„CAROL DAVILA", UNIVERSITY OF MEDICINE AND PHARMACY, BUCHAREST
DOCTORAL SCHOOL
MEDICINE

The study of malignancy and prognostic factors in gastrointestinal stromal tumors

SUMMARY OF THE DOCTORAL THESIS

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2022

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LIST OF ARTICLES PUBLISHED IN SPECIALTY JOURNALS:

1. **Rosulescu A**, Costache M, Dumitru A, Sajin M. Expression of PD-L1 and immune cells in gastrointestinal stromal tumours. Arch Balk Med Union. 2021; 56(3):284-290. https://doi.org/10.31688/ABMU.2021.56.3.01
2. **Roşulescu A**, Pechianu N C, Hortopan M, Mihai M, Dima S O, Stroescu C, Zamfir R, Brașoveanu V, Leonard D, Vasilescu C, Popescu I, Herlea V. Gastrointestinal stromal tumors of the colon and rectum. Polish Journal of Pathology. 2020; 71(3):200-206. https://doi.org/10.5114/pjp.2020.99786
3. **Roşulescu A**, Croitoru A, Iorgescu A, Becheanu G, Procop A, Tomulescu V, Croitoru V, Herlea V. Mutational Analysis in Gastrointestinal Stromal Tumors - A Series of Three Cases. Surg. Gastroenterol. Oncol. 2020; 25(4):213-220. https://www.sgo-iasgo.com/pdfs/2020-4-213.pdf

Co-autor:

4. Herlea V, **Roșulescu A**, Calotă VC, Croitoru V, Stoica Mustafa E, Vasilescu C, Alexandrescu S, Dumitrașcu T, Popescu I, Dima S O, Sajin M. Combined Positive Score for Programmed Death Ligand-1 Expression and Inflammatory Microenvironment in Gastrointestinal Stromal Tumors. Medicina. 2022; 58 (2):174. https://doi.org/10.3390/medicina58020174
5. Herlea V, **Rosulescu A**, Iorgescu A, Dima SO, Dumitrascu T, Brasoveanu V, Stroescu C, Vasilescu C, Popescu I. The Immune Response in Gastrointestinal Stromal Tumors. J Cancer Res Immunooncol. 2020; 6:125. https://www.longdom.org/open-access/the-immune-response-in-gastrointestinal-stromal-tumors.pdf

1. INTRODUCTION

Gastrointestinal stromal tumors (GIST) are the most common subtype of mesenchimal tumors located at the digestive tract level, originating in interstitial cells of Cajal. They represent approximately 1% of the total malignant tumors with this location and have a variable aggressivity and evolution [1].

The incidence of GIST is between 4 and 12 per 1 million persons, from the available studies, varying according to the geographic area. The highest rate is registered in patients between 60-65 years old, although they can affect any age [2].

The pathogenesis, in the majority of cases, is determined by activating mutations of KIT tyrosin-kinase, mostly in four exons: 11 (most frequently), 9 (associated with a decreased sensitivity for imatinib), 13 and 17. PDGFRA belongs to the class of tyrosine-kinases, and mutations at this level are mutually exclusive with kit in GIST. These mutations are identified in 10-15% of cases and affect predominantly the exons 18, 12 and 14. Subsequently, different types of mutations were discovered like those in NF1, BRAF, KRAS, SDH etc. The genotyping of GIST is important from the therapeutical point of view, different types of mutations showing a higher or lesser sensitivity to tyrosine-kinase inhibitors (TKI) and also a different clinical evolution [3-4].

This tumor type can be located at every level of the digestive tract, from the esophagus to the rectum, the stomach and the small bowel being the most common sites of involvement. There are cases located outside the gastro-intestinal tract, most frequently in the mesentery, omentum (80% of cases), retroperitoneum, etc. This sort of tumors were called EGISTs (extragastrointestinal stromal tumors) [5-6].

From the clinical point of view, the manifestations are very different, reflecting the location and dimension of the tumor, most signs and symptoms being related with digestive hemorrhage. In some cases the tumors are asymptomatic and discovered incidentally, during imagistic evaluation for other afflictions [7].

Macroscopicallly, gastrointestinal stromal tumors have variable dimensions, from less than 1 cm, to diameters larger than 20 cm. Usually, they are circumscribed tumors, surrounded occasionally by a pseudocapsule. Tumors can develop inside the lumen, being covered by an ulcerated mucosa in some instances, can be intramural or subserous. On the

cut sections, the colour can vary from whitish-grey to red-brown, depending on the vascularisation and the presence of haemorrhage. The aspect can be homogeneous or the tumor can vary in colour and consistency. Most of the times, it is solid, of elastic consistency, with cystic and necrotic areas. Large tumors can have central cystic degeneration, developing a pseudodiverticular aspect, the center of the tumor communicating with the intestinal lumen through a fistula. In some cases, the tumor can be hourglass-shaped, with both an extrinsic and an intraluminal component [8].

From the histopathological point of view, gastrointestinal stromal tumor are extremely heterogeneous, with a wide morphological spectrum. They can be composed of spindle cells (70% of cases), epithelioid cells (20%) or they can have a mixed pattern (10%). Features like pleomorphism, hypercellularity, sclero-hyalinisation, nuclear palisading, tumor necrosis, calcifications, cytoplasmic vacuolations, skenoid fibers etc, can characterise these tumors. Mitotic rate can have different values, tumors with <5/50 HPF having a better prognosis [1,9].

The microscopic differential diagnosis of gastrointestinal stromal tumors is made with other, mostly mesenchymal tumors, with digestive location. Among these are leiomyomas, leiomyosacomas, schwannomas, inflammatory fibroid polyps, inflammatory myofibroblastic tumors, desmoid fibromatosis, melanomas etc [10].

Immunohistochemically, the great majority of GIST are diffusely and intensely positive for CD117 (KIT), that is usually expressed in interstitial cells of Cajal, the cells of origin for these tumors. DOG1 has a comparable sensitivity and specificity with the previous marker, the pathologic role of the latter being incompletely elucidated. CD34 is positive in most gastric GISTs, but negative in most other locations. A significant percentage of cases can express smooth muscle markers (SMA, desmin, caldesmon) or neural markers (S100). Ki67 is used as an index of cellular proliferation, high values for it indicating a recurrence risk [11, 12].

A series of congenital syndromes associate GIST. Among these are Neurofibromatosis type I, Carney Triad (GIST, extra-adrenal paraganglioma and pulmonary chondroma), Carney-Stratakis Syndrome (GIST and paragangliomas) [13].

Regarding risk stratification, there are several classification systems. One is NIH (National Institutes of Health), developed by Fletcher et al in 2002, that classifies patients with GIST in four groups of risk: very low, low, intermediate and high, taking into account tumor dimension and mitotic rate. In 2006, Miettinen et al have proposed the classification system known as AFIP (Armed Forces Institute of Pathology) that adds tumor location, after

they found that gastric tumors have a lower recurrence risk by comparison with other locations, for the same dimension and mitotic rate. Consequently, 8 prognostic groups result (1, 2, 3a, 3b, 4, 5, 6a and 6b). In 2008, Joensuu suggests the revised NIH criteria, that additionally takes into account tumor rupture as a negative prognostic factor [14-17]. Mutational status also represents a prognostic factor. A better outcome is associated with exon 12 PDGFRA, exon 11 KIT and BRAF mutations. An adverse prognosis is associated with exon 18 PDGFRA and exon 9 KIT mutations [18]. According to some authors, the proliferation index Ki67 can be considered in determining the malignant potential in GIST [19].

In GIST treatment, a multidisciplinary approach is mandatory and includes the gastroenterologist, the radiologist, the surgeon, the pathologist and the oncologist, depending on the case [20]. The surgical treatment is potentially curable in the case of complete resection, with negative margins for neoplastic cells and without tumor rupture [21]. In high risk tumors, the adjuvant treatment with tyrosin-kinase inhibitors is indicated, first line being represented by Imatinib and is associated with an increase in overall survival and progression-free survival [22]. For imatinib resistant cases, therapeutic alternatives are represented by Sunitinib and Regorafenib [23, 24].

Tumorigenesis, in GIST, as in general, is a complex dynamic process, tumor microenvironment being closely correlated with neoplastic cells during tumor development [25]. Immune system has the role of monitoring and ensuring the maintenance the homeostasis by eliminating the cells with defects. Is a fact well known that tumors express antigens that can induce an immune response through the protein products they synthesize, which are perceived as non-self by the immune system. Three phases were described during the complex interactions between tumor cells and immune system: *elimination* – when tumor cells are destroyed, *equilibrium* – when immune cells antitumor and immunosuppressive functions are in balance and *escape*, when the tumor begins to progressively grow, becoming clinically apparent and tumor microenvironment is dominated by immunosuppression [26]. The intratumoral inflammatory infiltrate is variable from tumor to tumor from both the qualitative and quantitative point of view, but undoubtedly it is trying to counteract tumor progression through the so called immune surveillance. Numerous studies have correlated the intratumoral immune infiltrate with a better prognosis and a prolonged survival [27].

Studies regarding the intratumoral immune infiltrate are limited, showing that immune system is active in the tumor microclimate and enhances the effects of imatinib treatment. It was proved that the macrophages are the most frequent encountered subtype, followed by

CD3+ lymphocytes and also that macrophages are in a higher quantity in metastases compared with primary tumors. Also, the number of macrophages is superior in epithelioid subtype, in large tumors and in those with high mitotic rate [28-30]. Closely related with intratumoral inflammatory infiltrate is the expression of "check-point" molecules. Among these is PD-L1, present on the surface of neoplastic cells, a ligand for PD-1, expressed on the surface of activated cytotoxic T cells. The PD-1-PD-L1 interaction is followed by a decrease in the immune attack against tumor cells and so they are not destroyed. In GIST, PD-L1 expression was proven to be an adverse prognostic factor. On the other hand, an advantage could reside in the therapeutic approach of PD1-PD-L1 pathway, immune therapy associated with conventional treatment potentially improving the survival of patients with GIST, in selected cases. Still, in present times, this subject is only a hypothesis that needs to be verified in clinical studies [31].

All these characteristics bring out the complexity of GISTs and the importance of knowing the prognostic factors or those that can be approached therapeutically so that patient management would be as adequate and adapted to the particularities of the case as possible.

This current research approaches the before mentioned problem by studying the demographic, clinical, histological and immunohistochemical aspects of GIST and also by analysing the relationship between tumor cells and immune microenvironment, in particular PD-L1 expression on neoplastic cells and the characteristics of intratumoral inflammatory infiltrate. The first section of this doctoral study – The general part – is meant to present the theoretical notions that highlight what is known at the present time and the importance of studying more thoroughly the selected subject. The second section – The special part – brings to attention the studies that were conducted, with specific objectives and results. In the end the conclusions were stated. The results of this scientific research are meant to contribute to a better understanding of the clinical-pathological and immunohistochemical characteristics of GISTs, of the antitumor immune response for this entity and of the prognostic impact of the variables that were analyzed.

2. THE WORK HYPOTHESIS AND THE GENERAL OBJECTIVES OF THE RESEARCH

The aim of this doctoral research is to perform a detailed analysis of the gastrointestinal stromal tumors by investigating their clinical, morphological and immunohistochemical characteristics for a better understanding of prognostic significance of these variables. Also, this paper aims to draw attention to the role of tumor microenvironment on the disease dynamics by studying the intratumoral immune infiltrate and PD-L1 expression in tumor cells, followed by evaluation of the correlations between these and the prognostic factors.

The general objectives of the study include:

1. The analysis of the general characteristics, demographic and clinical, of the gastrointestinal stromal tumors – where will be detailed patients characteristics as gender, age, living environment, associated diseases/personal pathological history, signs and symptoms at presentation, and also tumor characteristics that influence the clinical picture like tumor location, primary/secondary nature of the tumor, uni-/multifocality, tumor maximal diameter. Also, will be evaluated the possible associations and correlations between these variables and the impact they have on survival.
2. The analysis of the morphological characteristics – where will be reported the most common gross features and will be detailed the microscopic aspects (tumor cell type, mitotic rate, the presence of tumor necrosis, ulceration, cellular pleomorphism and morphological particularities as cytoplasmic vacuolations, skenoid fibers, nuclear palisading), with representative pictures. Also, the existence of associations and correlations with other clinical-pathological and prognostic variables will be tested, as well as the way they influence the survival.
3. Assigning prognostic groups and risk classes to individual cases, according to diagrams proposed by AFIP and NIH, by considering tumor dimension, mitotic rate and location, with a thorough analysis of subgroups and search for associations and correlations with other clinical-pathological variables and the comparison of survival between categories.
4. The study of immunohistochemical expression of routinely used markers for positive diagnosis (CD117, DOG1, CD34) and differential diagnosis (SMA, S100) and also the proliferation index Ki67. Subsequently, the correlations between the IHC expression and other clinical-pathological and morphological variables will be tested and also will be evaluated the survival.

5. The morphological and immunohistochemical study of the intratumoral inflammatory infiltrate with the description of the pattern and distribution in variable tumor categories, and emphasising the immune profile of the cells (CD3+, CD4+, CD8+, CD20+, CD68+) and quantifying these with a specialised software. Consequently, the correlations and associations of this data with clinical-pathological, morphological, immunohistochemical features, prognostic and survival, will be tested.
6. The study of PD-L1 expression in tumor cells by immunohistochemical testing, quantification of positivity and determination of the relationship with intratumoral inflammatory infiltrate and other clinical-pathological, morphological, immunohistochemical and prognostic variables and also the influence PD-L1 expression has on survival.

3. GENERAL RESEARCH METHODOLOGY

This doctoral study is based on the retrospective analysis of cases of gastrointestinal stromal tumors diagnosed in two major university hospitals in Bucharest: Bucharest University Emergency Hospital and Fundeni Clinical Institute, over a period of 4 years (2015-2018), treated in the surgery departments of the mentioned hospitals and histopathologically diagnosed in the departments of Pathology of the same institutions. The cohort consisted of 96 cases. From the accompanying papers of the biological products, histopathological records and internal hospital databases, were extracted the main clinical data (age, sex, living environment, anatomical location of the tumors, date of diagnosis), macro- and microscopic aspects of the tumors, histopathological diagnosis, immunohistochemical expression, prognostic group and the risk of disease progression, information that was gathered to create a working database.

Microscopic analysis was performed using an optical microscope, following details such as cell type, mitotic rate, cyto-nuclear pleomorphism, presence of necrosis, ulceration, cytoplasmic vacuolations, skenoid fibers and nuclear palisading, intratumoral inflammatory infiltrate. At the same time, representative photos were taken with the cameras connected to the microscopes.

Immunohistochemical analysis was required primarily to confirm the diagnosis of GIST, and finally, all cases included in the study had this confirmation, by the positivity of CD117 and / or DOG1. Where tumor particularities required additional tests, supplementary markers were used for differential diagnosis (SMA, S100, CD34, Desmin, AE1 / AE3, etc.).

From this main group, a secondary group was selected consisting of 90 cases of GIST for which morphological and immunohistochemical analysis of intratumoral inflammatory infiltrate was performed, and a group of 89 cases for which PD-L1 expression was tested in the tumor cells. After all the tests were performed, the database was completed and represented the basis for the statistical analysis.

Inclusion criteria:

- The cases registered in the Pathology Departments of the Bucharest University Emergency Hospital and of the Fundeni Clinical Institute
- Cases diagnosed histopathologically as gastrointestinal stromal tumors.
- Cases with immunohistochemical confirmation of histopathological diagnosis

Exclusion criteria:

- Cases registered outside the period 2015-2018
- Cases in which essential clinical-pathological information was not accessible
- Cases in which the histopathological diagnosis has not been confirmed immunohistochemically

For statistical analysis, the variables of the studied sample were collected in a database in the Microsoft Excel 2016 program. The statistical analysis was performed in the IBM program SPSS Statistics 20. The Chi-Square test, the corresponding corrections when the criteria were not met (Likelihood ratio, Fisher test) and the Phi and Cramer V parameters were used to check the size of the effect; the t-independent test, with respect to the degree of freedom, the difference in averages, the Levene test for homogeneity testing, the Kolmogorov-Smirnov and Shapiro-Wilk normality tests for subgroups under 30 respondents. The confidence interval has been set to 95%. For the non-parametric Mann-Whitney U and Kruskal-Wallis non-parametric test, the normality test conditions were not used. The Pearson r test was used to test the correlations. The results were presented numerically and graphically. Kaplan-Meier curves for categorical variables and Cox regression for continuous variables were performed for the survival study.

4. SPECIAL PART - SYNTHESIS OF CHAPTERS

4.1.Study I: Clinical and epidemiological study of gastrointestinal stromal tumors

The present study (elaborated in chapter 7 of the doctoral thesis) aimed to analyze in detail the demographic and clinical features of GISTs in order to better understand the prognostic implications of these variables. Thus, the age and the sex of the patients, the living environment, the signs and symptoms, the pathological history of the patient, the uni-multifocality of the tumor, the primary/ secondary character of the tumor, the location and size of the tumor were evaluated. We also tested the existence of correlations or associations between variables as well as how they influence survival. The results were compared with those in the literature.

The study was conducted on a cohort of 96 patients diagnosed with GIST. They ranged in age from 28 to 78 years, with an average age of 58.2 years. The number of cases was slightly increased in favor of males (52.1% men versus 47.9% women). In females there were ages between 31 and 73 years, with an average of 57.3 years, and in males the ages were between 28 and 78 years, with an average of 58.9 years. Most patients came from urban areas (69.8%). The most common signs and symptoms in the cohort were those associated with gastrointestinal bleeding (39.5%).

In the category of primary tumors, the highest number of cases was registered at the gastric level (N = 50), followed by the small intestine (N = 18), the extragastrointestinal location (N = 9), the colon (N = 2) and the rectum (N = 2). The group also included 7 cases of tumor recurrences and 8 metastases. The dimensions of the gastrointestinal stromal tumors from the studied group were between 0.5 and 21 cm, with an average of 6,953 cm.

Most tumors had a unifocal character, more often those of the digestive tract, multifocality often characterizing secondary determinations and EGISTs. In addition, multifocal tumors were larger than unifocal tumors (mean 9.18 cm versus 6.44 cm). There were no other significant associations and correlations between the analyzed variables and these were not shown to have an impact on survival in the studied group.

4.2.Study II: The morphological study of gastrointestinal stromal tumors

The variability of GISTs is not only found in terms of clinical behaviour and disease progression, but also in terms of morphology. In this context, the present study (elaborated in chapter 8 of the doctoral thesis) aimed to analyze in detail the macro- and microscopic aspects of gastrointestinal stromal tumors belonging to the selected group, to assess the morphological features, to ascertain their frequency and to test the existence of associations and correlations between them and other clinical-pathological variables, as well as the impact on survival. The results were subsequently compared with the existing data in the literature, mostly being consistent with them. The present study included a total of 96 patients diagnosed with GIST.

Macroscopically, most GISTs in the digestive tract have been described as nodular, circumscribed, intramural tumors with submucosal localization, extending under the serosa, and even invading surrounding organs. Recurrences, metastases, and EGISTs have been described as nodular tumors. Particular aspects were represented by cystic areas, hemorrhage, necrosis, abscesses and ulceration.

Microscopically, GISTs belonging to the studied group consisted, in the vast majority of cases, of spindle-shaped cells (65.6%), followed by the mixed cellularity (28.1%), and on the last place the epithelioid cell type (6.3 %). Tumor cell type was significantly associated with tumor size, mixed cell tumors having larger dimensions. The presence of cytoplasmic vacuolations is more common in primary, unifocal, gastric tumors and less frequently in secondary determinations. Nuclear palisading was recorded in 13.5% of the total studied group with a higher frequency in gastric tumors, followed by those of small intestine and EGIST. The presence of skenoid fibers was found in 7.3% of all cases in the studied group in gastric and small intestine tumors.

Marked cyto-nuclear pleomorphism was found with a higher frequency in EGISTs (among primary tumors), and more often in secondary tumors (recurrences and metastases) compared to primary tumors. Marked pleomorphism was also more common in large tumors.

Intratumoral necrosis was often found in the cases of the studied group (45.8%), both in primary tumors, regardless of location, and in secondary tumors, more frequently in large tumors. Ulceration was also more common in larger tumors. An increased mitotic rate (> 5 mitoses / 50HPF) was more common in secondary determinations (recurrences + metastases) compared to primary tumors. The analyzed variables were found not to significantly influence survival in the studied group.

4.3.Study III: The study of prognostic groups and the risk of disease progression

Gastrointestinal stromal tumors have a broad biological spectrum, from small, benign tumors to fatal sarcomas [1]. The role of risk stratification is to assess the possibility of an unfavourable evolution and to select patients who could benefit from adjuvant treatment. The prognostic classification of gastrointestinal stromal tumors is currently based on features such as anatomical location, tumor size, mitotic rate, tumor rupture, and mutational status [32]. However, this is not ideal, as the role of other tumor variables is not fully elucidated. The most widely used prognostic systems today are AFIP and revised NIH criteria, which delimit prognostic groups, respectively risk classes, corroborating the location, size and number of mitoses / 50HPF [15-17]. The present study (elaborated in chapter 9 of the doctoral thesis) was performed on a batch of 81 primary tumors, aiming to stratify the risk using both classification systems and to assess the existence of associations and correlations with other clinical-pathological variables, in an attempt to deduce a possible prognostic role of the latter. The impact of prognostic groups and risk of disease progression on survival was also tested.

Regarding the division into prognostic groups, according to Miettinen / AFIP, the studied group included cases belonging to all 8 classes (1, 2, 3a, 3b, 4, 5, 6a, 6b), most representatives being included in group 2. Morphologically, the mixed cell type was significantly associated with a higher prognosis group. Tumors with marked pleomorphism were also associated with high prognostic groups; likewise, tumors characterised by necrosis and ulceration.

Following the division of primary tumors into risk progression classes, according to the revised NIH criteria, the studied group had representatives in all 4 categories (very low, low, intermediate and high risk), dominating the low risk. Morphologically, tumors with spindle-shaped tumor cells were associated with a lower risk of disease progression, while mixed cellularity was associated with a higher risk of disease. Moderate and high cyto-nuclear pleomorphism has been associated with an increased risk. The presence of necrosis was more common in high-risk tumors, as well as tumor ulceration. Multifocal tumors were significantly more frequently associated with high risk than unifocal tumors.

4.4.Study IV: The immunohistochemical study of gastrointestinal stromal tumors

Due to morphological heterogeneity, immunohistochemical confirmation is mandatory in gastrointestinal stromal tumors. For the positive diagnosis of GIST in current practice, CD117 and DOG1 are used, markers with high sensitivity and specificity and also CD34, with high sensitivity but low specificity. To rule out other differential diagnoses, other markers are often included in the panel as S100 - positive in tumors of nerve sheath origin and SMA - which highlights smooth muscle differentiation. Ki67 proliferation index may have a broad spectrum of values in this tumor entity, with an incompletely elucidated prognostic impact [33].

In the present study (elaborated in chapter 10 of the doctoral thesis), there were no statistically significant differences between CD117-positive and CD117-negative tumors in terms of morphopathological features, except for cyto-nuclear pleomorphism, which was predominantly moderate in CD117 + cases. The positive SMA expression was more common in males. SMA negative tumors had more often large dimensions. Also, the risk of disease progression was significantly different in relation with the expression of SMA, with positive SMA cases being more frequently low risk. S100 expression did not show statistically significant associations with other prognostic variables.

Regarding the value of the Ki67 proliferation index, it was significantly higher in secondary determinations, in large tumors, in association with epithelioid and mixed cell types, in tumors with marked pleomorphism. On the other hand, Ki67 had lower levels in the presence of morphological features such as cytoplasmic vacuolations and skenoid fibers. Ki67 has been associated with intratumoral necrosis, the latter being more frequently present in tumors with a high proliferation rate. The Ki 67 value was also significantly associated with the mitotic rate, at a rate above 5 mitoses / 50HPF the percentage of Ki67 being increased. In relation to the prognostic group and the risk of disease progression, the percentage of Ki67 increased in the same direction. Multifocal tumors had a higher proliferation index than unifocal tumors.

There where no statistically significant differences regarding survival between patients having GISTs with distinct immunohistochemical expression in the studied group.

4.5.Study V: The study of inflammatory infiltrate in gastrointestinal stromal tumors

The role of the inflammatory microenvironment is still incompletely elucidated in cancer. The antitumor immune response varies depending on the type of tumor but also on the particularities of the patient. Studies on this subject have found implications in determining the prognosis of the disease and in the development of immunotherapy [34].

In the present study(elaborated in chapter 11 of the doctoral thesis), the tumor-associated inflammatory infiltrate was analyzed in a total of 90 cases. In the first phase, the evaluation of the inflammatory cells was general, performed on the slides stained with the routinely used coloration: Hematoxylin and eosin. For this purpose, a representative slide was selected for each case and only the intratumoral inflammatory infiltrate was evaluated. Immunohistochemical tests were also performed on the corresponding block to determine the phenotype of inflammatory cells (CD3, CD4, CD8, CD20 and CD68). Qualitative analysis involved assessing the type of inflammatory cells (PMN, macrophages, lymphocytes), density and distribution pattern (diffuse, nodular, perivascular). For the quantitative analysis, 5 photographs were taken of each field with high magnification power (400X) on the same slide, with representative aspects of the case. Subsequently, inflammatory cells from the selected fields were counted using specialized image analysis software (ImageJ). For each case, an average number of immune cells was calculated (for all types of immune cells together, evaluated on H&E stain and for each subtype separately – immunohistochemically highlighted), the resulting number being assigned to the case.

The results of the qualitative analysis showed a variable distribution, either diffuse, with isolated and grouped cells, or in the form of aggregates that are predominantly perivascular and in the periphery of the tumor. In most cases, several patterns were found in the same tumor. The main type of immune cells identified was represented by lymphocytes, followed by histiocytes and less often by plasma cells, mast cells, eosinophils. Neutrophils have been found predominantly in the vicinity of ulcerated areas.

In the quantitative analysis, performed on the H&E stained slides, the number of inflammatory cells was variable and did not register statistically significant differences between various tumor locations, dimensional categories, mitotic rates, prognostic groups, risk classes, age categories, etc. The only statistically significant difference found was related to the patient's sex, with male patients having a higher amount of intratumoral immune cells.

Overall, the most common mononuclear inflammatory cell subtype was represented by T lymphocytes, followed by histiocytes, with B lymphocytes occupying the last place.

From the category of T lymphocytes, the CD4 + were in a higher quantity than the CD8 +. There were no statistically significant differences between the total number of CD3 + cells and other clinical-pathological and prognostic variables. On the other hand, the number of CD8 + T lymphocytes was significantly higher in ulcerated tumors compared to those covered by mucosa with no injuries and significantly lower in tumor recurrences. A CD4 / CD8 < 1 ratio was more common identified in tumors located in the small intestine and in those with SMA positivity, and a CD4 / CD8 >1 ratio was more commonly associated with a mitotic rate > 5 mitosis / 50 HPF and a larger tumor size.

Regarding the number of intratumoral B lymphocytes, it was lower in multifocal tumors, compared to unifocal tumors and also in tumor recurrences, unlike the other categories. The highest values of CD20 + cells were recorded in primary tumors, particularly in EGISTs. No other statistically significant associations were identified between the number of B lymphocytes and other morphological and prognostic variables.

The number of intratumoral histiocytes varied according to the size of the tumor, being fewer in small tumors, and more in those with increased diameter. CD68+ cells concentrations were statistically significantly higher at mitotic rate values exceeding 5 mitoses / 50HPF. This type of inflammatory cells was associated with the risk of disease progression, the highest amount of intratumoral histiocytes being recorded in high-risk tumors, and low amounts in low-risk tumors. Similarly, there were significant differences among the prognostic groups. Regarding the tumor cell morphology, the tumors with mixed cellularity had the highest degree of infiltration with histiocytes, at the opposite pole being those with fusiform cellularity. Tumors with marked and moderate pleomorphism were also significantly associated with more CD68 + cells compared with those with minimal pleomorphism. The presence of skenoid fibers in tumors has been associated with a significantly lower number of CD68 + cells. Related to the Ki67 proliferation index, intratumoral histiocytes had significantly lower values in Ki67≤5% tumors.

Regarding the relationship between the various categories of intratumoral immune cells, the titer of immune cells counted in the H&E stained sides was positively correlated with the values of CD3 +, CD20 + and CD8 +. Also, CD3 values correlated significantly, strongly and positively with CD4, CD20, CD8 values and CD4 values correlated significantly, strongly and positively with CD8 and CD68 values. The number of intratumoral immune cells did not significantly influence the duration of survival.

4.6.Study VI: The study of PD-L1 expression in gastrointestinal stromal tumors

PD-L1 is a checkpoint molecule and a ligand for PD-1. It is expressed both in immune cells (lymphocytes, macrophages, dendritic cells) and in non-immune, tumor cells. This molecule is involved in blocking the antitumor immune response and is associated with a negative prognosis in many cancers. PD-1-PD-L1 axis blockade may be a therapeutic strategy for PD-L1-expressing tumors. Testing for the presence of this marker has clinical utility, being approved in some cancer types to verify the response to treatment with PD-1 inhibitors [35-37].

In this study (elaborated in Chapter 12 of the doctoral thesis) the expression of PD-L1 in gastrointestinal stromal tumors was evaluated in relation to clinical-pathological variables, prognostic groups, risk of disease progression and intratumoral inflammatory infiltrate. In the cases of the studied group (89 cases), it was shown that the expression of PD-L1 is positively correlated with the age of the patients, this increasing with the advancing age of the patients. Among the morphological characteristics, it has been shown that nuclear palisading is significantly associated with increased PD-L1 values. Immunohistochemically, CD117 expression was significantly associated with PD-L1 expression. In relation to intratumoral inflammatory infiltrate, PD-L1 values were significantly correlated with the number of immune cells counted on the routinely used staining (H&E). Following the evaluation of immune cell subtypes in relation to PD-L1 expression, it was found that PD-L1 values correlate significantly with the number of CD3 +, CD4 +, CD8 +, CD20 + and CD68 + cells. The survival study did not show statistically significant differences between positive and negative PD-L1 tumors.

5. CONCLUSIONS AND PERSONAL CONTRIBUTIONS

The present doctoral research has achieved its aim and objectives proposed in the beginning, which focused on the characterization of gastrointestinal stromal tumors from a clinical, morphopathological, immunohistochemical point of view, the relationship with the inflammatory microenvironment and the identification of the prognostic role of the variables of interest. For this purpose, the existence of associations and correlations between various categories of variables and especially with the prognostic group and the risk of disease progression was tested. Thus, the mixed cell type was associated with a higher prognostic group. Also increased cyto-nuclear pleomorphism, the presence of necrosis and ulceration. Similarly, the same variables were associated with an increased risk of disease progression. In addition, tumor multifocality has been more frequently associated with high risk. From the immunohistochemical point of view, the Ki67 value increased in the same direction as the degree of the prognostic group, respectively the risk of disease progression.

An element of actuality nowadays was represented by the study of intratumoral inflammatory infiltrate, proving that immune cells are an important component of the tumor microenvironment in most cases. The dominant subtype was represented by T lymphocytes. The amount of histiocytes was associated with the prognostic group and the risk of disease progression, being higher in the superior groups and in the high risk classes. The other types of inflammatory cells have not been associated / correlated with current grading systems and have not been shown to influence survival.

Another matter of actuality was represented by the PD-L1 expression in tumor cells, which is an issue that opens the way for immunotherapy in various types of cancer. GISTs were immunohistochemically tested for this marker and have been shown to express it in varying amounts. Furthermore, it was proven that the level of expression correlates with the number of intratumoral immune cells, regardless of subtype. However, no association was identified between the PD-L1 value and the prognostic group / risk of disease progression and was not shown to influence survival.

None of the assessed variables were found to have statistically significant influence on survival, but these results should be interpreted with caution as the analysis was performed on a relatively small, heterogeneous population (primary and secondary tumors, different tumor locations), the retrospective nature of the study leading to a inevitable loss of data on the case.

The present doctoral study faced a series of limitations, on the one hand of material nature, not having access to genetic investigations, which would have completed the picture of tumors evaluated up to the molecular level, or exhaustive immunohistochemical testing. On the other hand, regarding the patients' history and clinical picture, the available information was not always complete and subsequently the follow-up was limited. The low incidence of gastrointestinal stromal tumors meant that the total number of cases that formed the investigated group was relatively low, although it took place over a period of 4 years and included patients diagnosed in two major university hospitals in Bucharest. Thus, it was not possible to obtain information of statistical significance for various tumor subcategories. The retrospective nature of the study inevitably led to data loss. Also, the observational character did not make it possible to highlight causal relationships between the analyzed variables, but only association trends.

Although considerable progress has been made in recent decades in understanding the biology of gastrointestinal stromal tumors and later in their treatment, with multiple options and therapeutic regimens available, there is still a subset of treatment-resistant cases. These cases require additional research aiming to discover treatment alternatives. Immunotherapy could be a solution, but studies on this topic are just at the beginning and in small number. Thus, the expression of PD-L1 and inflammatory infiltrate in GIST should be further investigated, in larger cohorts and supplemented with clinical trials. New directions of research could be oriented towards:

- PD-L1 expression in inflammatory cells
- development of a standardized system for the quantification of PD-L1 and other biomarkers and also for inflammatory cells
- response to PD-1 inhibitor treatment of PD-L1 positive tumors
- The significance of the inflammatory infiltrate at the edge of the invasion and the relationship with the intratumoral inflammatory infiltrate
- Other types of inflammatory cells in GIST (Natural killer, plasma cells, eosinophils, etc.)
- the contribution of artificial intelligence in the diagnosis of GIST

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