Tumor infiltrating lymphocytes can help to identify CD8+ tumor infiltrating lymphocytes and histopathologic subtypes of ovarian carcinoma

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Abstract. Ovarian carcinoma is the common cause of most death cases in female genital malignancy. Ovarian carcinoma is considered as one of the carcinomas that can evoke immune responses. One of the immune responses is characterized by lymphocyte infiltration of a tumor (tumor infiltrating lymphocytes). This study aims to determine the association of density of tumor infiltrating lymphocytes (TILs) with CD8+ tumor infiltrating lymphocytes and histopathologic subtypes of ovarian carcinoma. This study was a cross-sectional study. Samples were taken based on five main histopathologic subtypes of ovarian carcinoma from January 1, 2015, until December 31, 2017, comprising 50 cases. All preparations were stained with CD8 monoclonal antibody. The density of intratumoral and stromal TILs was subsequently evaluated on histopathologic preparations and immunohistochemical examination. The association between the density of TILs, CD8+ tumor-infiltrating lymphocytes and histopathologic subtypes of ovarian carcinoma was evaluated using Spearman, Kruskal-Wallis, and Mann-Whitney test. The density of TILs and intratumoral CD8+ TILs were associated with histopathologic subtypes, especially high-grade serous carcinoma that had a different density value compared to other histopathologic subtypes. In addition, the density of TIL and intratumoral CD8+ TIL had a meaningful association with histopathologic subtypes of ovarian carcinoma.

1. Introduction
Ovarian carcinoma is considered as a major cause of most death cases in female genital malignancy [1,2]. Ovarian carcinoma occurs only about 23% of all female genital malignancies with a mortality rate of about 47% of all death cases due to cancer in female genital according to FIGO in 2015 [2]. The new diagnosis of ovarian carcinoma according to WHO in 2014 occurred about 225,000 events each year, and 140,000 cases were associated with death worldwide [3].

Ovarian carcinoma is a heterogeneous neoplasm with diverse outcomes. Currently, there is evidence that some ovarian carcinomas evoke immune response with infiltration of lymphocytes in tumors that affect prognosis [4–6]. The first study on tumor infiltrating lymphocytes (TILs) was conducted by Zhang et al. in 2003 which proved infiltration of intratumoral lymphocytes particularly CD3+ as a favorable prognostic factor, followed by a study by Raspollini et al. in 2005, Tomsova et al. in 2008, and Strickland et al. in 2016. Meanwhile, Sato et al. et al. in 2005, Haminishi et al. et al. in 2007, Clarke
et al. in 2009 and Kroeger in 2016 showed that CD8+ TILs can increase life expectancy [7,8]. Although the results of the previous studies are still conflicting, most suggest CD8+ TILs as a prognostic factor which can increase life expectancy [5].

Some studies assess association between TILs and histopathologic subtypes. TIL is a prognostic factor that can improve survival in high-grade serous carcinoma, endometrioid carcinoma and mucinous carcinoma [9]. Moreover, James et al. considered that stromal TILs are associated with high-grade serous carcinoma and endometrioid carcinoma, while intratumoral TILs have no association with histopathologic subtypes [6].

Biomarkers as prognostic factors in ovarian carcinoma are rarely studied and limited in their clinical applications due to heterogeneous nature of ovarian carcinoma. One of the biomarkers considered to have a consistent and profitable value is the infiltration of lymphocytes or tumor infiltrating lymphocytes (TILs). TILs can be assessed through both histopathological and immunohistochemical examinations. One marker used is CD8 to evaluate infiltration of cytotoxic T lymphocytes as favorable prognostic factors [10].

This study aims to determine the association between the density of TILs histopathologically with CD8+ TILs and histopathologic subtypes of ovarian carcinoma at the Anatomic Pathology Department of Dr. Moh. Hoesin Public Hospital Palembang/Faculty of Medicine, Universitas Sriwijaya.

2. Methods
This study was analytic observational study with cross sectional design which was conducted on May 15, 2018 - June 15, 2018 at the Anatomic Pathology Department of Dr. Moh. Hoesin Public Hospital Palembang/Faculty of Medicine, Universitas Sriwijaya. The samples included 50 cases in which each case consisted of 10 main histopathological subtypes of ovarian carcinoma, namely high-grade serous carcinoma, low-grade serous carcinoma, endometrioid carcinoma, mucinous carcinoma and clear cell carcinoma that had been diagnosed histopathologically and selected at random. The examination of TILs density histopathologically was performed from the slides with H&E staining and immunohistochemical examination using CD8 primary antibody to observe the morphology of cytotoxic T lymphocytes.

Observation, calculation and interpretation of lymphocyte density were performed using BX51 microscope and photographed with Olympus cameras. The results were analyzed using image raster software of Miconos™. Interpretation of lymphocyte density was performed with weak magnification (40x) to select 10 foci consisting of 5 intratumoral areas and 5 stromal areas with the highest TIL density. Furthermore, with strong magnification (400x), the number of lymphocytes and CD8+ T lymphocytes in each focus were calculated. Density analysis was done by adding up all the lymphocyte cells from each focus and the mean was then determined.

After the data were completely collected, univariate analysis was performed to determine the distribution of ovarian carcinoma patients’ characteristics. Subsequently, the bivariate analysis was conducted to determine the association between the density of TILs with CD8+ TILs and histopathologic subtypes of ovarian carcinoma.

3. Results
The mean age of ovarian carcinoma patients at diagnosis was 45.4 ± 11.4 years with the age range of 21 years and the oldest was 71. The tumor size varied with the median of 15.5 cm and a range of 6.5 cm to 42 cm. Positive lymphovascular invasion was present in 18% cases and mostly occurred in high-grade serous carcinoma for five cases. The most FIGO classification staging was at the classification III (54%). Clinicopathological characteristics can be seen in table 1.

Distribution of intratumoral TILs density based on the median value was 5.2 (range: 1.2 to 50.2), while the median value of stromal TILs was 16.4 (range: 1.2 to 114.2). Distribution based on the median value of intratumoral CD8 TIL density was 4.7 (range: 0.0 to 56.4), while the median of stromal CD8 TILs was 7.25 with a range of 0.2 to 40.6 (table 2). Intratumoral TILs density (p = 0.020) and CD8+ TILs both intratumoral (p = 0.046) and stromal (p = 0.03) had a significant association with histopathologic subtypes of ovarian carcinoma.
Table 1. Clinicopathological characteristics of study subjects.

| Clinicopathological characteristics | Histopathologic subtypes | N | % |
|-------------------------------------|--------------------------|---|---|
| Age                                 |                          |   |   |
| <40                                 | 0                        | 4 | 12 |
| 40-49                               | 3                        | 4 | 12 |
| 50-59                               | 6                        | 0 | 0  |
| > 59                                | 1                        | 2 | 2  |
| Tumor size                          |                          |   |   |
| <10cm                               | 6                        | 0 | 0  |
| 10-20 cm                            | 4                        | 8 | 1  |
| > 20cm                              | 0                        | 2 | 0  |
| Clinical stage (FIGO)               |                          |   |   |
| I                                   | 0                        | 5 | 1  |
| II                                  | 0                        | 0 | 0  |
| III                                 | 7                        | 5 | 1  |
| IV                                  | 3                        | 0 | 0  |
| Lymphovascular invasion             |                          |   |   |
| positive                            | 5                        | 1 | 2  |
| negative                            | 5                        | 9 | 8  |

Intratumoral TILs density in high-grade serous carcinoma had a difference of comparison with the density of TILs in low-grade serous carcinoma (p = 0.019), mucinous carcinoma (p = 0.026) and clear cell carcinoma (p = 0.015) based on a post hoc analysis with the Mann-Whitney test. However, no differences were found related to intratumoral TILs density in endometrioid carcinoma (p = 0.939). Meanwhile, the density of intratumoral CD8+ TILs on high-grade serous carcinoma had a difference with the density of intratumoral CD8+ TIL in low-grade serous carcinoma (p = 0.013), mucinous carcinoma (p = 0.008), and clear cell carcinoma (p = 0.049).

Table 2. Association of intratumoral and stromal TILs density with histopathologic subtypes.

| TILs location | subtypes | TILs density | P* |
|---------------|----------|--------------|----|
| Intratumoral TILs | CCC | 5.080 | 4.6257 | 3.900 | 1.2 | 17.0 | 0.020 |
| | EC | 13.140 | 13.9294 | 8.000 | 2.6 | 50.2 |
| | LGSC | 5.120 | 3.3786 | 4.300 | 1.8 | 13.0 |
| | MC | 5.338 | 3.0442 | 4.495 | 1.4 | 12.4 |
| | HGSC | 12.359 | 9.8837 | 9.100 | 3.6 | 36.0 |
| Stromal TILs | CCC | 20.620 | 22.7568 | 11.500 | 4.0 | 69.4 |
| | EC | 37.300 | 34.3351 | 27.400 | 4.4 | 114.2 |
| | LGSC | 17.059 | 14.2447 | 12.200 | 1.2 | 50.4 |
| | MC | 16.739 | 11.5101 | 14.700 | 3.4 | 35.8 |
| | HGSC | 37.780 | 32.8561 | 22.300 | 7.0 | 108.8 |

*Kruskal Wallis, significant if p<0.05

Spearman correlation test was conducted to determine whether there was a correlation between the density of stromal and intratumoral CD8 TILs. TILs density with CD8 generally indicated that there was a correlation between CD8 TILs with both intratumoral and stromal (p <0.05) with weak to moderate correlation degree. The strongest correlation was found between the density of intratumoral CD8 TILs and the density of stromal CD8 TILs (r = 0.554, p = 0.00), which means that
the higher the density of intratumoral CD8 TILs, the higher the density of stromal CD8 TILs. The results can be seen in Table 3.

**Table 3. Correlation between TILs and CD8 TILs.**

| The correlation between | n  | *p       | r   |
|-------------------------|----|----------|-----|
| Intratumoral TILs       | 50 | 0.000    | 0.477 |
| Intratumoral TILs       | 50 | 0.020    | 0.329 |
| Stromal TILs            | 50 | 0.098    | 0.237 |
| Stromal TILs            | 50 | 0.001    | 0.471 |
| Intratumoral TILs       | 50 | 0.026    | 0.316 |
| Intratumoral CD8 TILs   | 50 | 0.000    | 0.554 |

**Figure 1.** Intratumoral TILs (H&E staining, 400x) in high grade serous carcinoma.

**Figure 2.** Intratumoral CD8 TILs (400x) in high grade serous carcinoma.

**Figure 3.** Stromal TILs (H&E staining, 400x) in high grade serous carcinoma.

**Figure 4.** Stromal CD8 TILs (400x) in high grade serous carcinoma.

4. **Discussions**

The concept of cancer immunoediting describes how tumor cells interact with the immune system. There are three phases in this concept, namely elimination, equilibrium and escape. In the elimination phase, destruction of tumor cells by T lymphocytes cell occurs. The population of tumor cells that are resistant to the immune system is formed in the equilibrium phase. Further pressure on the immune system occurs without interruption. Tumors in the escape phase are able to evade the immune system and cause destruction. This can be caused by loss of antigens in tumor cells, secretion of cytokine inhibitor or downregulation of major histocompatibility molecular complex [11,12].
Ovarian carcinoma can generate an immune response characterized by the infiltration of lymphocytes (TILs). TILs can be assessed by histopathological examination although the standard method for assessment of TIL in ovarian carcinoma has not been established such as in breast carcinoma. Histopathological examination and visual analysis method are known as affordable and more accessible examination [6]. Histopathological examination of TILs density in this study used slides with haematoxylin and eosin staining from patients operating results.

The prevalence of ovarian carcinoma generally increases rapidly starting from the age of 45 [2,13]. Age increase as risk factor that initiates the malignancy can be caused by the onset of the accumulation of somatic mutations that increase in accordance with increasing age [12]. Mean age in this study was 45.4 ± 11.4 years, the youngest was 21 years old and the oldest was 71. The median value of 15.5 cm for tumor size in this study with the smallest tumor size of 6.5 cm was in high-grade serous carcinoma. The size of a small tumor can be found in 10% -20% of advanced stage high-grade serous carcinoma cases with dominant ovarian surface involvement [3]. Meanwhile, the tumor size of larger than 20 cm could be seen in the mucinous carcinoma and the largest size reached 42 cm. Lymphovascular invasion would increase the risk of disease metastasis to lymph node which is mainly characterized by the presence of tumor cells out of their original place and into circulation [12,14]. However, lymphovascular invasion in ovarian carcinoma has not been widely studied. Ovarian carcinomas are generally diagnosed at the advanced stage, while the lymphovascular invasion is an early sign of the spread of tumor. Chen et al. found that the lymphovascular invasion has an association with the advanced stage, high grade serous carcinoma subtype, and lymph node metastasis [14]. The results of this study also showed that lymphovascular invasion occurred in 18% of cases, and mostly occurred in histopathologic subtypes of high-grade serous carcinoma in the stages III and IV. Stages III and IV according to FIGO classification show that there has been metastasis, one of which is lymph nodes metastasis [2]. Meanwhile, lymphovascular invasion was not found in mucinous carcinoma. Generally mucinous carcinoma subtypes histopathologically form cystic structures with thin fibrocolagen stroma, making it difficult to identify lymphovascular invasion. Although the lymphovascular invasion was not found, when it was seen from the data according to the FIGO clinical stage, it turned out that there were three cases of mucinous carcinoma in the stage IIIC which showed that there has been an invasion and even metastasis. The most found clinical stage according to FIGO classification in this study was 54% stage III, mainly IIIC.

The examination on intratumoral TILs density and histopathologic subtypes of ovarian carcinoma based on Kruskal-Wallis test results showed that there was a significant association between intratumoral TILs density and the histopathologic subtypes (p = 0.020). However, there was no significant association found between stromal TILs and histopathologic subtypes of ovarian carcinoma. The results of this study are different from the study conducted by James et al. with the results indicating that the histopathologic subtypes are associated with stromal TILs density, not with intratumoral TILs [6]. After further tested with the Mann-Whitney test, it was evident that the intratumoral TILs in high-grade serous carcinoma had differences with intratumoral TILs density in low-grade serous carcinoma, clear cell carcinoma and mucinous carcinoma. However, intratumoral TILs density in high-grade serous carcinoma had no difference with the intratumoral TILs density in endometroid carcinoma. The immune system plays an important role in ovarian carcinoma. In addition, various subtypes of ovarian carcinoma also have various immune responses and different immune responses may help identify different subtypes [6].

Furthermore, the density of both stromal and intratumoral CD8 TILs had significant association with histopathologic subtypes (p <0.05). After further tested with the Mann-Whitney test, the result showed that the density of intratumoral CD8 TILs in high-grade serous carcinoma had difference with the density of intratumoral CD8 TILs on other histopathologic subtypes. However, stromal CD8 TILs had no differences with mucinous carcinoma subtype. These results are consistent with the study conducted by Goode et al. who found that 83% of high-grade serous carcinoma had higher levels of CD8 TILs than other histopathologic subtypes of ovarian carcinoma. This can be caused by an increase in antigen which can further enhance the immune response such as T cell lymphocytes to recognize the tumor cells as an antigen [9].
TILs density with both intratumoral and stromal CD8 TILs showed a significant association (p <0.05) with degree of the association varying from weak to moderate. This shows that the higher the density of TILs, the higher the density of CD8 TILs. Thus, TILs density in histopathological assessment allows for assessment of TILs density especially CD8 [6]. However, further research needs to be conducted to establish standardization of TILs histopathological assessment.

This study has several limitations. The first one is the variation of TILs density mainly on histopathological examination due to the wide area on the specimen. In addition, some cases indicate a higher density value of CD8 TILs than TILs density value on histopathological examination. The most likely cause is the existing difference in TILs density on the slides before and after the cutting of paraffin blocks used for immunohistochemical examination.

5. Conclusions
This study has shown that stromal and intratumoral TILs levels are associated and that their levels are associated with clinical variables, such as tumor histopathologic subtypes. TILs density on histopathological examination also shows increased levels of both intratumoral and stromal CD8 TILs.

6. References
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