Gastric cancer progression in correlation with distribution and density of T-lymphocytes

Edina Lazović Salčin1*, Mirsad Babić, Ago Omerbašić2, Nina Čamdžić1, Svjetlana Radović1, Mirsad Dorić1, Suada Kuskunović-Vlahovljak1, Haris Čampara1

1Department of Pathology, Faculty of Medicine, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
2Department of Medical Physics and Biophysics, Faculty of Medicine, University of Sarajevo, Sarajevo, Bosnia and Herzegovina

ABSTRACT

Objectives: The main objective of the study was to determine whether the distribution and level of density of tumor infiltrating CD4+ and CD8+ T-lymphocytes correlates with standard prognostic factors for gastric cancer and whether it has impact on tumor progression.

Methods: The study included 60 tissue samples of operable gastric carcinomas of known regional lymph node status, stained by standard hematoxylin eosin and immunohistochemical method in order to determine standard pathologic prognostic factors for gastric cancer and to evaluate distribution and density of tumor infiltrating T-lymphocytes.

Results: CD8+ T lymphocytes were predominantly distributed along the margin of carcinoma infiltration, while inside the cancer tissue there were generally few. CD4+ lymphocytes were few in almost all three analyzed zones (margin of carcinoma infiltration, cancer stroma and cancer tissue). The density of CD8+ showed significant positive correlation with CD8+ T lymphocytes within the cancer's stroma. There was statistically significant difference in density of CD4+ T lymphocytes distributed along the margin of carcinoma infiltration and histological tumor grade, as well as in tumor grade according to Goseki.

Conclusion: CD8+ T lymphocytes are densely arranged along the margin of carcinoma infiltration and they correlate with histological grade of gastric carcinoma.

Keywords: gastric cancer, tumor microenvironment, T-lymphocytes

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INTRODUCTION

Acquired immunity is usually protective and limits tumor progression, while innate immunity enhances the progression of the disease. Interaction, between tumor environment cells of innate and acquired immunity and cancer cells, is of great importance for tumor outcome in the lines of tumor progression or regression (1, 2). Conditions of aberrant immune response serve the tumors, but also, an adequate immune response can suppress or eliminate the tumor through immune control (3). Infiltration of tumors with T lymphocytes is associated with more favorable prognosis in colorectal carcinoma, melanoma, ovarian cancer and breast cancer (4, 5, 6). The contribution of immune cells in solid tumors, in pathogenesis and defense, especially in gastric carcinoma is not clear yet (3).

The most of functional immune cells that infiltrate gastric carcinoma are T lymphocytes, macrophages, natural killer (NK) cells, dendritic cells (DC) and MCs (7). T lymphocytes are generally divided in CD8+ cytotoxic T lymphocytes and CD4+ supporting “helper” T lymphocytes (8). Cytokines of tumor infiltrating lymphocytes (TIL) have not been studied systematically, but in some tumors (Kaposi’s sarcoma, Hodgkin disease, bronchial carcinoma, and cervical carcinoma) they produce mostly IL-4 and IL-5 but not interferon γ (IFN-γ) (9). IL-4 and IL-5 are cytokines associated with Th2 reactions. Polarization of Th2 is mostly inefficient in tumors as well as in viral infections. Signalization through T cell receptors is, also, inefficient in TIL (10).

Prevalence of Th2 cells is common in tumors and suggests that polarization can be the main strategy for preventing immunological response against tumors (11).

MATERIAL AND METHODS

The study included analysis of tissue samples of gastric carcinoma of 60 patients that had undergone Bill-
roth II resection. Operable tumors, of known regional lymph node status and without evidence of distant dissemination at the time of diagnosis were included. Samples of normal gastric tissue, sampled from no less than 5 cm distance from tumor, were used as control group. Samples of carcinoma tissue, regional lymph nodes and gastric mucosa from no less than 5 cm distance of tumor tissue were fixed in 10% buffered neutral formalin, on room temperature, and afterwards embedded in paraffin blocks, cut on four-micron sections and stained by standard hematoxylin eosin (HE) method. There were 42 male and 18 female patients, ranging from 48 to 81 years (mean 63,52 years). Observation of prepared samples included the assessment of: anatomical localization of the tumor inside the stomach, tumor size (pT), the histological type of gastric carcinoma according to Laurens classification, grade according to the degree of tumor differentiation (according to WHO classification), Goseki grade (determined by the assessment of ratio between mucinous secretion and creation of tubular formations in carcinoma tissue), macroscopic type of carcinoma by Borrmann and the presence of lymphovascular invasion, regional lymph node status (pN). Immunohistochemical protein expression of examined cell types was carried out in accordance with manufacturer protocol for each individual antibody: CD4 (Dako, clone 4B12) and CD8 (Dako, clone C8/144B). For immunohistochemical staining, tissue was fixed in neutral, buffered formalin, paraffin-embedded and cut on four-micron sections and after staining, microscopically evaluated. Assessment of cellular density that implies a number of examined cells on a unit of surface, was performed using semi-quantitative methods. Evaluation of CD4+ and CD8+ T cells was based on their density and localization. Modified method of Naita Y. et al. was used (12). In order to avoid mistakes in statistical analysis and to simplify data processing, distribution of CD4 and CD8 lymphocytes were quantified through three zones by those:

a. that were distributed along the margin of carcinoma infiltration, marked as A zone;  
b. that infiltrated tumor's stroma, marked as B zone;  
c. and those that infiltrate carcinoma tissue, marked as C zone.  

The stain sections were screened at x100 magnification under light microscope (BX40F4, OLYMPUS, Japan) to identify the three regions of the section with the highest number of immunoreactive cells. Cells were counted in these areas at x400 magnification and their average numbers recorded, determined and marked from 0-3 (0, 1-19, 20-49, and over 50). Semi-quantitative score of infiltration intensity was divided in four groups: 0= no cells; 1= mild; 2= moderate; 3= strong (13).

Statistics  
Statistical analysis was performed in the IBM SPSS Statistics v. 21.0 for Windows. The normal distribution of continuous numerical variables was performed using the Kolmogorov-Smirnov’s test. The analysis of categorical variables was carried out using Pearson’s χ²-test or Fisher’s exact probability test. Non-parametric distributed numerical variables were analyzed using the Mann-Whitney U test. Analysis of non-parametric values between several groups was performed by Kruskal-Wallis test with subsequent Bonferroni correction. The statistical significance was at the conventional level α = 0.05.

Results  
Semi-quantitative analysis has shown that CD8 lymphocytes in 38 (50%) of gastric adenocarcinoma tissue samples, have the strongest density along the margin of carcinoma infiltration (CD8A), while CD4 lymphocytes were mostly present like low density infiltrate (Figures 1,2,3 and 4). Correlational analysis of CD4 and CD8 lymphocytes density showed statically significant positive linear correlation between values of CD8A and CD8B/C (p<0.001), CD4A (p<0.01) and CD4B/C (p<0.05). With increase in number of CD8 positive lymphocytes, distributed along the margin of carcinoma infiltration, increases the number of CD8 lymphocytes in two remaining analyzed zones (cancer’s stroma and amongst the cancer cells) and CD4 lymphocytes in all three analyzed zones of distribution. (Figures 5, 6, 7). Statistically significant positive linear correlation was also observed between number of CD8B and: CD8C (p<0.001), CD4A/B (p<0.01), and CD4C (p<0.05),
LAZOVIĆ SALČIN ET AL: GASTRIC CANCER PROGRESSION CORRELATES TO DISTRIBUTION AND DENSITY OF T-LYMPHOCYTES

Figure 1. (A) Absence of CD8A lymphocytes between tumor cells (100x). (B) CD8A lymphocytes stained and distributed along the margin of carcinoma infiltration (200x).

Figure 2. Distribution of CD8B positive lymphocytes inside carcinoma stroma (A:100x). Distribution of CD8C lymphocytes amongst the carcinoma cells (B:100x).

Figure 3. Absence of CD4+ lymphocytes along the margin of carcinoma infiltration (A:100x). CD4A lymphocytes along the margin of carcinoma infiltration (B:200x).

Figure 4. CD4 positive lymphocytes inside the gastric carcinoma stroma (A:200x). CD4 positive lymphocytes distributed among carcinoma cells (B:200x).
which means that linearly with increase in number of CD8 lymphocytes, distributed inside the tumor's stroma, increases the number of CD8 lymphocytes between cancer's cells and CD4 lymphocytes in all three analyzed zones of distribution. (Figures 8., 9.).

A statistically significant positive linear correlation was observed in number of CD8C and CD4B ($p<0.01$). With an increase, in number of CD8 lymphocytes, between carcinoma cells, increases the number of CD4 lymphocytes inside of cancer's stroma (Figure 10.).

Statistically significant positive linear correlation was also observed between density of CD4A lymphocytes and CD4B/C ($p<0.001$). Correlation means that with an increase in number of CD4 lymphocytes, distributed along the margin of carcinoma infiltration, linearly increases the number of CD4 lymphocytes in other examined zones of distribution (tumor stroma and between carcinoma cells) (Figures 11, 12.). A statistically significant positive linear correlation was also observed between the values of CD4B/C lymphocytes ($p<0.001$), which means that with an increase of the number of CD4 lymphocytes, distributed inside the tumor's stroma, linearly increases the number of CD4 lymphocytes between carcinoma cells (Figure 13.).

A statistically significant negative correlation was found between patient's age and CD8C lymphocytes ($p<0.01$), which means that increase of patients' age decline the number of CD8 lymphocytes distributed in between the carcinoma cells (Figure 14.).

Correlational analysis showed statistically significant difference in the number of CD8 lymphocytes (distributed along the margin of carcinoma infiltration -

Figure 6. The ratio between values of CD8+ lymphocytes distributed along the margin of carcinoma infiltration and CD8+ lymphocytes that infiltrate carcinoma tissue.

Figure 7. The ratio between values of CD8+ and CD4+ lymphocytes distributed along the margin of carcinoma infiltration infiltration.

Figure 8. The ratio between values of CD8+ lymphocytes distributed inside the tumor's stroma and CD8+ lymphocytes that infiltrate carcinoma tissue.

Figure 9. The ratio between values of CD8+ lymphocytes distributed inside the tumor's stroma and CD4+ lymphocytes distributed along the margin of carcinoma infiltration infiltration.
CD8A) in gastric carcinoma of histological grade 2 and 3 (p=0.041) and CD4 lymphocytes (distributed along the margin of carcinoma infiltration - CD4A) in gastric carcinoma of histological grade 2 and 4 (p=0.024) (Figure 15.).

After omnibus tests, Bonferroni correction was performed (p <0.008), and the difference, observed between gastric carcinomas of histological grades 2 and 3, didn’t reach the level of statistical significance for CD8 (p=0.010) and CD4 (p=0.013) lymphocytes, distributed along the margin of carcinoma infiltration. However, there is a statistically significant difference in the number of CD4 lymphocytes, distributed along the margin of carcinoma infiltration, between histological grade 2 and 4 for gastric carcinoma (p=0.007) (Figures 16 and 17).

Correlation analysis of medians has shown a statistically significant difference between the number of CD4

Figure 10. The ratio between values of CD8+ lymphocytes that infiltrate carcinoma tissue and CD4+ lymphocytes distributed inside the tumor’s stroma.

Figure 11. The ratio between values of CD4+ lymphocytes distributed along the margin of carcinoma infiltration infiltration and CD4+ lymphocytes distributed inside the tumor’s stroma.

Figure 12. The ratio between values of CD4+ lymphocytes distributed along the margin of carcinoma infiltration infiltration and CD4+ lymphocytes that infiltrate carcinoma tissue.

Figure 13. The ratio between values of CD4+ lymphocytes distributed inside the tumor’s stroma and CD4+ lymphocytes that infiltrate carcinoma tissue.

Figure 14. The ratio between patient age and values of CD8+ lymphocytes that infiltrate carcinoma tissue.
lymphocytes distributed along the margin of gastric carcinoma infiltration (p=0.035), and CD4 lymphocytes distributed among the carcinoma cells (p=0.023), according to Goseki grade (Figure 18). After omnibus tests, Bonferroni correction was performed (p<0.008) and statistically significant difference was observed for CD4A (p=0.004) and CD4C (p=0.003) lymphocytes between gastric carcinoma of Goseki grade 1 and 3 (Figures 19 and 20.)

Figure 15. The ratio of CD4 and CD8 lymphocytes and histological tumor grade

Figure 16. The ratio of CD8+ lymphocytes distributed along the margin of carcinoma infiltration and gastric carcinoma histological grade (all p>0.008).

Figure 17. The ratio of CD4+ lymphocytes distributed along the margin of carcinoma infiltration infiltration and gastric carcinoma histological grade (p=0.007).

Figure 18. The ratio of CD4 and CD8 lymphocytes and gastric cancer grade according to Goseki grade

Figure 19. The ratio of CD4+ lymphocytes distributed along the margin of carcinoma infiltration infiltration and gastric cancer grade according to Goseki grade.

Figure 20. The ratio of CD4+ lymphocytes that infiltrate carcinoma tissue and gastric cancer grade according to Goseki grade.
Discussion

In the most of the carcinoma tissue samples, CD8+ T lymphocytes were distributed along the margin of carcinoma infiltration. Inside the cancer tissue there were generally few. CD4+ lymphocytes were few in almost all three analyzed zones. The density of CD8+ has a significant positive correlation with CD8+ T lymphocytes within the cancer’s stroma. There was no statistically significant correlation of T lymphocytes density and patient’s sex, anatomical localization of carcinoma, pT and pN tumor stages, macroscopic cancer type according to Borrmann, histological cancer type according to Lauren and presence of lymphovascular invasion. We noted the statistically significant difference in density of CD4+ T lymphocytes distributed along the margin of carcinoma infiltration and in histological tumor grade, as well as in tumor grade according to Gosoki. Other correlations of T lymphocytes were of no statistical significance.

Similar to our results, Ohno et al. (14), monitored the expression of CD8+ lymphocytes. They analyzed the density through different localizations and their ratio to carcinoma cells, correlated with clinical-pathological parameters, and also researched their influence on survival rate in patients with an advanced form of gastric carcinoma. In results, they state that most of CD8+ T cells were distributed along the margin of carcinoma infiltration, but the presence of these cells was observed in between carcinoma cells as well. Infiltration with CD8+ T cells has had no correlation with age and sex of the patients, tumor invasion, presence of metastatic lymph nodes, size of the tumor, and also with Lauren’s or Borrmann’s classification of gastric carcinomas (14), which is almost completely identical to our results. Ohno et al., in the study from 2002., have investigated the influence of the amount of carcinoma stroma and CD8+ lymphocytes on the occurrence of relapses in patients with gastric carcinomas after curative resection. They published that density of CD8+ lymphocyte infiltration has uppermost predictive value in the assessment of relapse occurrence. However, using multivariate analysis they reported that amount of carcinoma stroma is not a significant predictive factor, but could be one of limiting factors for the density of CD8+ T cell infiltration in carcinoma tissue. It is considered that extracellular matrix forms a barrier, that joined with CD8+ lymphocytes, supports the progression of carcinoma. The extracellular matrix is involved in the storage of growth factors, initiates tumor neovascularization and so accelerates tumor growth. In that regard, for the success of immunotherapy based on actions of T lymphocytes, complementary treatments are necessary for the destruction of local barriers, incorporated into tumor stroma. Several elements of tumor stroma can be highlighted as a target of research on anti-carcinoma therapy (15). Despite numerous studies were conducted with the goal to examine the role of tumor infiltrating lymphocytes (TIL) and their influence on survival rates, it is still very little known about the relationship between stromal development and TIL in carcinomas (16, 17, 18).

Authors have in their earlier study (19) reported that small groups of CD8+ lymphocytes can be significant prognostic factors in patients with gastric carcinomas. Naito Y. et al. have in 1998. (18), researched CD8+ TIL and their role in prognosis in patients with colorectal carcinomas. They conducted a clinical-pathological analysis of lymphocytes through their three zones of distribution, which is equal to our approach to analysis. In their conclusion, they state, that both, monovariant and multivariant statistical analysis show, that CD8+ TIL are significantly linked to more favorable prognosis in patients with colorectal carcinomas. Granzyme B+ cytoplasmic granules, that have been detected in TIL, have confirmed their activated cytotoxic phenotype. Authors further gather that infiltration of colorectal carcinoma with CD8+ T lymphocytes can be used as a prognostic factor (18).

Kawai O. et al. (2008) have published the results of a study, where they researched the significance of infiltration of stage IV non-microcellular lung carcinoma with macrophages and CD8+ T lymphocytes. In conclusion, they state, that the density of infiltration and wide range of distribution of macrophages and CD8+ lymphocytes in carcinoma tissue, as opposed to carcinoma stroma, in correlation with more favorable prognosis in patients with stage IV of non-microcellular lung carcinoma (20). CD8+ cytotoxic T lymphocytes (CTL) pursue active antitumor role and patients with high CTL infiltration show better prognosis in gastric carcinomas (21). However, there are papers that show that higher level of CD8+ CTL does not indicate favorable outcome when it comes to the progression of metastatic disease due to development of adaptive immunological resistance in gastric adenocarcinomas (22). In the meantime, research has been published that showed that CTL can produce IL-17 that stimulates inflammation, thus resulting in a poor prognosis for patients with gastric carcinoma (23).

Conclusion

CD8+ T lymphocytes are densely arranged along the margin of carcinoma infiltration and they are in correlation with histological grade of gastric carcinoma. CD4+ lymphocytes are mostly missing in the tissue of gastric adenocarcinoma.
LAZOVIĆ SALČIN ET AL: GASTRIC CANCER PROGRESSION CORRELATES TO DISTRIBUTION AND DENSITY OF T-LYMPHOCYTES

REFERENCES:


