NM23HI as Marker of Metastasis in Invasive Ductal Breast Cancer

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ABSTRACT

Aim: to examine the presence of metastasis marker in various histological malignancies of ductal breast carcinoma using NM23HI protein. Methods: paraffin blocks were obtained from 97 patients with invasive breast ductal cancer with 1, 2, 3 grade of malignancy from 2000 to early 2006 in several hospitals in Jakarta and Bandung. Examination began with histophatologic examination of eosin hematoxylin slides to diagnose the case of invasive ductal cancer and to get the data on the degree of histologic malignancy, metastasis or non-metastasis cases. It then continued to immunohistochemistry examination of NM23HI, and cytokerinat. Results: subjects were 29 - 75 years old with the mean of 48.19 years; most subjects were 40 – 45 years old with malignancy grade 1 numbering 18.56%; grade 2, 45.36%; and grade 3, 36.1%. Ninety seven paraffin blocks were examined from 2000 to 2006. There was a significant relationship between NM23HI expression in primary tumor with the possibility of inhibition of invasion and metastasis 11 times of those of negative expression of NM23HI. The ROC curve showed that NM23HI expression was strongly correlated (r=0.816) sensitive and specific as metastasis marker. NM23HI expression did not show significant relationship with histologic degree of invasive ductal carcinoma. Conclusion: NM23HI expressions can be used as invasion and metastasis markers, but cannot be used as markers for the degree of histologic malignancy of invasive ductal cancer.

Key words: breast cancer, NM23H1.
INTRODUCTION

The incidence of breast cancer occupies the first position in the world\(^1\) and second in Indonesia lagging to cervical cancer.\(^2\) Many cancer patients visited their doctors at advanced stage of cancer, so the treatment will be difficult. The worst of all is that, in Indonesia, most of cancer patients are still young and productive.\(^3,4\)

Based on the records in Dr. Cipto Mangunkusumo hospital, invasive ductal cancer is the breast cancer subtype with the highest incidence, i.e. 85%-95% (Mangunkusumo R, personal communication). Early detection of invasive ductal cancer in its early stadium can give the patients maximum and affordable treatments. The discovery of sensitive of invasion and metastasis of invasive ductal cancer in its early stadium is crucial.\(^5\)

Invasion and metastasis determine the choice of therapy. Patients without invasion and metastasis may need only primary tumor removal without axillary lymph node removal, radiation, and chemotherapy. Patients with possibility of metastasis need further treatments including axillary lymph node removal, radiation, and chemotherapy. Markers, NM23H1, can save patients from the emergence of loco regional metastasis so that doctors can still preserve the patients’ productivity and better ensure patients’ recovery.\(^5\)

Previous studies concluded that the presence of metastatic marker, until now, is not yet clear, while therapy and prognostic factors of patients are very dependent on the prediction of metastasis.\(^5\) Therefore, it is necessary to develop method that can detect early metastasis, as well as to predict and estimate the prognosis of patients in early stages of breast ductal cancer. These examinations should be accurate, affordable, and available with a combination of clinical, histopathological and immunohistochemical examinations currently being developed rapidly. This study aims to learn the description of NM23H1 expressions to predict the emergence and metastasis. These examinations are expected to detect or predict the potential of local and distant metastasis.

METHODS

The study was conducted in the Department of Anatomic Pathology, Faculty of Medicine, University of Indonesia, Cipto Mangunkusumo Central National Hospital, from November 1st to December 31st, 2006. Initiated by collecting medical records, H&E slides of secondary and primary tumor tissues, assessing the morphological descriptions of the tumor tissues and the grade of malignancy of primary tumors. Assessment of metastasis in lymph nodes and patients’ slides was performed. Paraffin blocks were obtained from Department of Anatomic Pathology of the respective hospitals. New Hematoxylin Eosin (H&E) staining was then carried out to describe the histopathologic description, grade of malignancy, and metastasis, in both primary tumors and lymph nodes.

The protocol of the study has been approved by The Committee of The Medical Research Ethics of the Faculty of Medicine University of Indonesia.

Samples

The samples were all H&E slides and paraffin blocks of breast mastectomy cases from 2000 to 2006 that met the inclusion criteria, i.e. all breast cancer of female patients that had been histopathologically diagnosed as invasive ductal breast carcinoma with low grade of malignancy (grade of malignancy I) with and without metastases in lymph nodes, as invasive ductal breast cancer with grade of malignancy II, with and without metastases in lymph nodes, as well as invasive ductal breast cancer with high grade of malignancy (grade of malignancy III).

Calculation of Sample Size

The samples were selected using consecutive sampling. With NM23H1 examinations, it was expected that the prediction would be more significant. It was expected that the accuracy of the prediction would reach ninety percent.

Slide preparation and immunohistochemical staining\(^5,8\)

The paraffin blocks were cut and immunohistochemically stained; blocks of primary tumors and their metastases in lymph nodes were stained with NM23H1, while blocks of lymph nodes without metastasis were stained with cytokeratin to make sure that there was no metastase in lymph nodes.

Immunohistochemical staining used the labeling streptavidine Biotin Complex method. As a primary antibody of NM23H1 and Cytokeratin were identified using mouse monoclonal
antibody, which was mouse monoclonal anti NM23H1 antibody, Ig:1:100 (Novocastra) and for cytokeratin were anti A/E3 antibody, Ig:1:100 (Daco). Every outward appearance included negative controls using the same breast cancer tissue, and each running consisted of 8 - 10 cases with a positive control of breast cancer in-situ, as the outward appearance and standard assessment techniques.

Assessment NM23H1 protein expression was performed in 500 tumor cells from 5 large fields of view (400 x) of different and random. Each region was represented by 100 cell tumors. NM23H1 positivity outward appearance was seen firmly in the brown coloration cytoplasm of tumor cells. Degree of positivity of NM23H1 was assessed with a score system and included in the percent positive value (the value of color intensity by Nichols). The assessment of protein expression of cytokeratin was done on the entire field of view (magnification 100 x) and with a large field of view (magnification 400 x). The positivity of cytokeratin outward appearance was seen as brown coloring on the intra-tumor cell cytoplasm. The degree of positivity of cytokeratin was assessed in the same manner as above.

Data were retrieved from the archives of the Department of Anatomic Pathology, Faculty of Medicine University of Indonesia/General Hospital Cipto Mangunkusumo, Kramat 128 Hospital Jakarta, Jakarta Breast Centers, Public Hospitals Hasan Sadikin Bandung, Jakarta Islamic Hospital and the Darmanugraha Hospital Rawamangun, Jakarta. The data retrieved were: the hospital of specimen origin, age, sub-type of tumor, tumor grade, and lymph node metastasis.

The data recorded from the immunohistochemical staining results were the positivity and expression strength of NM23H1 in primary tumors, and metastases in lymph nodes in invasive ductal breast cancer.

The data were entered into a main table, and data analyses were done using SPSS version 13 and Med Calc version 9.6.4.0. software. Chi square test was used to calculate the Odds Ratio (OR) and 95% confidence interval (95% CI) for NM23H1 positivity as well as expression strength on the occurrence of metastatic tumors compared to non metastatic tumors. In addition, the OR and 95% CI for tumor NM23H1 positive expression on the occurrence lymph node positive compared to negative expression were calculated. Further, diagnosis test to get the sensitivity and specificity value from the ROC curve using NM23H1 positivity was done. Presentation of data was done in tables, pictures and graphs.

RESULTS

Forty-eight cases of non-metastatic ductal breast cancer and 49 cases of metastatic ductal breast cancer were included in the study. Subjects were 29-75 years old with the mean of 48.19 years; most subjects are 40 – 45 years old, with malignancy grade 1 numbering 18.56%; grade 2, 45.36%; and grade 3, 36.1%. Distribution frequency of less than 50 years of age were 58.76% and equal to or more than 50 years were 41.24%. The most frequent histopathologic sub type was solid tubular and squir 22.2% followed by a solid tubular 18.9% and solid tubular with papilobular tubular as much as 12.2%. While the degree of malignancy was as follows: grade 2, were 45.36%, followed by grade 3 in 36.1% and grade 1 in 18.9%. Histopathology results of lymph nodes metastasis were 48.9% and 51.1% non metastatic. The distributions of the primary tumor NM23H1 negative were 43.3%, weakly positive 44.3% and moderate positive was 29.1%.

Table 1 shows the comparison of the expressions of NM23H1 in non-metastatic and metastatic primary tumor in patients with invasive ductal breast cancer. The chance of positive expression of NM23H1 to prevent metastasis was 11.3 times of negative expression of NM23H1, 95% CI 4.29-30, p <0.001.

Regarding the comparison of NM23H1 expression grade, the chance of weak positive NM23H1 expression to prevent metastasis was eight times as much as that of negative expression, 95% IC, 2.94-21.4, p <0.0001. The chance of moderate positive NM23H1 expression to prevent metastasis was 50 times as many as that of negative expression (95% CI 5.82 – 439; p<0.0001).

Because of the minimum number of samples and the presence of zero in strong positive NM23H1 expressions, strong positive NM23H1 expression could not be evaluated. The trend
test revealed increasing Odds Ratio from weak positive to moderate positive, from 8 to 50 times (Table 2).

Table 3 showed an increase of NM23H1 expressions in lymph nodes compared with a 34-time decrease of NM23H1 expressions in the primary tumors, 95% CI 0.00079-0.208, p >0.05. There was disagreement between 95% CI and p value because there was a “0” (zero) in one column.

ROC curve was made to illustrate the concordance of sensitivity and specificity of marker - NM23H1 – with their metastasis and their histologic grade of malignancy.

The chart can predict a sensitivity of 69.4% and a specificity of 83.3% with a cut-off point ≤0 and area under the curve of 0.827 appear in the curve of concordance between the results of NM23H1 expression and sensitivity (Figure 1).

The Results of Immunohistochemistry Staining

The results of immunohistochemistry staining of NM23H1 can be seen in Figure 2-3. Two pathologists’ calculation of immunohistochemical

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**Table 1.** Chi square test of NM23H1 expressions in non metastasic (n=48) compared to metastatic primary tumors (n=49) using Chi square test

<table>
<thead>
<tr>
<th>Expression</th>
<th>Primary Tumor</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM23H1</td>
<td>Non Metastasic, %</td>
<td>Metastasic, %</td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>40 (83.3%)</td>
<td>15 (30.6%)</td>
<td>11.3</td>
</tr>
<tr>
<td>negative</td>
<td>8 (16.7%)</td>
<td>34 (69.4%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>48 (100%)</td>
<td>49 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

p value x² 26.74 Trend test p < 0.0001

**Table 2.** Results of gradation test of NM23H1 expressions in non metastatic primary tumor (n=48) compared with NM23H1 expressions in metastatic primary tumors (n=49) using Chi square test

<table>
<thead>
<tr>
<th>NM23H1 Expression</th>
<th>Primary Tumor</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non Metastasic, %</td>
<td>Metastasic, %</td>
<td></td>
</tr>
<tr>
<td>Strong positive</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>3.89</td>
</tr>
<tr>
<td>Moderate positive</td>
<td>12 (25%)</td>
<td>0 (0%)</td>
<td>50.6</td>
</tr>
<tr>
<td>Weak positive</td>
<td>28 (58.3%)</td>
<td>15 (30.6%)</td>
<td>7.93</td>
</tr>
<tr>
<td>Negative</td>
<td>8 (16.7%)</td>
<td>34 (69.4%)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>48 (100%)</td>
<td>49 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

p value x² 26.74 Trend test p < 0.0001

**Table 3.** Chi square test of NM23H1 expressions in primary tumors’ metastasis (n=49) compared to lymph node metastasis (n=49)

<table>
<thead>
<tr>
<th>NM23H1 expression in lymph nodes</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive %</td>
<td>15 (34.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Negative %</td>
<td>28 (65.1%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>43 (100%)</td>
<td>6 (100%)</td>
</tr>
</tbody>
</table>

p value x² = 6.39 p < 0.001
reading gives likelihood ratio of 4.31 prediction of the occurrence of metastasis is four.

In lymph nodes, likelihood calculation is not performed because the purpose will be different. Metastasis prediction before and after given immunohistochemistry with a priori probability comparison method with a posteriori according to Veneis method show an increase of 49.27% for NM23H1.

**Figure 4** shows that weak positive NM23H1 expressions in primary tumors had positive NM23H1 expressions in lymph nodes; roughly one-third of which had moderate positive expressions while the rest (approximately two-thirds) had weak positive expressions in their lymph nodes. Negative NM23H1 expressions in primary tumors had negative, weak positive, strong positive NM23H1 expressions.

**DISCUSSION**

Among carcinoma sub types, the invasive ductal breast cancer has the highest incidence rate. As a reference hospital, Dr. Cipto Mangunkusumo’s patients hospitalised can represent breast cancer patients in Indonesia. During the search for specimen, various problems encountered (such as the tumor mass was run out, eaten by mice, the paraffin block was gone or was damaged, etc) so that the expected minimum sample size required was not fulfilled. That is why some samples were obtained from Hasan Sadikin hospital, Bandung, Kramat 128 hospital, Jakarta and/or Jakarta Breast Center, Jakarta, Islam hospital, Jakarta, and Dharma Nugraha hospital, Rawamangun, Jakarta.

This study used histopathologic and immunohistochemical examinations with the availability of good or liable paraffin blocks as the inclusion criterion and the unavailability of non-liable paraffin blocks (e.g. broken paraffin blocks or paraffin blocks whose tumor mass was cut or eaten by animals, etc) as the exclusion criterion. As many as 97 cases of liable paraffin blocks meeting the inclusion criterion were obtained. The HE staining was then carried out. Immunohistochemical slides were then compared with negative controls made for each case and positive controls made for each round of case making. The selection and the reading of immunohistochemical supplies were carried out by two anatomic pathologists, researchers and pathologists experienced in reading histopathologic supplies so that the internal validity was expected to be good.

The specimens used were mastectomy cases of invasive ductal breast cancer with grade malignancy one, two, and three. In situ ductal breast cancer cases were not used because of the scarcity of patients. In situ ductal cancer gives strong positive expressions, and micro-invasion
has not occurred yet so that in this study, in situ ductal carcinoma was used as positive controls of NM23H1 immunohistochemistry staining.

All invasive ductal cancer cases used in this study were obtained from mastectomy with metastasis only to axillary lymph node, although it is known that metastasis occurs not only in axillary lymph nodes but also in internal mammary lymph node, areas of clavicula, or other organs. Far metastasis is contra indication for mastectomy.

Clinical application of this study is to detect metastase in breast cancer. If the expression of NM23H1 is negative, we can assume that there were metastasis. To confirm the metastasis we should do mastectomy and histopathological examination for the primer tumor and lymphnode Level 1,2,3. And if the lymphnode contains tumor mass, Immunohistochemistry (IHC) for NM23H1 expression should be done. Negative expression of NM23H1 in lymphnode specimen suggests that there is gene mutation, therefore, the patient should not be given targeting therapy. But if the expression of NM23H1 is positive at the tumor mass in the lymphnode specimens, we suggest that there is Epigenetic/Hypermethylation in the breast cancer. Therefore, the patient should be given targeting therapy and the goal of therapy was improved prognosis. Yet the targeting therapy must be combined with hormonal therapy, or with another chemotherapy.

**Table 2** shows an increase in Odds Ratio from weak positive, i.e. 8 times, to moderate positive, i.e. 50 times. These results were in agree with one of NM23H1 work mechanisms that is as metastasis suppressor that reduces cell motility, lowers cell differentiation, thus reducing or preventing invasion and metastasis.

In this study, analysis of NM23H1 expression in lymph nodes was higher compared to NM23H1 expression in primary tumor that decreases 34 points, so we could see that there was an Epigenetic.

The time when NM23H1 in lymph nodes increase can be explained by Kowalczyk et.al., 1994, who found that decreased NM23H1 expression in invasive ductal carcinoma causes carcinoma to develop and grow to form metastasis both in lymph nodes and far sites. Cancer cells can re-express their NM23H1 protein as soon as they reach the far sites. Re-expressed cancer cells can even be found in all metastasis of ductal cancer and their expression level can be as high as or higher than their expression level in the primary tumors.

ROC curve revealed the concordance, between markers NM23H1 expression and metastasis. Area under the curve was 0.81 with optimal specificity of sixty-nine point four percent and specificity of 83.3%. The curves show that NM23H1 expression is accurate and has added value as a metastasis marker of breast cancer.

It can be concluded, based on all analyses, that NM23H1 is a good invasion and metastasis marker.

A priori and a posteriori probability comparison shows the percentage of metastasis prediction before and after immunohistochemistry is administered. In this study an increase of NM23H1 appears from a priori of fifty-point-five percent to a posteriori of ninety-nine point nine percent; that is forty-nine point four percent. In this study the effectiveness of immunohistochemistry usage also appears to predict the occurrence of metastasis with the presence of an increase in significant a priori and
a posteriori probabilities.\textsuperscript{10}

These results agree with the application of immunohistochemistry markers by Veneis in his research about cancer. The aim was to make the golden rules of clinical results in use; that was the immunohistochemical occurrence of metastasis. The golden rule that P. Veneis obtained was also obtained in this study.\textsuperscript{10}

The limitation of this study was that we did not assess the clinical characteristics of the patients. Generally, in diagnostic or prognostic study, multivariable determinants were assessed, starting from the clinical characteristics of patients, clinical additional examination, up to the prognosis of patient, however, our study can help to determine diagnosis, therapy and prognosis of the patients.

The use of odds ratios in cross sectional studies in all researches, and included in this study may allow the estimation results a bit excessive.

CONCLUSION

There was a significant relation between the expression of NM23H1 and metastases in the lymph node. There was an inverse relationship between the degree of malignancy with expression of NM23H1. There was no linkage between the expression of the NM23H1 with the age of the patient of ductal breast cancer.

The most valuable result of this study is that the chance of weak positive NM23H1 expression to prevent metastasis is eleven times as much as the chance of negative NM23H1 expression.

Most disorders occurring in NM23H1 are epigenetic disorders so that these disorders are expected to be treated with demethylation therapy which now widely spreads.\textsuperscript{14,15} These hypermethylated genes are expected to be normal again.\textsuperscript{16} Further studies using RT-PCR to differentiate which case is mutation and which case is epigenetic/ hypermethylation are urgent so that doctors can give optimum treatments to their patients.\textsuperscript{16,17}

Further studies are needed with combination of the clinical characteristics of patients to predict metastasis of invasive ductal breast cancer and to determine therapy and prognosis of the patients.

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