emergency Hospital (SUUB) were retrospective researched for EGIST cases. The included cases must have a confirmed diagnosis of EGIST and sufficient material for an elementary immunohistochemistry (IHC) testing and digital analysis. The research was based on paraffin embedded tissue and corresponding slides benefiting from Ethics and Scientific Research Committee of INCDVB Bucharest approval no. 83/2020.

Immunohistochemistry was performed to highlight the lymphoid cells and structures using monoclonal antibodies. We used antibodies against cluster of differentiation 5, 20 and 45 (CD5, CD20 and CD45 or leucocyte common antigen). For the proliferative index we estimate the fraction of active tumoral cells marked with Ki67 (clone Mib1). We have preferred monoclonal antibodies from current laboratory stock utilizing clones 4C7, L26 and X16/99 to detect T, B and Common Leukocyte Antigen from Leica Novocastra. Test were performed on a Leica Bond II automated immunostainer. Epitope retrieval was performed fallowing producer recommendations, utilizing Bond epitope retrieval solution 1 and 2. For detection a polymer kit (Bond Polymer-Refine-Detection) was used, and the stain was

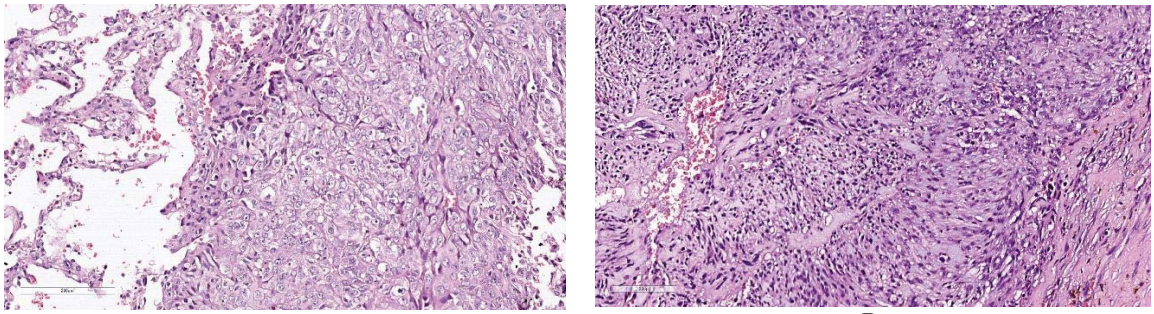
performed using 3,3-diaminobezen (DAB) as chromogen. Counterstain was performed as a regular hematoxylin. Positive control slides with tonsillar tissue were performed in parallel with each batch.

Resulting slides were scanned using Leica Aperio LV1 IVD. Resulting files were imported into QuPath[4] software projects. A total of 10 areas of interests totalizing at least 2mm were chosen for each tumor. Small samples were process in totality. A cell detection step followed by positive count cell corresponding to each type of investigated cell lineage was performed. Review of the positive cell detection was made by two pathologists adjusting their morphometric parameters and excluding false positive cell detections. To evaluate tumor-infiltrating lymphocytes (TIL) for the series we used a modified version of the recommendations TILs Working Group 2014. All leucocytes were taken into account, reporting distinctively: lymphocytes B, T and other leukocytes (mainly histiocytes). Neutrophils and plasma cells were not accounted, as they were considered secondary actors and not markedby our immune pannel. The necrotic areas and surrounding leucocytes were avoided. Borders of the invasive tumors were included in the evaluation. Lymphoid tertiary structures (TLS) were reported as numbers per sample. The individual cell population of TLS was not quantified when reporting the density of TIL.

For statistical analysis software we opted for Excel Office 365 with extend statistical package ad-in. A corrected Kruskal-Wallis Test was performed to determine if median and rank of lymphocytes density per sample (T, B and a total number of including histocytes) is different for the three different morphological variants (epithelioid, mixt and spindle cell). Correlation between proliferative index and TIL specific lineage (B cells and T types) were calculated using the Pearson correlation coefficient. The chi squared values were calculated to retrieve the corresponding p value.

RESULTS

We selected 22 cases from a series of cases of EGIST resulted from prior investigations of our institutional database research (work in prepress). The average age of the EGIST lot was 51 years (interval 27-78 years old, with a median of 52 years). The gender was equally distributed with M/F ratio of 1 to 1. Histologically, most of the lot was composed of spindle cell morphology EGISTs (14 cases, 63%) while the remaining cases were composed of epithelioid (3 cases 14%) and mixed type cells morphology (5 cases, 23%). Histological examination showed in most of the cases areas of multiple foci of interstitial hemorrhages of recent type, see figure 1. Tumor front was of bulky type, some cases comporting to a pseudo-encapsulation made by compressing the nearby connective tissues. TILs were barely perceptible in HE, stains more obvious in the periphery of the tumor, but difficult to estimate as a density parameter.

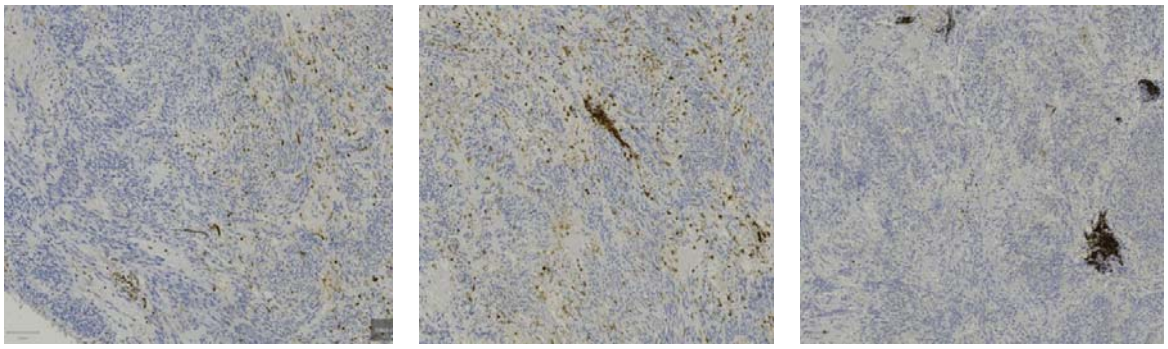


A. B.

Figure 1. Histologic types of EGIST. A. Photography of an epithelioid lung EGIST. Some examples of TIL are pointed by light blue arow heads. They are barely visible in HE stains and reduced in numbers in the epithelioid types of our series. Periphery of a bulky tumoral expansion. On left part of the panel, lung alveolar walls remnants are readily identifiable. Scale bar 200µm, lower left corner B. Spindle cell variant of EGIST. TILs are more abundant, sometimes forming lose aggregates around small vessels pointed by orange arrow (left upper quadrant). HE stains, scale bar 500µm, 100X.

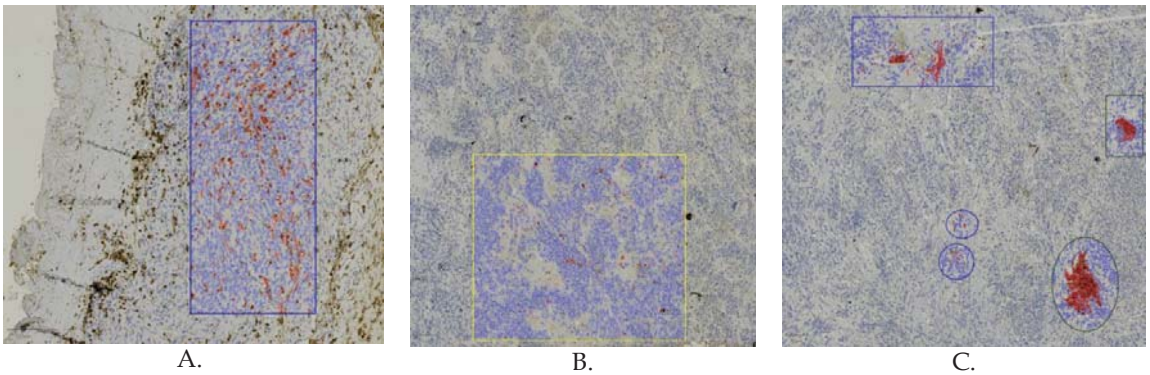
IHC stains showed a proliferation index with Ki67 marking tumoral cells between 1% and 65% with an average 19%. The epithelioid cell type EGISTs comported an average Ki67 of 28%. The mixed types comported a slightly lower value of 26% activated tumor cells. The spindle cell variant comported the lowest ki67, with an average value of 14%.

IHC staining with CD5 CD20 and CD45 clearly delimited the TLS from lose or compact aggregates of lymphoid cells. Their numbers per sample was recorded with an average of 2,41 foci / sample (varying from 0 to 8 with a median value of 2 TLS), see figure 2 and 3.



A. B. C.

Figure 2. Immunohistochemical detection of TIL. A. Spindle cells type EGIST exhibiting variable distribution of intra-tumoral leucocytes detected with CD45 (T and B lineage TIL as well as TAM (green arrow). Intravascular lymphocytes were avoided be selecting an appropriate region of interests (dark blue arrowhead left bottom corner). B. Picture is highlighting a compact aggregate of forming a true TLS (orange arrow). Distribution of isolated intra- tumoral leucocytes is relative diffuse and homogenous. Detection with anti CD45 antibody. C. Immunohistochemical detection of B lineage TIL using CD20antibody lights a few isolated cells as well as two compact TLS (green arrow heads) difficult to consider due to numerous overlaps. All stains were made with DAB, counterstain Hematoxylin. All photographs correspond to screen snapshoots exported from QuPath and contains a scale bar of 100µm located at bottom-left corner.



A. B. C.

Figure 3. Digital image analysis of TIL. A. Periphery of a cystically degenerated EGIST. Immunohistochemical detection of T lineage TIL using CD05 antibody. Region of interest marked by blue border rectangle. In translucent red are colored positive cell detection (with brown background as control). Tumoral cell marked as light blue cell shapes. Periphery of the sample contains lose clusters of T-cell TIL , overlapping each other making difficult to estimate. B. Central region of spindle cell type EGIST. Region of interest marked by yellow border rectangle. In translucent red are colored positive cell detected with CD05 antibody. Notably in this case it was a homogenous distribution and low density of T-cells TIL compared to case presented in figure 3A. C. Immunohistochemical detection of B-cell lineage TIL in the same EGIST case as in figure 2C. Positive B cell detected by QuPath software are marked in red. Regions of interest ellipse and rectangle marked with blue borders while the discarded All stains were made with DAB, counterstain Hematoxylin. All photographs correspond to screen snapshoots exported from Qu-Path and contains a scale bar of 100μm located at bottom-left corner

Image data analysis showed a predominance of T cell lineage TIL versus the B cell lineage. In average, the positive CD5 was 1,6*103 cells/mm (range 0,148-6,5*103/mm2; a trimmed average exclusion of 5% extreme values was 1,4*103/mm2). TIL positive for CD20 showed an average density of 263 B cells / mm2 (range 0,04-2,6*103/mm2; a trimmed average with exclusion of 5% extreme values was 153 B cells/mm2). TAM counting using nuclear size restriction parameters and CD45 detection did not yielded confident results.

Statistical analysis of correlation between the tumor type and number of T cell lineage and the other TIL revealed a p higher than the proposed α. Pearson correlation between number of T and B cell TIL with r=-0,156; t=0,674 and a p =0,5077. Same correlation coefficient was calculated to test the association between type of TIL and the ki67 values showing a r =0,096 with t=0,41; and a p =0,68 for T cell TIL density. The B cell density associated with the proliferative index has r=-0,268; t=1,14; and a p value of 0,2675 in our EGIST series.

The Kruskal–Wallis test to compare difference between histological variants of EGIST with T cell type TIL density resulted in H=0,00485 with a p=0,99 (two tailed test, df=2). Same test to compare the rank differences in B cell type TIL density and the three histological variants of EGIST, presented a H= 1.099 with p=0,577; see figure 4.

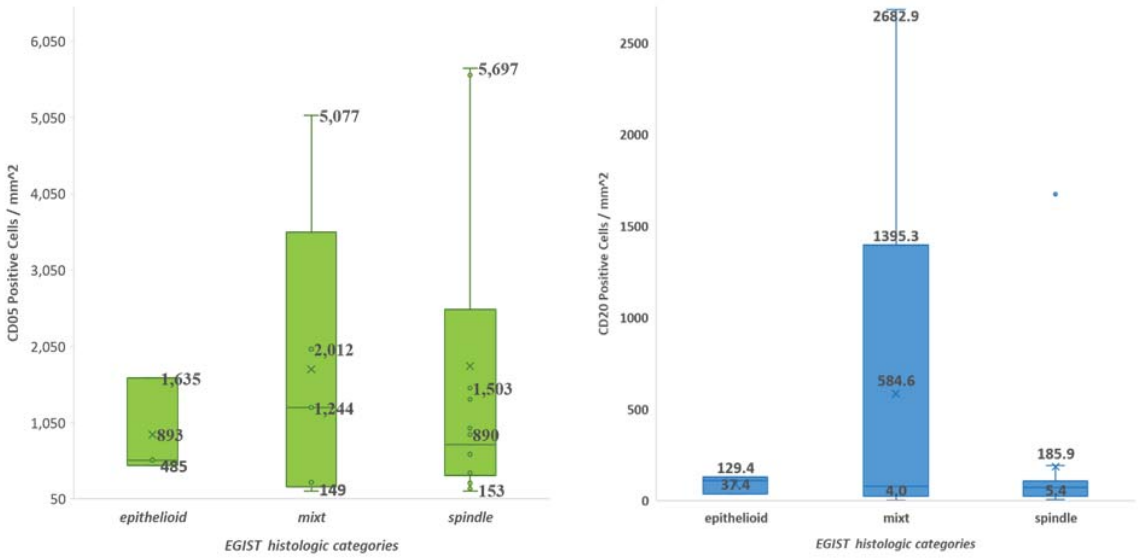


Figure 4. Graphical representation of the number of tumor T and B lymphocytes in the EGIST case series. Left panel: T lymphocyte (positive for CD05) count per square millimeter by histological categories. Values with an order of magnitude ranges from hundreds to a maximum 5,7 thousand. Further testing did not corelate the parameter with the histological category (see details in text). Right panel: Number of B lymphocytes (CD20 positive) per square mm, by EGIST histological categories. Inhomogeneous distribution with values spanning between tens and maximum 2.7 thousand, much lower than T lineage lymphocytes

DISCUSSIONS

The term GIST was first attributed by Mazur et Clark [5] in 1983 for tumors arising in the gastrointestinal tract that on electron microscopy showed both aspects of smooth muscle and nerve cell-like organelles. EGIST was documented as the localization of this type of neoplastic cells outside the digestive tract. It has been proven on small series that they do behave more aggressively that their counterpart in the digestive tract but share a common immunophenotype as well the same molecular defects.

Like other neoplasms, these sarcomas have a microenvironment with a varied composition. The tumor microenvironment contains three major structural components: microvessel (lymphatic and blood neoformation) tumor stroma, and host immune cells. To these, are added histological structures of the body that are incorporated by the neoplastic process as well as various precursor cells that take part in the process of tissue repair and construction (fibroblasts, myofibroblasts, etc.). Various cytokines and inflammatory factors along with various substances with a hormonal role in promoting and developing the neoplastic process are considered part of the tumor microenvironment. In recent years, the therapeutic advancement of immunotherapy has led to extensive morphological, proteomic, and genomic investigation of the entire tumor environment. From this point of view, sarcomas are difficult to investigate due to the low number of annual cases and the high number of histological types. While GIST is the most common sarcoma of digestive tract, the EGSIT remains an elusive target. The tumor infiltrating leucocytes contain both B and T cell lineages lymphocytes, as well as several macrophages recruited from circulating monocytes. Other inflammatory events that take part in the tumor development (cystic degeneration, necrosis, etc.) contribute to the tumoral environment, in addition to neutrophils and other myeloid derived leukocytes.

The mini-series of 22 EGIST cases that we selected in our study, showed a high density of T cell TIL compared to the B lineage by a factor of 6. Our initial hypothesis that T cell lineage TIL number and subsequent their density, could be correlated to the rate of

multiplication of the tumor and/or its histological variant showed a p>0,005 that could not reject the null hypothesis.

T cell lymphocytes are ubiquitous in all neoplasia. They play a central role in adaptive immune response and are the newest of the CART cell therapies. As a rule, their apparent role is to limit the tumor development as their presence results in better outcomes and prognosis correlating with increased amounts of T cells. This was shown in numerous studies for melanoma, breast cancer colorectal carcinoma, as well as for sarcomas. The immune-mediated cell death response in GIST was associated with presence of CD8 positive cells T cell, so called cytotoxic types. Their relationship with tumoral associated macrophages (TAM) is incompletely understood as their activity is normally enhanced by activated macrophages. In the context of GIST, some studies suggested that administrations of anti- CD40 or PDL 1 ligands activates TAM to an M1 form. The activated TAM increase secretion of diverse lymphokines that stimulates the cytotoxic activity of TIL. Furthermore the benefits of imatinib therapy is partially explained by it's indirect effect on decreasing tumoral expression of the indoleamine 2,3-dioxygenase in vitro models [6]– [8].

Using digital image analysis our investigations confirmed the general trend of a low B cell lineage TIL in EGIST. Their presence was scarce, leading to an apparent homogenous distribution. In our study we noticed they were more abundant intratumoral in the form of small aggregates around capillaries. Occasionally, intravascular spots were prominent, possibly as a result of a vascular marginalization and adhesion due surgical maneuvers. Their leukodiapedesis ability trending to form local TLS, has to be revealed by further studies. It is well accepted that B cell lymphocytes are the principal actors in humoral immune response. CD20 marks a plethora of B-cell types, excepting the very immature forms and highly differentiated plasma cells. In neoplasia development, their role remains elusive, an in general they are reduced in number compared to T cell lineage and located predominantly inside TLS. In neoplasia, it seems to fulfil the role of ambivalent agents, acting as regulators and as promoters of antitumoral activity. Their function as direct cytotoxic actor as well as stimulating T cell to secrete diverse cytokines was investigated in several studies [2], [9]. On the other hand, suppressive effects were noted when acting as B cell regulators, interfering with the functions of TAM and T cell TIL [3]:

In our study we tried to assess the presence on TAM. We have applied following technical setups to identify their presence. First, we setup the cell detector procedure to identify cells with a surface area bigger than 250 μm2, a nucleus dimeter bigger than 10μ, and membranous staining for CD45. These lead to some positive results, but the number of cells selected in these setups showed to be higher than the number of cells obtained after subtracting total TIL from the sum of fully positive cells with a cell diameter bigger or equal to 8 μm. This inadvertence can be partially explained by the difference between the section plans. Their presence in the context of neoplasia has been associated with poor prognosis [10]. In the case of the neoplasia, they will transform from attracted monocytes into TAM. Theoretically they can serve as a backbone for presenting tumoral antigens and triggering the immune response. Due to local secreted factors (mainly cytokines like IL6 IL8 IL10 as well as PDL1) in solid tumors their active role is suppressed [11], [12]. Acting like inhibitor cells that suppress the potential response of T cell has been shown in various studies [13], [14]. In GIST, the macrophages were investigated as potential targets for therapies, and they are characterized as the second most prominent immune cell population after the T cells [15], [16]. Till now, there are no studies that investigated their prevalence in EGIST.

In our pursuit to quantify the tertiary lymphoid structures (TLS) using digital analysis of slide images we faced the first impediment to distinguish them from lymphoid aggregates. Lack of cellular morphology and zonal distribution of antigen-specific B and T lymphocytes, facilitated their classification. TLS are found in solid tumors a microenvironment reaches in

inflammatory factors and cells. Their presence is associated with better prognosis and a better response to immune therapies in melanoma, breast carcinoma and sarcomas including GIST [17]– [19].

From our perspective, the study of TIL using the digital slides and corresponding software is the best approach in reporting immune components, as well as for gathering information about the immunophenotype status in the immune check points. The study is however limited in these aspects due to lack of multiplex IHC and subclassification of the TIL. Another drawback and time-consuming event were the identification of small foci of interstitial hemorrhages as they were prone to DAB precipitations leading to false positive identifications of TIL by the software. Very small amounts of individual red blood cells found in the interstitial spaces where more easily identifiable in visceral localization than in (pseudo)cystic degenerated structural parts of EGISTs. This was address by increasing the amount of saturation in the blue channel hist, searching for the presence of a central nucleus (blue), as well as revisiting manually each portion or region of interest on the physical slide by pathologists. Despite this, similarities with other studies and past experience of the authors recommends these method over the use of less accurate expression like "brisk" and "non-brisk TIL" [20]– [22].

CONCLUSIONS

In our study we were able to highlight the variable morphological immunophenotype spectrum of TIL EGISTs. The main cellular actor in the immune response to EGIST presence are T-cell TIL. The TIL and TLS disposition is inhomogeneous in EGIST. We underlined the potential of TIL density as a histological parameter independent from morphological variant as well as from proliferative rate in our EGIST.

The benefits of digital image analysis of TIL is enormous facilitating regions of samples to be inspected and supervised. Regardless of that, multiplexing methods are further necessary as they can precisely identified and localize the main immune cell actors in this act, leaving to proteomics tests the role to describe scenery.

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