Correlation Between IFNγ+ and CTLA-4+ Tumor Infiltrating Lymphocytes In Luminal And Non-Luminal Breast Carcinoma

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Research Article

Correlation between IFNγ+ and CTLA-4+ tumor infiltrating lymphocytes in luminal and non-luminal breast carcinoma

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ABSTRACT

The role of tumor infiltrating lymphocytes (TIL) in breast carcinoma depends on the molecular subtype, especially the expression of the estrogen receptor. A greater mutation load in the non-luminal subtype leads to continuous activation of the immune system resulting in exhausted T lymphocytes. Observational research with a cross-sectional approach was conducted on 40 formalin fixed paraffin-embedded tissue from breast carcinoma at the Anatomical Pathology Laboratory of Dr. Soetomo General Hospital Surabaya during January 2017 - December 2018. Samples were divided into two groups based on their status of ER expression. The parameter was a positive percentage of TIL immunoreactivity against IFNγ and CTLA-4 antibodies. Percentage of IFNγ+ TIL is higher in the luminal subtype (p =0.001), whereas the percentage of CTLA-4+ TIL is higher in the non-luminal (p =0.001). These expressions were significantly correlated with the molecular subtype of breast carcinoma (p=0.001). A significant correlation between IFNγ+ and CTLA-4+ TIL were found (rs=-0.350, p=0.027). Exhausted T lymphocytes express some inhibitor molecules such as CTLA-4. CTLA-4 (Cytotoxic T-Cell Lymphocyte Associated Protein-4) suppresses immune system function including the activity of IFN-γ as an important molecule in anti-tumor immunity and forms an immunosuppressive and pro-tumor microenvironment. Different level of expressions of IFNγ+ (p=0.001) and CTLA-4 + (p=0.001) TIL were proven to be related to the molecular subtype of breast carcinoma (rs=-0.683, p=0.001; rs=0,501, p=0.001, respectively). The negative correlation between IFNγ+ and CTLA-4+ TIL shows the role of CTLA-4 as an inhibitory molecule to the immune system (rs=-0.350, p=0.027).

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ABSTRAK

Peran *tumour infiltrating lymphocytes* (TIL) pada karsinoma payudara berhubungan erat dengan subtipe molekuler, terutama ada atau tidaknya ekspresi reseptor estrogen. Beban mutasi yang besar pada subtipe non-luminal menyebabkan pengaktifan sistem imun terjadi terus menerus dengan hasil akhir terbentuknya subset limfosit T yang kelelahan. Penelitian observasional analitik dengan pendekatan potong lintang ini menggunakan sampel 40 blok parafin dari penderita karsinoma payudara di Laboratorium Patologi Anatomi RSUD Dr. Soetomo Surabaya periode 1 Januari 2017 – 31 Desember 2018 yang dibagi menjadi 2 kelompok berdasarkan ada atau tidaknya ekspresi reseptor estrogen. Parameter penilaian adalah jumlah persentase TIL area tumor invasif yang terpulas positif dengan antibodi IFNγ dan CTLA-4. Ekspresi IFNγ+ TIL didapatkan lebih tinggi pada subtipe luminal (p=0,001), sedangkan ekspresi CTLA-4+ TIL didapatkan lebih tinggi pada subtipe non-luminal (p=0,001). Analisis statistik menunjukkan adanya korelasi signifikan antara ekspresi IFNγ+ TIL dengan CTLA-4+ TIL (rs=-0,350, p=0,027). Salah satu sifat dari sel limfosit T kelelahan adalah mengekspresikan molekul inhibitor sistem imun antara lain CTLA-4 (*Cytotoxic T-Cell Lymphocyte Associated Protein*-4). CTLA-4 akan menekan fungsi sistem imun dan berdampak pada penurunan aktivitas IFN-γ yang merupakan salah satu molekul penting dalam imunitas anti-tumor sehingga terbentuklah lingkungan mikro yang imunosupresif dan pro-tumor. Hasil penelitian menunjukkan adanya perbedaan ekspresi IFNγ+ TIL (p=0,001) dan CTLA-4+ TIL (p=0,001) pada kedua kelompok yang secara bermakna berhubungan dengan subtipe molekuler karsinoma payudara (secara berurutan mendapatkan nilai (rs=-0,683, p=0,001 dan rs=0,501, p=0,001). Korelasi negatif antara IFNγ+ TIL dengan CTLA-4+ TIL menunjukkan adanya peran CTLA-4 sebagai molekul inhibisi terhadap sistem imun (rs=-0,350, p=0,027).

Kata kunci : tumour infiltrating lymphocytes, IFNγ, CTLA-4, karsinoma payudara, subtipe molekuler luminal

INTRODUCTION

The ability of the immune system to recognize and destroy abnormal cells has great potential in inhibiting the progression of various malignancies but in some circumstances tumor cells 'trick' the immune system to escape and expand. Breast carcinoma was initially considered as a poorly immunogenic tumor type, but recently, many emerging evidence report that certain cases of breast carcinoma are strongly infiltrated by immune cells and those infiltrations showed significant prognostic and predictive values. Dense lymphoplasmacytic infiltration, labeled as tumor infiltrating lymphocytes (TIL) were found in non-luminal breast carcinoma and significantly correlated with prognosis and survival of patients, but on the other hand, this subtype is also known to have more aggressive nature, with a poor prognosis and lower survival rate (Kotoula et al., 2015; Staton and Disis, 2016; Ravelli et al., 2017; Sucipto et al., 2018). This condition is related to the phenotype of TIL and a state of exhausted T-cell. This subset expresses some inhibitory molecules including CTLA-4 (Cytotoxic T-Cell Lymphocyte Associated Protein-4) that suppress the anti-tumor activity of the immune system and leads to decreasing in interferon gamma (IFNγ) production, therefore the microenvironment in non-luminal breast carcinoma is thought to be more immunosuppressive compared to luminal. This also suggests that non-luminal breast carcinoma is more immunogenic than its counterpart and becomes a potential target for immunotherapy against immune inhibitory molecules such as CTLA-4.

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METHODS

This is analytic observational research with a cross-sectional approach conducted on breast carcinoma cases in Anatomical Pathology Laboratory of Dr. Soetomo General Hospital during January 2017–December 2018. Inclusion criteria: (1) MRM specimens with no history of chemotherapy or radiotherapy, (2) diagnosed as primary breast carcinoma and underwent histopathological and estrogen receptor (ER) immunohistochemistry (IHC) examination in Anatomical Pathology Laboratory of Dr. Soetomo General Hospital. Cases with additional malignancy other than carcinoma and inadequate paraffin blocks were excluded. Formalin-fixed, paraffin-embedded tissues from 40 breast carcinoma cases were collected, consisted of 20 luminal and 20 non-luminal.

Immunohistochemistry examinations using antibodies against IFNγ (LLO6Z: sc-74108, Santa Cruz Biotechnology, dilution 1:200) and CTLA-4+ (F-8: sc-376016, Santa Cruz Biotechnology, dilution 1:200) were performed in all samples. Expressions were assessed for immunoreactivity in stromal and intra-tumoral TIL (the cytoplasm for IFNγ, both membrane and cytoplasm for CTLA-4). Two pathologists and one anatomical pathology resident reviewed and assessed all specimens in the blinded fashion. Any disagreement was solved by interobserver agreement. The final result was average percentages of positive expression in TIL from five different fields with 400x microscope magnification.

Statistical analysis was calculated with the help of SPSS version 21.0. A comparison of expression in luminal and non-luminal subtype was analyzed using Mann Whitney U and paired T test. The correlation was analyzed using the Spearman correlation test, with a significance level < 0.05 ($p<0.05$). This research had been reviewed and approved by the Institutional Ethical Committee of Dr. Soetomo General Hospital (1190/KEPK/V/2019).

RESULTS

The samples were obtained from 40 patients. All patients were female. The mean age at diagnosis was 51.6 years in the range from 32 to 79. Most patients were 41 – 50 years of age group (32.5%). Invasive carcinoma of NST (IDC NOS) was the most common histological subtype found in this research, accounting for 85% of cases. Most cases were grade three (65%), followed by 30% of cases were grade two. Approximately twenty-nine cases (72.5%) were pathologically staged T2 (tumor > 20mm - ≤ 50mm) and twenty cases (50%) were N0 (without lymph node metastasis) at the time of diagnosis. Baseline characteristics of all samples (n=40) are summarized in table 1. The expression of IFNγ+ TIL and CTLA-4+ TIL in luminal and non-luminal breast carcinoma

The mean percentage of TIL density was found high in non-luminal breast carcinoma (68±16.1). Mann-Whitney U test was conducted and found a significant difference between these two groups ($p=0.003$). Expression of IFNγ+ TIL was found higher in luminal breast carcinoma (74.1±16.9), while expression of CTLA-4+ TIL (47.9±23.3) was found lower compared to non-luminal. Mann-Whitney U was conducted on the difference of IFNγ+ TIL and showed a significant difference ($p=0.001$). A similar result was found in the analysis of CTLA-4+ TIL, in which paired T-test found a significant difference between luminal and non-luminal breast carcinoma ($p=0.001$). The results are shown in table 2. Correlation between IFNγ+ TIL and CTLA-4+ TIL in luminal and non-luminal breast carcinoma.

Spearman correlation test showed significant correlation between both expressions (IFNγ+ TIL and CTLA-4+ TIL) and molecular subtype of breast carcinoma ($rs=-0.683$, $p=0.001$; $rs=0.501$, $p=0.001$, respectively). The correlation between IFNγ+ TIL and CTLA-4+ TIL was further analyzed and also showed a significant correlation (figure 4).

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Table 1. Baseline clinicopathological characteristic of samples (n=40)

Characteristics		Total (n=40)	
		Quantity	Percentage
Age (y.o.)	31 – 40	7	17.5
	41 – 50	13	32.5
	51 – 60	10	25
	61 – 70	8	20
	71 – 80	2	5
Histopatological subtype	Invasive Carcinoma of NST (IDC	34	8
	NOS)	1	2.5
	Invasive Lobular Carcinoma (ILC)	1	2.5
	Invasive Carcinoma with	3	7.5
	Medullary feature	1	2.5
	Mixed IDC – ILC		
	Mixed IDC – Mucinous		
Grade	Grade 1	2	5
	Grade 2	12	30
	Grade 3	26	65
Pathological stage	T1	5	12.5
	T2	29	72.5
	T3	6	15
	T4	0	0
	N0	20	50
	N1	15	37.5
	N2	4	10
	N3	1	2.5

Table 2. Comparison of TIL density , IFNγ+ TIL and CTLA-4+ TIL between luminal and non-luminal breast carcinoma

Variables	Molecular subtype	Mean	SD	Minimum	Maximum	p
TIL density	Luminal	48	21.6	10	80	0.003
	Non-luminal	68	16.1	30	90	
IFNγ+ TIL	Luminal	74.1	16.9	27	92	0.001
	Non-luminal	43.1	16.9	6	80	

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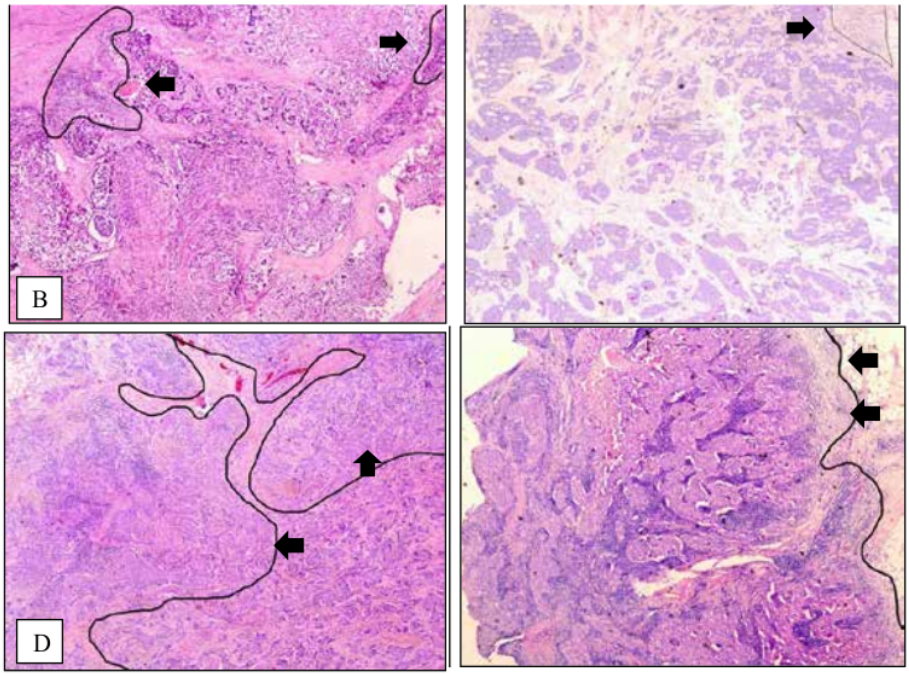

Figure 1. TIL density in breast carcinoma, 40x magnification. Area inside the marker highlighted by arrows show the stromal component that highly infiltrated by lymphocytes (A: 10% in luminal; B: 30% in non-luminal; C: 60% in luminal; D: 90% in non-luminal).

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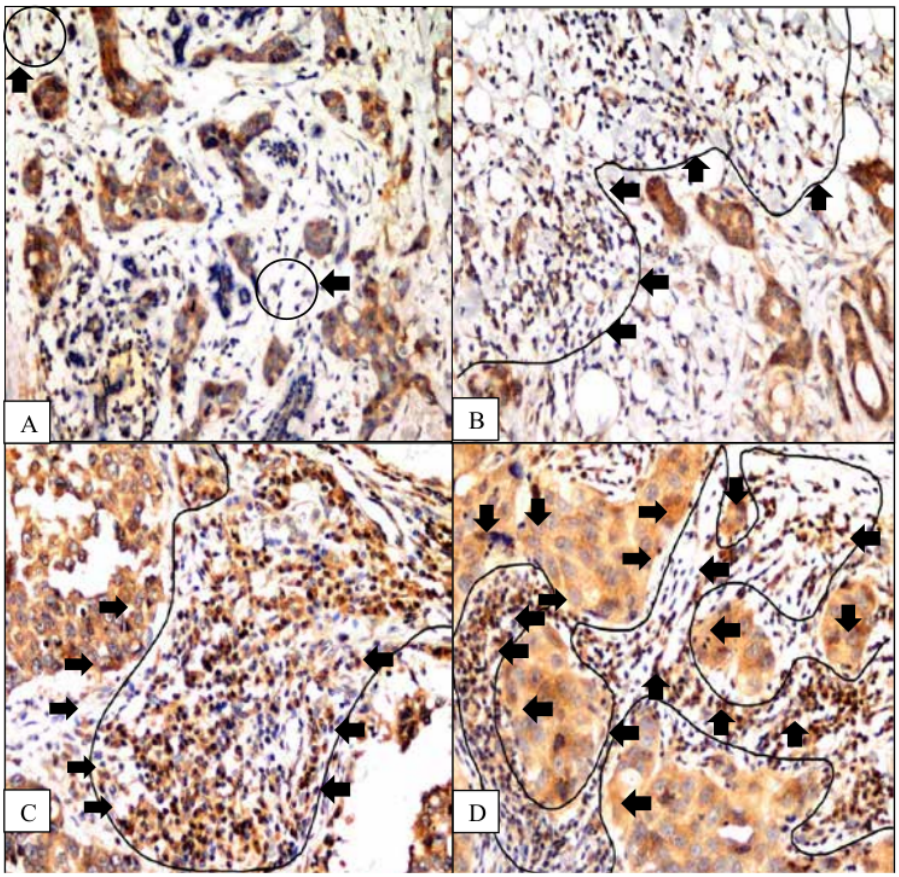

Figure 2. IFNγ+ TIL in breast carcinoma, 400x magnification. Note the positive stain in the cytoplasm of lymphocytes inside the marker highlighted by arrows (A: 10% in non-luminal,; B: 40% in non-luminal; C: 80% in luminal; D: 90% in luminal).

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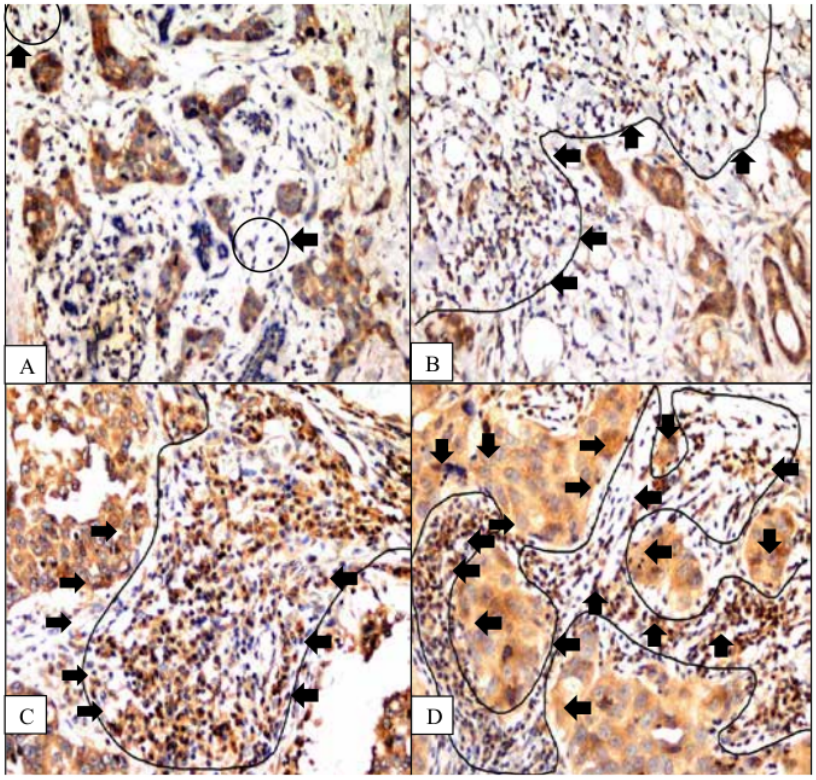

Figure 3. CTLA-4+ TIL in breast carcinoma, 400x magnification. Note the positive stain in the membreane and cytoplasm of lymphocytes inside the marker highlighted by arrow (A: 10% luminal; B: 50% in luminal; C: 80% in non-luminal; D: 90% in non-luminal).

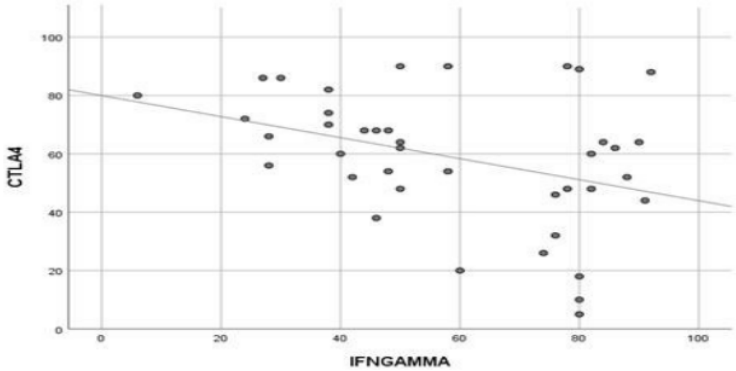

Figure 4. Scattered plot of correlation between IFNγ and CTLA-4.

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DISCUSSION

Breast carcinoma is still one of the major concerns regarding health around the world. Global Cancer Statistics 2018 estimated about 2.1 million new cases of breast carcinoma diagnosed in 2018, covering 1 of 4 cases of malignancy in women(Bray et al., 2018). Indonesian National Health Research (Rikesdas) in 2013 found that breast carcinoma is the most common malignancy in women, contributed 30% from all malignancies in Indonesia (Ayu et al., 2015). Breast carcinoma is a highly heterogeneous disease, with various genetic profiling, biological behavior, morphology, prognosis, and molecular subtypes. Estrogen Receptor (ER) is the most powerful single predictive factor compared to other predictive factors found in breast carcinomas (Allison and Sam, 2012; Dai et al., 2016). The age of patients participated in this research ranged from the age of 32 to 79 years (mean 51.6). Most belonged to a group of 41 to 50 years (32.5%), followed by 51 to 60 years (25%). This finding is in line with previous research conducted in Yogyakarta (2014), Malaysia (2016) and China (2015) (53,15 ± 10,89; 52,57 ± 10,54; and 49,5 ± 11.3, respectively). The mean age of diagnosis in Asian countries tends to be younger than in Western. This is due to ethnic and genetic differences and environmental factors such as lifestyle, reproductive factors and nutritional intake (Widodo et al., 2014; Si et al., 2015; Nizuwan, Nurdianah and Musa, 2016; Bray et al., 2018). Invasive carcinoma of NST (IDC NOS) was diagnosed in 85% cases. The terminology of this most common histological subtype in breast carcinoma has changed from invasive ductal carcinoma not otherwise specified (IDC NOS) in the 2003 WHO classification to invasive carcinoma of no special type (NST) in 2014 WHO classification. This group consists of all invasive breast cancers that do not have a distinct feature to be included in specific invasive carcinoma diagnosis groups (Sinn and Kreipe, 2013; Makki, 2015). Most cases diagnosed in this research were grade 3 (65%), pT2 (72.5%) with no regional lymph node metastasis (50%). The breast carcinoma in Asia generally is diagnosed at a late stage (the size of the tumor is greater and has metastasized). These findings are relatively different from Western countries where more cases are diagnosed in the early stages. Public awareness and compliance with early detection procedures are the biggest problems regarding this matter A large multicentre research conducted in China found that this is also heavily influenced by the socioeconomic factor (Widodo et al., 2014; Si et al., 2015; Nizuwan, Nurdianah and Musa, 2016).

TIL and breast carcinoma

The role of the immune system against cancer has long been the center of attention. Tumors infiltrating lymphocytes (TIL) which defined as infiltrate of mononuclear immune cells in the tumor microenvironment are considered to represent the quality of immune response. Breast carcinoma originally was not categorized as immunogenic tumor, but in the last few years some new pieces of evidence emerged that found intensive infiltration of immune cells and their significant correlation with prognosis and survival of patients in certain molecular subtypes (Staton and Disis, 2016; Hammerl et al., 2017; Yu et al., 2017). The density of TIL in breast carcinoma is highly influenced by the molecular subtypes. Some previous research stated that TIL is denser in more aggressive subtypes including those with HER2 amplification (HER2) and triple negative breast cancer (TNBC), while in luminal breast cancer, TIL is found fewer (Kotoula et al., 2015; Staton and Disis, 2016; Ravelli et al., 2017). This study also showed similar results. A significant difference between the percentage of TIL in two groups was proven, and TIL was denser in non-luminal subtype ($p < 0.05$).

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Correlation between IFNγ+ and CTLA-4+ TIL in luminal and non-luminal breast carcinoma

Interferon-gamma (IFNγ+) is one of the cytokines which plays an important role in anti-tumor immunity. The expression of IFNγ+ TIL in luminal breast cancer was significantly higher compared to non-luminal ($p<0.05$). Luminal breast carcinoma is known for the high IFNγ level, characterized by over-expression of the IFN gene (Dai et al., 2016). These results indicated that the IFNγ level in non-luminal breast carcinoma was lower and raised a crucial question regarding the quality of TIL. Non-luminal breast carcinoma was proven to have a higher percentage of TIL compared to luminal, but on the other hand, it also well-known to have more aggressive behavior with poorer prognosis and low survival rate. Some researchers suggested that not only the number but also the phenotype of TIL play an important role, while others highlighted the state of exhausted T-cell (Staton, Adams and Disis, 2016).

The naive T-lymphocytes are activated and differentiated into the effector T-lymphocytes within 1 – 2 weeks after infection or vaccination. This process is accompanied by massive proliferation, transcription, epigenetic and metabolic reprogramming to form the well-functioning effector T-cell for eradication. Most of the active T cells die after completing the task, leave a subset that will transform into T-cell memory. This subset will then suppress the activation of effector T-cell but still maintains the ability to quickly return into an active mode in time of re-stimulation. This physiological pathway becomes drastically changed in the state of chronic infections and malignancy. Continuous exposure to antigens and inflammation creating different subset called as exhausted T cell. One of their characteristics is the expression of inhibitory receptors, such as CTLA-4(Wherry and Kurachi, 2016; Catakovic et al., 2017). Cytotoxic T Human antigen-4 (CTLA-4, also known as CD152) is a surface protein that serves as a negative regulator for the proliferation and activation of T cells. The expression of CTLA-4 in naive T cells is very low but will be upregulated within two to three days after T-cell activation begin (Mao et al., 2010; Rowshanravan et al., 2017). This study found a significant difference between the expression of CTLA-4+ TIL in the luminal and non-luminal molecular subtype ($p < 0.05$). The expression CTLA-4+ TIL in luminal breast cancer is lower compared to non-luminal subtype.

Correlation between expression of IFNγ TIL, CTLA-4 TIL and molecular subtype of breast carcinoma ($p< 0.05$) were found in this research. This difference is caused by differences in mutational loads. Higher mutational loads lead to a greater amount of tumor-associated antigen (TAA) formed. Cancer Genomic Atlas (2012) stated that mutational loads in breast carcinoma depend on molecular subtypes. The rate of mutation was found lowest in the luminal subtype, particularly luminal-A (Spurrell and Lockley, 2014; Varn et al., 2016; Law et al., 2017; Hammerl et al., 2017). TAA then becomes the target of the immune system, presented by APC with the help of MHC class II and later recognized by the TH1 (CD4+) cells. This bond between the TCR and MHC/peptide causes a release of various cytokine, including IFNγ resulting in the activation of cytotoxic T lymphocytes. The initial signal caused by the bonding between TCR and MHC/peptide alone is not strong enough to elicit a T-cell immune response, and an additional signal known as "second signal" is needed. This second signal formed as the result of interaction between the CD28 receptor on the surface of T cells with CD80 or CD86 ligands on the APC surface. CTLA-4 and CD28 share the same ligands (CD80 and CD86) but CTLA has higher affinity than CD28 against both ligands (Rowshanravan

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et al., 2017). Several hours after the activation of T cell as a result of TCR and CD28 activity, CTLA-4 is up-regulated and binding CD80/ CD86 with a stronger affinity than CD28, and finally suppresses the immune system (Mao et al., 2010; Zhao et al., 2018).

The study found a significant negative correlation between IFNγ + TIL expression and CTLA-4 + TIL in breast carcinoma. CTLA-4 inhibits IFNγ production with several mechanisms, such as altering the activation signal of T-lymphocytes to the opposite direction by binding CD80 and CD86 ligands so that the anti-tumor function of the immune system decreases and the production of IFNγ also decreases (Pandiyan et al., 2007; Rudd, 2012; Grosso and Jurekunkel, 2013; Mo et al., 2017; Karachaliou et al., 2018). The inhibition of IFNγ production by CTLA-4 on breast carcinoma especially in the non-luminal subtype indicates that in the state of malignancy, continuous exposure of antigen interfere the T cell differentiation process forming a subset of exhausted T-cell which expresses inhibitory molecules, such as CTLA-4.

CONCLUSION

Expression of IFNγ+ TIL was higher in luminal breast carcinoma (p=0.001), while expression of CTLA-4+ TIL was lower (p=0.001) compared to non-luminal. These expressions were significantly correlated with the molecular subtype of luminal and non-luminal (rs=-0.683, p=0.001; rs=0,501, p=0.001, respectively). There was also a significant negative correlation between IFNγ+ TIL expression and CTLA-4+ TIL (rs=-0.350, p=0.027) which suggests the role of CTLA-4 in inhibition of anti-tumor immune system, especially in non-luminal breast carcinoma. This finding proved that non-luminal breast carcinoma has immunosuppressive microenvironment, therefore, it can be a potential target for immunotherapy.

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