# TUMOR INFILTRATING LYMPHOCYTES PECULIARITIES IN DIFFERENT HISTOPATHOLOGICAL AND MOLECULAR SUBTYPES OF GASTRIC CARCINOMA

**Museridze N., Tutisani A., Chabradze G., Beridze N., Muzashvili T.**

*Tbilisi Medical Academy Named After P. Shotadze*

Gastric cancer represents the third major cause of cancer related mortality worldwide [1]. Two major histological subtypes of gastric carcinoma are so called enteric type carcinoma and diffuse type gastric carcinoma, the latter being more aggressive [2]. There have been many advances in treatment of gastric cancer. However, the prognosis of these patients remains poor [2]. Some studies indicate that treatment with immunotherapeutic agents may improve the prognosis of gastric cancer [3]. Therefore, the understanding of tumor microenvironment in different types of gastric cancers, represents the major research issue. Tumor infiltrating lymphocytes (TILs) has recently emerged as not only important prognostic factor in different types of solid cancers, but also as a good predictor of immunotherapy response [4]. Solid tumors, including gastric cancer can be classified into two major subgroups based on lymphocytic infiltration: so called "hot" tumors, with high number of TILs and "cold" tumors with the low number of TILs [5]. It has been shown that TILs, which are mainly represented by T lymphocytes and macrophages, are associated with tumor growth, progression and metastases[6]. However, the results of TILs studies in different tumors are conflicting. TILs in gastric carcinoma are not very well characterised. Also, there is limited information of the TILs distribution in different histological and molecular subtypes of gastric cancer. Even though there is a possibility to immunohistochemically characterise different types of TILs in tumors, it is not well known if this method provides any additional information to standard evaluation of TILs on H&E stained specimens. Moreover, immunophenotyping of TILs requires an extra work and additional tissue material, which is frequently limited in everyday diagnostic process. Therefore, researchers nowadays dedicate their effort to characterise the TILs based on the evaluation of H&E specimens in different types of solid tumors. Furthermore, some studies already have shown that TILs evaluation on H&E specimens harbour prognostic as well as predictive information [7]. In our current study we have characterised the distribution patterns of TILs in different histological and molecular subtypes of gastric carcinoma. Histological subtypes included enteric type and diffuse type gastric carcinoma and molecular subtyping was based on the immunoexpression of CDH1, Ki67, p53 and Her2.

**Material and methods.** Study included formalin-fixed and paraffin-embedded tissue material obtained from 50 patients at the Diagnostic, Research and Teaching Centre of Tbilisi State Medical University. Ten cases out of 50 were a control group of normal gastric tissue, 20 cases were diffuse gastric carcinoma and 20 cases – enteric type gastric carcinoma.

*Evaluation of tumor infiltrating lymphocytes (TILs).* TILs were evaluated in standard H&E stained specimens, based on semi quantitative method in three different areas of the lesion, including central part of the tumor, margins of the tumor and tumor adjacent stroma. The presence of TILs was categorised as follows: total absence of TILs, aka negative (0), minimal infiltration, including less than 10% of tumor area (1), moderate infiltration – TILs covering 10-50% of tumor area (2) and extensive infiltration – TILs covering >50% of tumor area (3).

*Immunohistochemistry.* 4μ FFPE tissue sections were deparaffinized in xylene, rehydrated by using serial dilutions of ethanol (96%, 80%, 70%), and heat mediated antigen retrieval has been performed. Ready-to-use antibodies against the following antigens were used: CDH1 (MCH-38, Invitrogen), Ki67 (EP5, Bio SB), p53 (DO-7, Leica) and Her2 (EP3, Bio SB). Staining and visualisation has been performed using BOND Polymer Refine Detection system. The number of positive cells was counted in 10HPF. Proliferation index was defined based on the ratio of Ki67 positive tumor cells to total number of tumor cells at 10HPF. Proliferation index >30% was considered as high and proliferation index ≤30% was considered as low. Her2 evaluation was based on Hofmann 4-tier scoring system, as following: membranous positivity is not detected – negative (0); weak membranous positivity in about 10% of cells – negative (1+); moderate membranous positivity in >10% of cells – borderline positivity (2+) and sharp membranous positivity in >10% of cells – positivity (3+).

Correlations were assessed using Pearson correlation and X2 test. Comparisons between groups were assessed using Kruskal-wallis test. P value <0.05 was considered as significant in all tests. All statistical analyses have been performed using SPSS V.19.0 software.

**Results and discussion.** All 10/10 (100%) cases of normal gastric mucosa showed a low number of TILs. In enteric carcinoma 4/10 (20%) of cases showed minimal TILs, 10/20 (50%) cases showed moderate TILs and 6/20 (30%) cases showed high number of TILs. In enteric type adenocarcinoma of the stomach 7/20 (35%) of cases showed low TILs, 5/20 (25%) of cases showed moderate TILs and 8/20 (40%) cases showed high number of TILs.

The analysis of the results of TILs distribution in different histological subtypes of gastric carcinoma showed, that low TILs are more characteristic to diffuse type gastric carcinoma compared to the enteric type, whilst moderate TILs are more frequently present in enteric carcinoma compared to diffuse type gastric carcinoma. With regard to high TILs, it is more pronounced in diffuse type gastric carcinomas.

Normal stomach tissue was equivocally characterised with CDH1+/Ki67 low/P53-/Her2- molecular phenotype, meaning all 10 cases of normal stomach epithelium were positive for CDH1, low in Ki67 proliferation index, were characterised with the absence of p53 mutations and were negative for Her2 expression. With regard to enteric carcinoma, CDH1 was positive in all 20/20 (100%) of cases, meaning the absence of CDH1 gene mutation. However, several molecular subtypes were identifiable based on the expression of Ki67, p53 and Her2 particularly: (1) CDH1+/Ki67 low/p53-/Her2- (8/20 cases 40%); (2) CDH1+/Ki67 high/p53+/Her2- (7/20 cases 35%); (3) CDH1+/Ki67 high/p53+/Her2+ (2/20 cases, 20%) and (4) CDH1+/Ki67 high/p53-/Her2- (3/20 cases, 15%).

*Table 1. Distribution of TILs, in enteric and diffuse gastric adenocarcinoma*

| | TILs | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Normal stomach | 10 (100%) | 0 (0%) | 0 (0%) |
| Enteric carcinoma | 4 (20%) | 10 (50%) | 6 (30%) |
| Diffuse carcinoma | 7 (35%) | 5 (25%) | 8 (40%) |

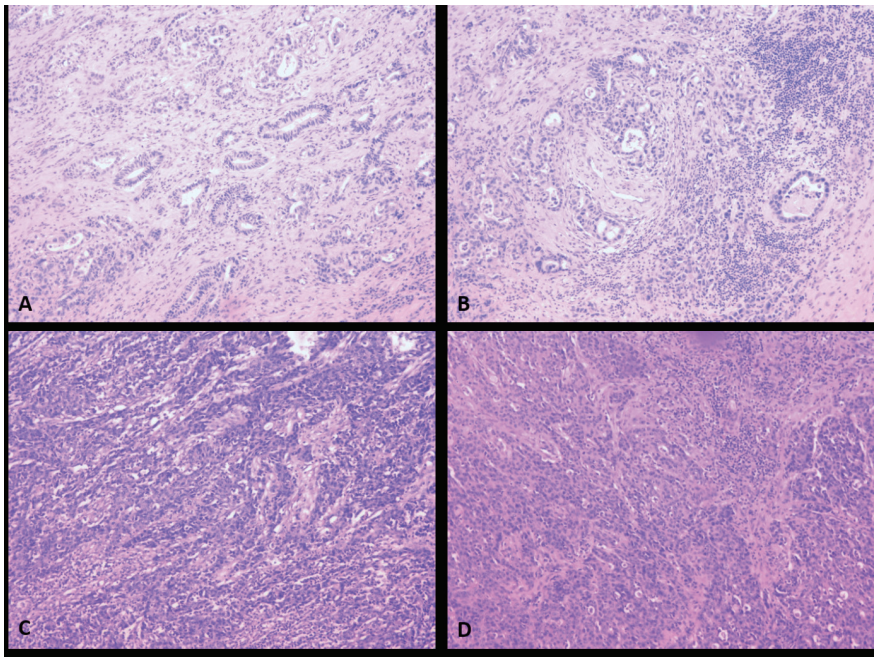
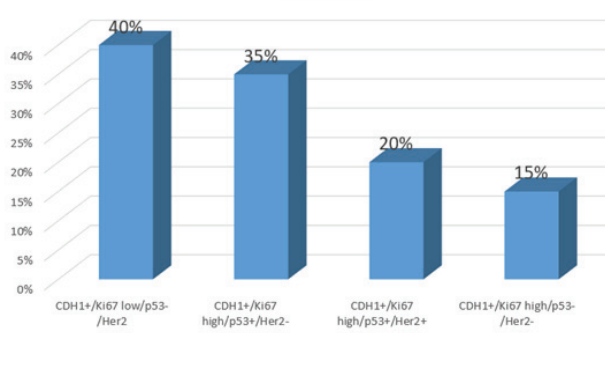
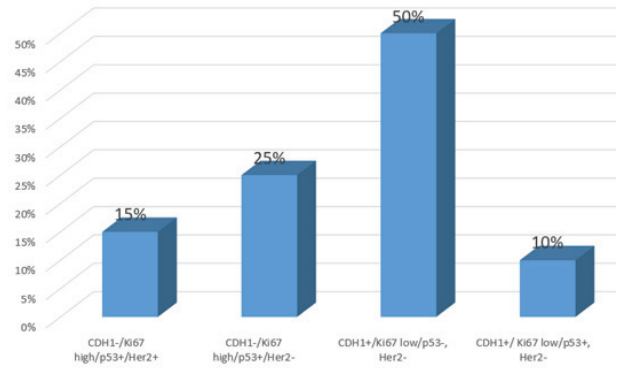



*Fig. The distribution of TILs in enteric and diffuse type gastric carcinoma: A. moderate TILs in enteric type gastric carcinoma, B. high TILs in enteric type gastric carcinoma, C. moderate TILs in diffuse gastric carcinoma and D. high TILs in diffuse type gastric carcinoma, H&E staining, x100*



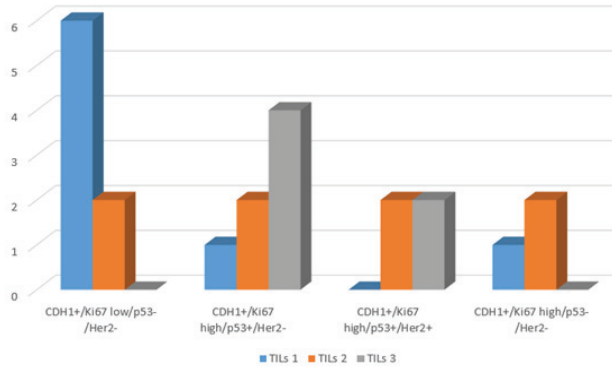

*Graph 1. Distribution of different molecular subtypes in enteric type gastric carcinoma*



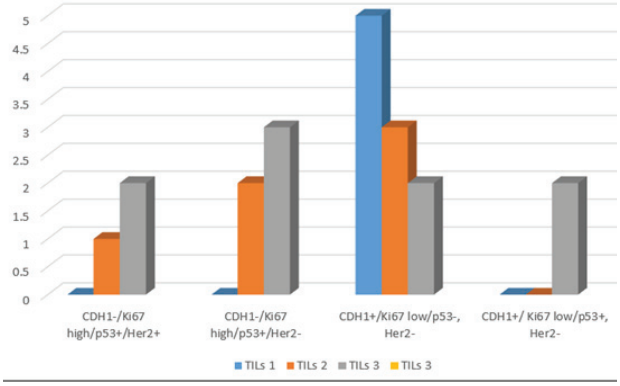

*Graph 2. Distribution of different molecular subtypes in diffuse type gastric carcinoma*

With regards to diffuse gastric carcinoma, 8/20 (40%) of cases were characterised with CDH1 gene mutation, represented by the absence of CDH1 immunohistochemical expression. Therefore, the molecular subtypes of diffuse gastric carcinoma were as follows: (1) CDH1-/Ki67 high/p53+/Her2+ (3/20 cases, 15%); (2) CDH1-/Ki67 high/p53+/Her2- (5/20 cases, 25%); (3) CDH1+/Ki67 low/p53-, Her2- (10/20 cases, 50%), (4) CDH1+/Ki67 low/p53+, Her2- (2/20 cases, 10%).

The distribution of TILs in different molecular subtypes of enteric carcinoma showed the following results: in CDH+/Ki67 low/p53-/Her- group 6/8 (75%) cases were characterised with low TILs and 2/8 (25%) cases were characterised with moderate TILs. In CDH+/Ki67 high/p53+/Her- group 1/7 (14.3%) cases were characterised with low TILs, 2/7 (28.6%) cases were characterised with moderate TILs and 4/7 (57.1%) cases were characterised with high TILs. In CDH+/Ki67 high/p53+/Her+ group low TILs were not detected in any of 4 cases (0%), 2/4 (50%) of cases were characterised with the moderate TILs and 2/4 (50%) of cases were characterised with high TILs. In CDH1+/Ki67 high/p53-/Her2- group 1/3 (33.3%) case was characterised with low TILs and 2/3 (66.7%) of cases were characterised with moderate TILs. The presence of high TILs in this group was not detected.




*Graph 3. The distribution of TILs in different molecular subtypes of enteric carcinoma*




*Graph 4. The distribution of TILs in different molecular subtypes of diffuse carcinoma*

The comparative analysis of TILs distribution in different molecular subtypes of enteric carcinoma showed significant relationship of TILs with p53 mutation status. The highest number of TILs, represented by moderate and high expression was seen in p53 mutation positive group, which suggests that the presence of p53 mutations in gastric cancer might affect the presence of TILs. Similar results have been seen in breast carcinoma patients by Lee et al., who found that TILs status is related to the p53 mutation status and cases with p53 mutations contain higher number of TILs[8]are associated with high endoplasmic stress, and possess a high frequency of TP53 mutations. TP53 missense mutations lead to the production of mutant p53 protein and usually show high levels of p53 protein expression. Tumor-infiltrating lymphocytes (TILs. Based on our study results the proliferation marker Ki67 and Her2 oncogene are not related to TILs status in enteric carcinoma patients.

The distribution of TILs in different molecular subtypes of diffuse gastric carcinoma showed the following results: in CDH-/Ki67 high/p53+/Her2+ group 1/3 (33.3%) of cases were characterised with moderate TILs and 2/3 (66.7%) cases were characterised with high TILs. In CDH-/Ki67 high/p53+/Her2- group 2/5 (40%) of cases were characterised with moderate TILs and 3/5 (60%) cases were characterised with high TILs. In CDH+/Ki67 low/p53-/Her2- group 5/10 (50%) of cases were characterised with low TILs, 3/10 (30%) of cases were characterised with moderate TILs and 2/10 (20%) of cases were characterised with high TILs. In CDH+/Ki67 low/p53+/Her2- group total of two (100%) cases were characterised with high TILs.

The comparative analysis of TILs status in different molecular subtypes of diffuse gastric carcinoma showed, that highest number of TILs are present in molecular subgroups with p53 mutations, similar to enteric type adenocarcinoma.

To the best of our knowledge this is the first detailed characterisation of tumor infiltrating lymphocytes in different types of gastric carcinoma, based on evaluation of H&E stained tissue sections. In addition, the presented study is the first to identify different molecular subtypes of gastric carcinoma based on the expression of CDH1, Ki67, p53 and Her2.

**Conclusions.** TILs status varies significantly in different histological and molecular subtypes of gastric adenocarcinoma, which might be the reason for different prognosis in these patients.

High TILs status is significantly related to the presence of p53 mutations in both enteric type and diffuse type gastric carcinoma, which can be further explored for immunotherapeutic options in these patients.

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## SUMMARY

## TUMOR INFILTRATING LYMPHOCYTES PECULIARITIES IN DIFFERENT HISTOPATHOLOGICAL AND MOLECULAR SUBTYPES OF GASTRIC CARCINOMA

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*Tbilisi Medical Academy Named After P. Shotadze*

Gastric carcinoma represents one of the major causes of cancer related mortality worldwide. Recently, immunotherapeutic means gave the new promise for the treatment of gastric carcino-

ma, although, not all patients benefit from this type of treatment. Tumor infiltrating lymphocytes (TILs) are considered as one of the promising prognostic and predictive biomarkers in solid tumors. However, the presence of TILs is not well characterised in different types of gastric cancer. The aim of our study was to characterise TILs profile in different histopathological and molecular subtypes of gastric carcinomas. We used standard haematoxylin and eosin staining (H&E) for evaluation of TILs and immunohistochemistry to detect molecular markers, including CDH1, Ki67, p53 and Her2. The results of our study revealed that TILs status varies significantly in different histological and molecular subtypes of gastric adenocarcinoma, which might be the reason for different prognosis in these patients. Also, high TILs status is significantly related to the presence of p53 mutations in both enteric type and diffuse type gastric carcinomas, which can be further explored for immunotherapeutic options in these patients.

**Keywords:** Tumor infiltrating lymphocytes, CDH1, Ki67, p53, Her2.

**РЕЗЮМЕ**

**ОСОБЕННОСТИ РАСПРЕДЕЛЕНИЯ ИНФИЛЬТРИРУЮЩИХ ОПУХОЛЬ ЛИМФОЦИТОВ В РАЗНЫХ ГИСТОЛОГИЧЕСКИХ И МОЛЕКУЛЯРНЫХ ПОДТИПАХ КАРЦИНОМ ЖЕЛУДКА**

**Мусеридзе Н.Г., Тутисани А.А., Чабрадзе Г.Г., Беридзе Н.А., Музашвили Т.З.**

*Тбилисская медицинская академия им. П. Шотадзе, Грузия*

Карцинома желудка - одна из основных причин смертности от злокачественных опухолей. По последним данным использование иммунотерапии принесло новую надежду в лечении пациентов с карциномой желудка. Несмотря на позитивные результаты, известно, что часть пациентов не реагируют на иммунотерапию. Инфильтрирующие опухоль лимфоциты считаются одним из потенциальных прогностических и предиктивных биомаркеров для пациентов с солидными опухолями. Однако особенности распределения инфильтрирующих опухоль лимфоцитов в разных типах карцином желудка недостаточно изучены. Целью исследования явилось изучение распределения инфильтрирующих опухоль лимфоцитов в разных гистологических и молекулярных подтипах карцином желудка. Лимфоцитарная инфильтрация изучена в стандартных препаратах, окрашенных гематоксилином и эозином, а молекулярные маркеры CDH1, Ki67, p53 и Her2 - иммуногистохимическим методом. Результаты исследования показали, что распределение инфильтрирующих опухоль лимфоцитов меняется как в гистологических, так и молекулярных подтипах, что может быть причиной разных прогностических поведений этих опухолей. Избыточное наличие инфильтрирующих опухоль лимфоцитов, в основном, связано с мутациями p53 как в энтеральных, так и в диффузных типах аденокарцином. Этот показатель в дальнейшем возможно изучить в соответствии с иммуно-терапевтическими агентами в карциномах желудка.

რეზიუმე

სიმსივნის მაინფილტრირებელი ლიმფოციტების განაწილების თავისებურებები სხვადასხვა ჰისტოპათოლოგიური და მოლეკულური ქვეტიპის კუჭის კარცინომებში

ნ. მუსერიძე, ა. თუთისანი, გ. ჩაბრაძე, ნ. ბერიძე, თ. მუზაშვილი

პ.შოთაძის სახ. თბილისის სამედიცინო აკადემია, საქართველო

კუჭის კარცინომები წარმოადგენს ავთვისებიანი სიმსივნით სიკვდილიანობის ერთ-ერთ მთავარ მიზეზს. ბოლოდროინდელი მონაცემებით იმუნოთერაპიული საშუალებების გამოყენებამ შემოიტანა ახალი იმედი კუჭის კარცინომის მქონე პაციენტების მკურნალობაში. მიუხედავად დადებითი შედეგების არსებობისა, ცნობილია, რომ პაციენტთა ნაწილი არ ექვემდებარება იმუნოთერაპიით მკურნალობას. სიმსივნის მაინფილტრირებელი ლიმფოციტები ითვლება ერთ-ერთ პოტენციურ პროგნოზულ და პრედიქტულ ბიომარკერად სოლიდური სიმსივნეების მქონე პაციენტებში. თუმცა სიმსივნის მაინფილტრირებელი ლიმფოციტების განაწილების თავისებურებები სხვადასხვა ტიპის კუჭის კარცინომებში კარგად შესწავლილი არაა.

კვლევის მიზანს შეადგენდა სიმსივნის მაინფილტრირებელი ლიმფოციტების განაწილების თავისებურებების შესწავლა სხვადასხვა ჰისტოპათოლოგიური და მოლეკულური ქვეტიპის კუჭის კარცინომებში. ლიმფოციტური ინფილტრაცია შეფასებული იყო სტანდარტულ ჰემატოქსილინით და ეოზინით შეღებილ ანათლებში, ხოლო მოლეკულური მარკერები, როგორებიცაა CDH1, Ki67, p53 და Her2, გამოვლინდა იმუნოჰისტოქიმიური ტექნოლოგიის საშუალებით. კვლევის შედეგებმა აჩვენა, რომ სიმსივნის მაინფილტრირებელი ლიმფოციტების განაწილება მკვეთრად ცვალებადობს როგორც ჰისტოლოგიურ, ისე მოლეკულურ ქვეტიპებში, რაც შესაძლებელია წარმოადგენდეს ამ სიმსივნეების განსხვავებული პროგნოზული ქცევის მიზეზს. სიმსივნის მაინფილტრირებელი ლიმფოციტებით ჭარბი ინფილტრაცია მნიშვნელოვნად არის დაკავშირებული p53-ის მუტაციების არსებობასთან როგორც ენტერულ, ისე დიფუზური ტიპის ადენოკარცინომებში. ეს მახასიათებელი შესაძლებელია შემდგომში შესწავლილი იქნას იმუნოთერაპიულ საშუალებებთან მიმართებაში კუჭის კარცინომებში.