Expression of Biomarkers CXCR4, IL11-RA, TFF1, MLF1P in Advanced Breast Cancer Patients with Bone Metastatic: a Diagnostic Study

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ABSTRAK

Tujuan: *untuk menganalisis penanda biologi CXCR4, IL11-RA, TFF1 dan MLF1P, klinikopatologi dan profil ekspresi genetik mRNA sebagai penanda peningkatan kejadian metastasis tulang pada pasien kanker payudara stadium lanjut.* ***Metode:*** *studi ini merupakan penelitian potong lintang. Analisis dilakukan pada total 92 pasien kanker payudara, terdiri atas 46 pasien metastasis tulang dan 46 pasien dengan metastasis nontulang. Analisis imunohistokimia dan microarray, dilakukan pada 81 sampel formalin fixed paraffin embedded (FFPE) dari 81 pasien yang didapat. Data dikumpulkan melalui rekam medis, pemeriksaan imunohistokimia (IHK), dan microarray dengan nanoString nCounterTM.* ***Hasil:*** *artikel ini merupakan bagian satu dari dua tahap pelaporan hasil penelitian. Pada tahap satu diperoleh hasil analisis IHK, IL11-RA dengan cut-off ≥103,5 menunjukkan peningkatan kejadian metastasis tulang, dengan OR 3,803 (95 % interval kepercayaan [IK], 1,375-10,581), p=0,010, dan MLF1P dengan cut-off ≥83,0 menunjukkan peningkatan kejadian metastasis tulang, dengan OR 2,784 (95% IK, 1,009-7,681), p=0,048. Status ER+ menunjukkan peningkatan kejadian metastasis tulang, dengan OR 7,640 (95 % IK, 2,599-22,459), p<0,000. AUC gabungan IL-11RA, MLF1P dan ER+, mempunyai ketepatan hampir 80% (meningkat dibandingkan AUC masing-masing secara terpisah), untuk membedakan dan menjelaskan kejadian metastasis tulang, pada kanker payudara stadium lanjut.* ***Kesimpulan:*** *IL11-RA, MLF1P dan ER+, merupakan determinan peningkatan kejadian metastasis tulang pasien kanker payudara stadium lanjut.*

Kata kunci: *ER+, ESR1, IL11-RA, MLF1P, nanoString, profil ekspresi genetik.*

ABSTRACT

Aim: *to analyze expression of biomarkers CXCR4, IL11-RA, TFF1 and MLF1P, and clinicopathology in advanced breast cancer patients with bone metastatic.* ***Methods:*** *this is a cross-sectional study. Analysis was done*

against a total of 92 breast cancer patients, including 46 bone metastatic patients and 46 non-bone metastatic patients. Immunohistochemistry and microarray analysis was performed in 81 formalin fixed paraffin embedded (FFPE) samples from 81 patients were used. Data were collected through medical records, immunohistochemistry (IHC), and microarray with nanoString nCounterTM. ***Results:*** *this article is part one of a two stage reporting research results. In part one we got the results of the IHC analysis, IL11-RA with cut-off ≥103.5 showed OR 3.803 (95 % confidence interval [CI], 1.375-10.581), p=0.010, MLF1P with cut-off ≥83.0 OR 2.784 (95% CI, 1.009-7.681), p=0.048, and ER+ OR 7.640 (95 % CI, 2.599-22.459), p<0.000, were associated with bone metastastic incidences in advanced breast cancer, and were statistically significantly different. A combination of IL-11RA, MLF1P and ER+, showed an accuracy of approaching 80% to discriminate between bone metastatic and non bone metastatic in advanced breast cancer patients.* ***Conclusion:*** *IL11-RA, MLF1P, and ER+ were the determinants that were associated with increasing bone metastasis incidence.*

Keywords: *breast cancer, metastatic bone disease, ER+, ESR1, IL11-RA, MLF1P.*

INTRODUCTION

Breast cancer is the second leading cause of cancer death in women, and it is the top cancer among women in developed countries. For the last few years, breast cancer has become the leading cancer type on a global level.[1-4] Breast cancer mortality is mostly related to distant metastasis to other vital organs. Approximately 30-40% of breast cancer recurrence cases involved bone metastasis, while some autopsy studies report bone involvement in 70% of breast cancer related mortality. Previous study conducted in Dharmais cancer hospital showed a 24.4% bone metastasis incidence in advanced stage breast cancer. St Gallen consensus reported 14.9% and 40.8% cumulative incidence of bone metastasis in node positive breast cancer 2 and 10 years after diagnosis.[5,6]

Breast cancer is a heterogenous malignancy with complex and not fully understood mechanism. Anatomy variance, multiple clinicopathology and molecular biology factors still are unable to explain nor predict the possibility of bone metastasis. Cancer metastasis to certain organs, including bone is not a random event and we believed it proceeds in a systematic sequence.[7] Cancer cells are able to survive because of its capability to express normal genes related to bone, and by doing so, cancer cells has the ability to survive and proliferate in bone microenvironment. For example, osteopontin (OPN) produced by cancer is closely related to poor prognosis and survival.[8]

Certain subtypes such as intrinsic luminal breast cancer is well known to relapse not until 20 years after first being diagnosed and treated, and differs from HER-2 subtype and TNBC that have the tendency to relapse within the first 5 years of treatment.[9,10] Studies have discovered several immunohistochemistries as an early bone metastasis marker. Guise et al. discovered that PTHrP may have a role in osteolytic breast cancer process.[11] Kang et al.[12] found IL-11, CXCR-4, MMO-1, ostepontin and FGF5 have their own role in bone metastasis event such as angiogenesis, homing, invasion and osteolysis, yet those findings are not completely valid and some studies were inconsistent due to complex molecular base. There are no publications yet, covering clinicopathology, and biologic marker in breast cancer, that are able to identify valid factors that are related or can predict the possibility of a bone metastasis event.

METHODS

This is a cross-sectional study to evaluate expression of biologic markers, and clinicopathology in advanced breast cancer cases. This study first started in July 2012 and lasted until February 2015. This study has received approval from the ethics committee of the Medical Faculty of Universitas Indonesia, MTA (Material Transfer Agreement) from the Ministry of Health and this study was conducted in agreement with the declaration of Helsinki.

This study recruited 92 subjects with advanced breast cancer based on study criteria. Study subjects consisted of 46 bone metastasis

patient and 46 nonbone metastasis patient from 3 hospitals in Jakarta mostly collected from Cipto Mangunkusumo Hospital, Mochtar Riady Comprehensive Cancer Centre and Darmais Cancer Hospital. Pathology results were reviewed by staff of Pathology Department Medical Faculty University of Indonesia, who were specifically assigned.

This article is part one of a two stage reporting research. In part one we got the results of the immunohistochemistry (IHC) analysis and part two is microarray RNA expression. Formalin fixed paraffin embedded (FFPE) cutting result, optimation process, IHC examination dan result evaluation were done twice including supervised and validation. Intensity and immunohistochemistry evaluation were conducted againts CXCR-4, IL11RA, MLF1P and TFF-1. Results were scored based on 300 tumor cells from 5 HPF (High Power Field) 400 time magnification of light microscope, using H-score formula=Σ(Pi x i) or H-score=(% intensity of stained cell 1x1) + (% intensity of stained cell 2x2) + (% intensity of stained cell 3x3).[13]

Clinicopathology and microarray RNA expression data were obtained from medical and pathology records as per-operational definition and conducted at the National Cancer Center Singapore using NanoString nCounterTM protocol as per recommendation http://www.nanostring.com/support/video_nCounter_gex_protocol.php Editing process were conducted before data entry. Data were analyzed using SPSS for Windows v.20 (SPSS Inc, USA). Data analysis included univariate analysis to evaluate data distribution, bivariate analysis using Chi-square test or Fischer and multivariate analysis using logistic regression test. Variable tested in multivariate analysis were the ones with p value <0.25 during bivariate analysis.

RESULTS

Ninety two FFPE samples consisting of 46 cases of bone metastase and 46 case of nonbone metastasis were evaluated from 274 advanced breast cancer, collected from 6 major hospitals in Jakarta.

Tumor size and node status were not analyzed since most of the sample size were unmeasureable (subcutaneous lesion, bone lytic lesion, etc) and the node size were only based on clinical impression. Most, clinicopathology factor were not associated with a bone metastasis event (p value >0,050) but age >65 years, ductal invasive, low malignancy degree and relapse had higher bone metastases risk. Positive ER status (p value <0.001) and luminal subtype (p value=0.019) had higher risk rate, as well as PR positive had 2.5 times higher bone metastases risk than PR negative (p=0.033). However, PR+ and luminal type were not statistically significant (p value=0.055) in multi regression analysis, because of their interaction to ER+.

Evaluation of CXCR4, IL11-RA, TFF1 dan MLF1P with cut-off >200 showed no significant difference. We then analyzed to determine the optimal cut-off value. Receiver operating characteristic (ROC) was used to obtain cut-off value. Based on that analysis, we obtain a good area under the curve (AUC) on IL11-RA and MLF1P with each value of 0.652 and 0.670 and significant differences between bone and nonbone metastase. (**Figure 1**)

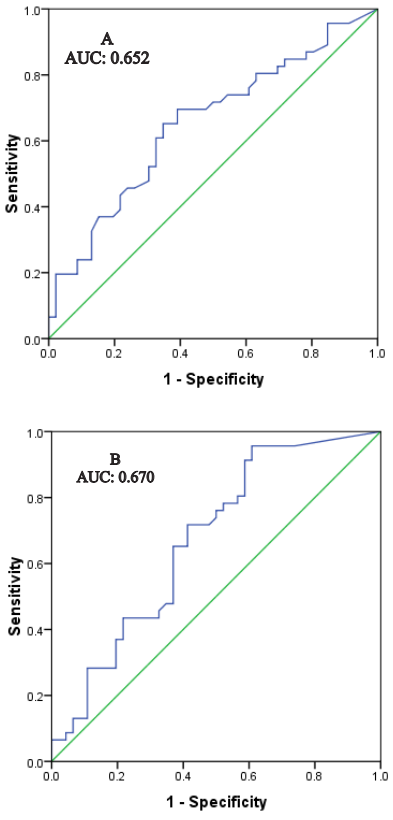

Figure 1. ROC with area under the curve (AUC) for prospective gene expression in breast cancer in: a) IL11-RA (AUC=0.652), b) MLF1P (AUC=0.670)

Table 1. Association of clinicopathology variable and immunohistochemistry with bone metastase and non bonemetastase in advanced breast cancer patient

	Metastasis		OR (95% CI)	p*
	Bone, n (%)	Non-bone, n (%)		
Clinicopathology				
Age				
- > 64	3 (33.3)	6 (66.7)	0.500 (0.105-2.379)	0.384
- 55 - 64 years	8 (47.1)	9 (52.9)	0.889 (0.270-2.925)	0.846
- 45 - 54 years	20 (55.6)	16 (44.4)	1.250 (0.473-3.303)	0.653
- 35- 44 years	15 (50.0)	15 (50.0)	Reff	
Histopathologi				
- Ductal Invasive/NST	37 (50.7)	36 (49.3)	1.142 (0.415-3.137)	1.000
- Lobular Invasive	9 (47.4)	10 (52.6)	Reff	
Malignancy degree				
- Low	13 (43.3)	17 (56.7)	0.328 (0.070-1.518)	0.154
- Intermediate	26 (50.0)	26 (50.0)	0.429 (0.099-1.841)	0.255
- High	7 (70.0)	3 (30.0)	Reff	
- Primary/ relapse				
- Primary	25 (43.9)	32 (56.1)	0.521(0.222-1.225)	0.198
- Relapse	21 (60.0)	14 (40.0)	Reff	
Immunohistochemistry				
Estrogen (ER)				
- Positive	39 (66.1)	20 (33.9)	7.243 (2.682-19.561)	< 0.001
- Negative	7 (21.2)	26 (78.8)	Reff	
Progesteron (PR)				
- Positive	33 (58.9)	23 (41.1)	2.538 (1.070-6.021)	0.033
- Negative	13 (36.1)	23 (63.9)	Reff	
HER-2				
- High expression	15 (40.5)	22 (59.5)	0.528 (0.227-1.229)	0.202
- Low expression	31 (56.4)	24 (43.6)	Reff	
Molecular subtype				
- Luminal	39 (58.2)	28 (41.8)	3.582 (1.319-9.726)	0.019
- TNBC	0 (0.0)	8 (100.0)	-	0.006
- HER-2	7 (41.2)	10 (58.8)	0.646 (0.222-1.878)	0.591
CXCR4				
- ≥190.0	32 (55.2)	26(44.6)	1.758 (0.746-4.142)	0.280
- <190.0	14 (41.2)	20 (58.8)	Reff	
IL11-RA				
- ≥103.5	33 (58.9)	23 (41.1)	2.538 (1.070-6.021)	0.033
- <103.5	13 (36.1)	23 (63.9)	Reff	
TFF1				
- ≥153.0	28 (53.8)	24 (46.2)	1.426 (0.623-3.263)	0.528
- <153.0	18 (45.0)	22 (55.0)	Reff	
MLF1P				
- ≥83.0	35 (60.9)	23 (39.7)	3.182 (1.306-7.752)	0.018
- < 83.0	11 (32.4)	23 (67.6)	Reff	

Chi square test, if p< 0.05, indicates significant statistic differences; Reff: represent group with the lowest risk

Analysis result showed statistically significant difference on variables IL11-RA (p=0.033) and MLF1P (p=0.018) as shown in **Table 1**. In the high cut-off value group, IL11-RA (cut-off ≥103.5) and MLF1P (cut-off ≥83.0), each had 2.54 and 3.18 times more bone metastasis risk compared to the low cut-off value group variabel (**Table 1**), that has p<0.25, and which was analyzed using multivariate analysis with logistic regression. Those variable were ER, PR, HER-2, IL11-RA, MLF1P, degree of tumor grade, and primary/relapse type. Luminal subtype variable were not analyzed further in multivariate analysis as part of positive interaction from estrogen positive.

Result of multivariate analysis showed that variables having significant association (p<0.05) with bone metastasis incidence in breast cancer were ER, IL11-RA, and MLF1P, as shown on **Table 2**.

Multivariate analysis yielded odds ratio (OR) 3.803 for IL11-RA, which means that breast cancer patients with IL11-RA ≥103.5 have almost four times the risk for bone metastasis compared to breast cancer patient with gene expression <103,5. This also applies for MLF1P ≥ 83.0 which showed 2.784 times higher risk for bone metastasis compared to <83.0. Result of multivariate analysis showed that variables having significant association (p<0.05) with bone metastasis incidence in breast cancer were ER+, IL11-RA, and MLF1P, as shown on **Table 2**.

Further, statistically significant variable in multivariate analysis, i.e. ER, MLF1P, and IL11-RA were analyzed using combined ROC curve for the three variables. The result showed that AUC for combination of ER, IL11-RA, and MLF1P was 0.80 in which better than each variable alone.

DISCUSSION

The main complication of breast cancer is the metastasis of cancer cells to surrounding tissues or other organs. It is known that the majority of primary cancer cells have the ability to circulate all the time and only few of it can survive specifically.[14] It is estimated that around 40% to 75% cases of breast cancer have bone metastasis.[3,6,15,16] The mechanism of metastasis of breast cancer cells to the bone is a complex process, involving proliferation, invasion, adhesion, and colonization in the bone.[17] This mechanism can only be done by breast cancer cells with specific characteristics and function, by interaction of cancer cells with molecules, growth factors, chemokines, and receptors which can did homing to the bone.

Table 2. Logistic regression for clinicopathology and immunohistochemistry variables with bone metastasis incidence in breast cancer

Variables	Coef. (B)	SE (B)	P*	OR (IK 95%)
Early Model				
- ER (+)	1.917	0.726	0.008	6.799 (1.637-28.233)
- PR (+)	0.071	0.638	0.911	1.074 (0.307-3.751)
- HER-2 (high)	-0.343	0.655	0.601	0.710 (0.197-2.564)
- IL11-RA (≥103,5)	1.331	0.564	0.018	3.786 (1.254-11.436)
- MLF1P (≥83,0)	1.280	0.607	0.035	3.598 (1.095-11.827)
- Tumor grade (rendah)	-1.658	0.935	0.076	0.191 (0.031-1.190)
- Tumor grade(moderate)	-0.853	0.640	0.183	0.426 (0.122-1.493)
- Primer/Relaps (primer)	-1.024	0.598	0.087	0.359 (0.111-1.160)
Final Model				
- ER (+)	2.033	0.550	0.000	7.640 (2.599-22.459)
- IL11-RA (≥103,5)	1.336	0.519	0.010	3.803 (1.375-10.518)
- MLF1P (≥83,0)	1.024	0.518	0.048	2.784 (1.009-7.681)

Multi regression logistic test, if p<0.05, implies statiscally significance

Based on clinicopathology data shown on **Table 1**, there was no significant difference for each variable (age, histopathology, grading, and tumor status, i.e. primary or recurrent). Around 72% of breast cancer patients were under 54 years, with age ranging from 45 to 54 years. The IBCSG (International Breast Cancer Study Group) study involving 6.792 breast cancer patients revealed that 55.6 % were above 50 years, whereas 25.6% were above 60 years.[18] The result implies that our study showed tendency to a younger age distribution, which is also found in Pakistan, India, and Srilanka.[3,19-21] The reason for difference in age distribution is not known, but it is suggested that ethnic, geographic nutrition, and genetic factors could possibly be the underlying factors.

Breast cancer patients with recurrent tumor have higher bone metastasis proportion (60%) than non-bone metastasis (40%). The recurrence indicates that the tumor has been treated and grew within a certain time period, and has the potential to change genetic characteristics and expression, thus eventually they tend to have metastasis to a particular secondary organ.[22,23] For primary tumors, the proportion of bone metastasis was lower (43.9%) compared to non-bone metastasis (56.1%). The higher non-bone metastasis in primary tumor is probably due to bone metastasis genotype was covered by other genotypes with more aggressive characteristics, such as TNBC or HER2, thus they tend to have metastasis to other organs,such as: lung, liver, and brain.[24-26]

Our result was different with the study of Koizumi et al.[27] which demonstrated that tumor size of 3 cm, positive lymph node, histopathologic type of invasive lobular and scirrhous have significant difference to bone recurrence in early stage, also with Mintempergher et al.[28] and Colleoni et al.[18] who showed lymph node status had significant association with long term recurrence, especially bone. These different findings were due to our subjects who were in very advanced stage and mostly came with unmeasurable lesion, thus tumor size and lymph node status were not suitable as predicting factors for recurrence.

Estrogen, progesterone, and luminal molecular subtype have significant difference in bone metastasis group compared to non-bone group, while HER2 showed insignificant difference. Similar result was shown by Wei B et al.[29] and Wei S et al.[30] demonstrating significant difference to positive increased estrogen in breast cancer patients with bone metastasis compared to non-bone metastasis. This result was consistent with NSABP B-14 findings, which showed higher ER expression as strong predictor for 5-year-recurrency.[9] Smid et al.[31] found around two-thirds of patients with bone recurrence were luminal cases (ER+) and only 7% found in basal type tumor.

Ryungsa Kim et al.[32] also showed no significant difference for HER2 and age, tumor size, grading, and histologic type between bone metastasis and non-bone metastasis. Furthermore, eleven patients (22.9%) with high expression of HER-2 showed insignificant increase of metastasis protein activated by HER-2, such as c-Met, VEGF, CXCR4 and pAKt. This showed the possibility of different mechanisms of bone metastasis in high expression HER2 cases. Although estrogen is known to have significant association with bone metastasis, the role of estrogen to bone metastasis still could not be explained completely.[28,33]

One explanation correlated ER with bone metastasis were overexpression of SRS+ (signature Src+).[10,15,25] Src is a tyrosin kinase which plays a role in progressivity and metastasis of cancer cells with slow growth. Specifically, it causes long term recurrence. Increased activity of Src (SRS+) is a response to TRAIL signal and also AKT as a response to CXCL12 and IGFI which are expressed in the microenvironment of bone metastasis, i.e. bone marrow stroma, thus it escapes apoptosis. Those characteristics make Src regarded as a mediator for anti-apoptosis mechanism.[10,15,25] Furthermore, Src increases activity of P3IK and Akt pathways as selective response to enrich primary tumor clonal which has the ablility for bone metastasis.

There were 3 requirements needed for a premetastatic selection to occur: hyperactive Src in primary tumor, heterogenous characteristic in primary tumor, and minimal concentration of CXCL12/IGFI as a selector for tumor cells

which will proliferate and survive.[10] Author also realize than none of the seven previous study were included Src as gene predictor.

IL11-RA and MLF1P variables showed significant difference in bone and non-bone metastasis, while CXCR4 and TFF1 did not reveal significant difference. Thomassen et et al.[34] proved that, MLF1P had significant difference on bone metastasis and consistently highly expressed, thus the gene remains a candidate for predictor gene to progresivity of cancer cells to bone. The MLF1P gene was bound to MLF1 which was a negative regulator of p53, coded by Tp53 gene. The gene has potency to disturb broad and complex functions of p53, including cells cycle "arrest", apotosis, angiogenesis, and DNA repair.[35]

IL11-RAand IL11 were known to have association with tumor progressivity, cells growth, and differentiation from several malignant tumors.[36,37] IL11 which was produced by various cells including bone marrow stromal as a response to inflammation will be bound to IL11-RA and then activate STAT3 pathway and facilitate EMT by breaking down ECM as an initial process of metastasis.[38] This is consistent with findings of Smid et al.[31] and Minn et al.[39] which demonstrated that IL11, together with other genes such as OPN, CTGF, TTF, CXCR4, had significant roles to bone recurrence incidences. Kang et al.[12] has also demonstrated that human breast cancer line, most of the gene with osteolytic function generally was expressed with a big difference. IL11 alone without RANKL, can directly induce osteoclast forming from the precursor in the bone marrow. IL11-RA can be found in cytoplasma and membrane, while IL11 is only found in cytoplasma.

All area under the curve (AUC) of those variables showed AUC less than 0.70, with smallest value of 0.537 and 0.603 in TFF1 and CXCR4 respectively. Those values showed the capability of TFF1 and CXCR4 to differentiate breast cancer patients which has been predicted to have unfavourable bone metastasis. Combination of ER+, MLF1P and IL11-RA yielded AUC almost 80% compared to separated IL11-RA and MLF1P with AUC of 0.652 and 0.670 respectively, which indicated that the combination of those factors yielded better performance to differentiate cases with bone and non-bone metastasis compared to individual factors.

CONCLUSION

There is an association between the high expression of biomarker IL 11-RA, MLF1P, and ER (+) with the increase in incidence of bone metastasis in advanced breast cancer. The combination of these markers resulted in AUC of almost 0.8, which can be used to differentiate between bones and non-bones metastasis.

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