ORIGINAL RESEARCH PAPER

Pathology

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# IMMUNOHISTOCHEMICAL EXPRESSIONS OF CK5 AND CK8/18 IN TRIPLE NEGATIVE BREAST CANCERS

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**ABSTRACT**

A cross-sectional study of 58 triple negative breast cancer (TNBC) patients' formalin-fixed paraffin-embedded tissue blocks was conducted to assess the immunohistochemical expressions of CK5 (basal cell marker) and CK8/18 (luminal cell marker) as well as their association with various TNBC clinicopathological parameters, such as patients' age, menopausal status, disease stage, tumor size, lymph node involvement, distant metastasis, histological type, grade, and mitosis. Statistical analyses were performed by using independent sample Student t, Pearson's Chi square and Fisher's exact test. Basal-like subtype identified by positive CK5 expression was observed in 43.1% of TNBC cases while negative CK8/18 expression was seen in 13.8% of cases. Both biomarkers expressions showed no significant association with observed clinicopathological parameters. However, CK5 was found to be significantly associated with mitosis (p = 0,032).

## INTRODUCTION

Triple negative breast cancer (TNBC) is immunohistochemical subtype of breast cancers that do not express estrogen receptor, progesterone receptor, and HER2 protein. This subtype accounts for 10-20% of all breast cancers and is best known for its heterogeneity.1,2 TNBC is an interesting research area for both researchers and clinicians because of its aggressive nature, various responses to chemotherapy and the absence of effective targeted therapy. Thus, it is associated with poor prognosis.[3,4]

Over the past few years, great attention has been focused on keratin as a marker of neoplastic differentiation and development of human breast epithelial cells. The expression of intermediate filament protein, especially cytokeratin (CK), which is a component of cell cytoskeleton, reflects the type of epithelial cell, tissue growth state, and differentiation.5 In normal breast tissue, CK5, CK14, CK17 are expressed in basal cells, while CK7, CK8, CK18, and CK19 are expressed in luminal cells. During the development of malignancy, the actual CK profile is considered stable so that different types of breast cancer can sometimes be classified according to the cell origin based on CK expression.[6]

Based on gene expression profiling, most of TNBC show basal-like phenotype, which are aggressive tumors with poor prognosis.7 Immunohistochemically, basal-like subtype was identified by using basal cell markers, such as CK5 (or CK5/6).8 The expression of this basal cell marker has been widely reported to be significantly associated with poor prognosis.9,10 Meanwhile, loss of CK8/18 expression is reported to be associated with clinicopathological features that indicate a poor prognosis by several researchers.11,12 However, co-expression of CK5 and CK8/18 has been noted in up to 84% of basal like breast cancers.13 Therefore, this study aimed to determine the dist ribution of CK5 and CK8/18 expressions in TNBC and their association with various clinicopathological parameters.

## MATERIALS AND METHODS

### *Sample selection*

This cross sectional study was conducted in Department of Anatomical Pathology, University of Sumatera Utara/ H. Adam Malik General Hospital, Medan and includes 58 cases of TNBC. All samples were obtained through surgical procedure, 31 (53.45%) cases from incision biopsy and 27 (46.55%) cases from mastectomy. Inclusion criteria were TNBC cases with adequate clinical data, available and undamaged formalin-fixed paraffin-embedded tissue block with sufficient tumor tissue. Detailed clinical data were obtained from medical records or pathology archives consisting of age, menopausal status, and stage. Histological type, grade, and mitotic count were determined independently by researchers through hematoxylin and eosin stained slides examination.

### *Immunohistochemistry protocol and interpretation*

The tissue sections were deparaffinized and rehydrated before pretreatment. Endogenous peroxidase was blocked with hydrogen peroxide followed by antigen retrieval. Keratin 5 (clone XM26, Thermo Scientific, Cheshire, UK) and Keratin 8/18 (clone 5D3, Thermo Scientific, Cheshire, UK) mouse monoclonal antibodies were used as primary antibody. Diagnostic BioSystems (Diagnostic BioSystems, Pleasanton, CA, USA) polymer kit was used for detection. The reaction was visualized with diaminobenzidine and counterstained with Mayer's hematoxylin followed by dehydration, clearing, and mounting. Positive control was skin tissue for CK5 and breast for CK8/18. Negative control was obtained by omission of primary antibodies.

CK5 and CK8/18 expressions were determined independently by researchers. Using the Allred scoring method, the percentage of stained cells (none, 0; <1%, 1; 1-10%, 2; 11-33%, 3; 34-66%, 4; 67-100%, 5) and the staining intensity (absent, 0; weak, 1; moderate, 2; and strong, 3) were summed to produces total score of 0 to 8. Score of ≥ 4 was taken as positive expression.

### *STATISTICAL ANALYSIS*

Statistical analysis was performed using SPSS software package version 22.0 (SPSS Inc., Chicago) with 95% confidence interval and Microsoft Excel 2010. Inter-rater reliability (IRR) among researchers for histological parameters and biomarkers expressions were calculated using Fleiss . Continue variables were presented in mean and standard deviation, while categorical variables were presented in frequency and percentage. Independent sample Student t test was applied to compare difference means among normally distributed variables. Pearson's chi square or Fisher's Exact test was applied to find out the association between CK5 and CK8/18 expressions with clinicopathological parameters. The p-values < 0.05 were considered significant.

## RESULTS

### *Patients' characteristics*

The mean age for TNBC patients was 46.9 (±7.69) years and 77.59% of cases were diagnosed at more than 40 years of age with premenopausal women predominance. Of all TNBC cases, 58.62% had stage III disease, 60.34% had T3 tumor, 56.89% with lymph nodes involvement, most of the cases (98.27%) were not accompanied with distant metastasis, 53.45% were of grade 2 tumor, and 44.82% were under low mitotic count category. Invasive carcinoma of no special type (70.69%) was the predominant histological type, but a meaningful number of cases exhibited special histological type, such as medullary (10.34%) and metaplastic (6.89%) features. Other special type carcinomas were invasive lobular carcinoma, carcinoma with apocrine differentiation, carcinoma with neuroendocrine feature and

mucinous carcinoma (Figure 1). IRR among 3 researchers revealed an almost perfect agreement for histological type, grade, and mitosis ( = 0.84, 0.93, and 0.89, respectively). Clinicopathological features were summarized in Table 1.

**Table 1. Clinicopathological features of triple negative breast cancers**

| Variable | Number of cases (%) |
|---|---|
| Age, mean ± SD (years) | 46,9 ± 7,69 |
| ≤40 | 13 (22.41) |
| >40 | 45 (77.59) |
| Menopausal status | |
| Pre-menopause | 45 (77.59) |
| Post-menopause | 13 (22.41) |
| Clinical stage | |
| Stage I | 0 |
| Stage II | 23 (39.65) |
| Stage III | 34 (58.62) |
| Stage IV | 1 (1.72) |
| Tumor size | |
| T1 | 0 |
| T2 | 3 (5.17) |
| T3 | 35 (60.34) |
| T4 | 20 (34.48) |
| Lymph node involvement | |
| Negative (N0) | 25 (43.11) |
| Positive (N1-3) | 33 (56.89) |
| Distant Metastasis | |
| M0 | 57 (98.27) |
| M1 | 1 (1.72) |
| Histological type | |
| Non-special | 42 (72.41) |
| Special | 16 (27.59) |
| Histological grade | |
| Grade 1 | 1 (1.72) |
| Grade 2 | 31 (53.45) |
| Grade 3 | 26 (44.82) |
| Mitosis | |
| ≤7/10 HPF | 26 (44.82) |
| 8-14/10 HPF | 21 (36.2) |
| ≥15/10 HPF | 11 (18.96) |

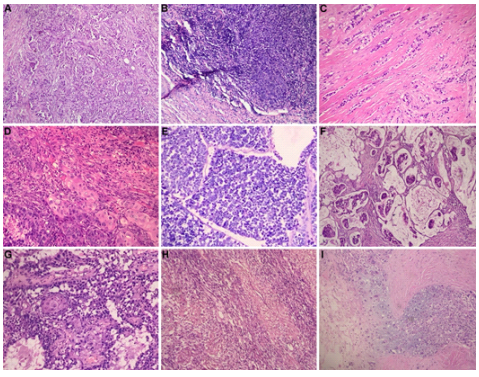

**Figure 1.** Histological type. A, Invasive carcinoma NST. B, Carcinoma with medullary feature. C, Invasive lobular carcinoma. D, Carcinoma with apocrine differentiation. E, Carcinoma with neuroendocrine feature. F, Mucinous carcinoma. G, Adenosquamous carcinoma. H, Spindle cell carcinoma. I, Metaplastic carcinoma with mesenchymal differentiation.

Basal-like subtype identified by positive CK5 expression was observed in 43.1% of TNBC cases, while negative CK8/18 expression was seen in 13.8% of cases (Table 2, Figure 2). The majority of cases (48.27%) were negative for CK5 and positive for CK8/18. Interestingly, 37.93% cases revealed co-expression of both biomarkers, of which 22 of 25 basal-like TNBC cases in this study showed positive expression of CK8/18. IRR among 3 researchers revealed a substantial agreement for CK5 expression ( = 0.80) and almost perfect agreement for CK8/18 expression ( = 0.87).

**Table 2. Samples distribution based on CK5 and CK8/18 expressions**

| Ekspresi imunohistokimia | Number of cases (%) |
|---|---|
| CK5expression | |
| Positive | 25 (43.1) |
| Negative | 33 (56.9) |
| CK8/18expression | |
| Positive | 50 (86.2) |
| Negative | 8 (13.8) |
| Combination of CK5 and CK8/18 expressions | |
| CK5 positive CK8/18 positive | 22 (37.93) |
| CK5 positive CK8/18 negative | 3 (5.17) |
| CK5 negative CK8/18 positive | 28 (48.27) |
| CK5 negative CK8/18 negative | 5 (8.62) |

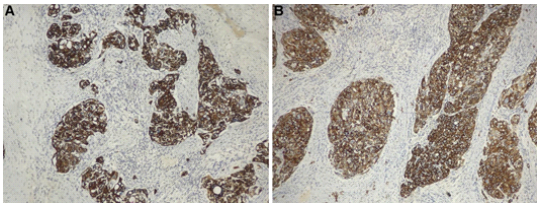

**Figure 2**. Immunohistochemical expression. A, CK5 positive. B, CK8/18 positive

### *Association of CK5 and CK8/18 expressions with clinicopathological parameters*

There was no significant age difference between the positive and negative CK5 expression groups. CK5 expression did not show any significant association with age group, menopausal status, clinical stage, tumor size, lymph node involvement, distant metastasis, histological type, and grade ($p > 0.05$). However, CK5 expression was found to be significantly associated with mitotic count ($p = 0.032$), which TNBC cases with mitosis ≥15/10 HPF have a greater tendency to demonstrate positive expression of CK5 (Table 3).

**Table 3. Association of CK5 expression with clinicopathological features of TNBC**

| Variable | CK5 expression | | p |
|---|---|---|---|
| | Positive (n = 25) | Negative (n = 33) | |
| Age | | | |
| Mean ± SB (years) | 46.28 ± 7.86 | 47.36 ± 7.65 | 0.6 |
| ≤40 | 6 (46.15) | 7 (53.85) | 0.801 |
| >40 | 19 (42.22) | 26 (57.78) | |
| Menopausal status | | | |
| Pre-menopause | 20 (44.44) | 25 (55,56) | 0.701 |

| | | | |
|---|---|---|---|
| Post-menopause | 5 (38.46) | 8 (61,54) | |
| Clinical stage | | | |
| Stage I | 0 | 0 | 0.556 |
| Stage II | 11 (47.83) | 12 (52.17) | |
| Stage III | 14 (41.17) | 20 (58.82) | |
| Stage IV | 0 | 1 (100) | |
| Tumor size | | | |
| T1 | 0 | 0 | 0.366 |
| T2 | 2 (66.67) | 1 (33.33) | |
| T3 | 16 (45.71) | 19 (54.29) | |
| T4 | 7 (35) | 13 (65) | |
| Lymph node involvement | | | |
| Negative (N0) | 12 (48) | 13 (52) | 0.512 |
| Positive (N1-3) | 13 (39.39) | 20 (60.61) | |
| Distant metastasis | | | |
| M0 | 25 (43.86) | 32 (56.14) | 1.000 |
| M1 | 0 | 1 (100) | |
| Histological type | | | |
| Non-special | 15 (35.71) | 27 (64.29) | 0.066 |
| Special | 10 (62.5) | 6 (37.5) | |
| Histological grade | | | |
| Grade 1 | 0 | 1 (100) | 0.136 |
| Grade 2 | 11 (35.48) | 20 (64.52) | |
| Grade 3 | 14 (53.85) | 12 (46.15) | |
| Mitosis | | | |
| ≤7/10 HPF | 7 (26.92) | 19 (73.08) | 0.032* |
| 8-14/10 HPF | 10 (47.62) | 11 (52.38) | |
| ≥15/10 HPF | 8 (72.73) | 3 (27.27) | |
| * p < 0.05 | | | |

Age did not differ significantly in both positive and negative CK8/18 expression groups. Unfortunately, there was also no significant association between all clinicopathological features and CK8/18 expression (p > 0.05) (Table 4).

**Table 4. Association of CK8/18 expression with clinicopathological features of TNBC**

| Variable | CK8/18 expression | | p |
|---|---|---|---|
| | Positive (n = 50) | Negative (n = 8) | |
| Age | | | |
| Mean ± SD (years) | 47.18 ± 7.56 | 45.13 ± 8.81 | 0.488 |
| ≤40 | 11 (84.62) | 2 (15.38) | 1.000 |
| <40 | 39 (86.67) | 6 (13.33) | |
| Menopausal status | | | |
| Pre-menopause | 38 (84.44) | 7 (15.56) | 0.669 |
| Post-menopause | 12 (92.31) | 1 (7.69) | |
| Clinical stage | | | |
| Stage I | 0 | 0 | 0.700 |
| Stage II | 19 (82.61) | 4 (17.38) | |
| Stage III | 31 (91.18) | 3 (8.82) | |
| Stage IV | 0 | 1 (100) | |
| Tumor size | | | |
| T1 | 0 | 0 | 0.241 |
| T2 | 3 (100) | 0 | |
| T3 | 28 (80) | 7 (20) | |
| T4 | 19 (95) | 1 (5) | |
| Lymph node involvement | | | |
| Negative (N0) | 20 (80) | 5 (20) | 0.272 |
| Positive (N1-3) | 30 (90.9) | 3 (9.1) | |
| Metastasis jauh | | | |
| M0 | 50 (87.72) | 7 (12.28) | 0.138 |
| M1 | 0 | 1 (100) | |
| Tipe histologi | | | |
| Non-special | 37 (88.1) | 5 (11.9) | 0.672 |
| Special | 13 (81.25) | 3 (18.75) | |
| Grade histologi | | | |
| Grade 1 | 1 (100) | 0 | 0.446 |
| Grade 2 | 28 (90.32) | 3 (9.68) | |
| Grade 3 | 21 (80.77) | 5 (19.23) | |
| Mitosis | | | |
| ≤7/10 LPB | 24 (92.31) | 2 (7.69) | 0.167 |
| 8-14/10 LPB | 18 (85.71) | 3 (14.29) | |
| ≥15/10 LPB | 8 (72.73) | 3 (27.27) | |

## DISCUSSION

TNBC can be divided into basal-like dan non basal-like.14 Positivity of CK5 (or CK5/6) is the most commonly used basal cell marker, either singly or in conjunction with CK14 dan CK17.[15] Basal-like subtype determined by CK5 expression was found in 43.1% of TNBC cases in this study. This subtype proportion varied from 6% to 81% in other studies.[16-20] The discrepancy between these studies may be due to the difference in the number of samples.

Basal-like subtype has a worse prognosis compared to non basal-like subtype. This can be seen from the tendency of basal-like subtype to occur in younger age, pre-menopause women, with larger tumor size, less frequent axillary lymph node involvement, more frequent visceral metastases, higher histological grade, brisk mitosis, and poorly differentiated invasive carcinoma.[21-23]

In the current study, the mean age of TNBC patients with positive CK5 expression was slightly younger than negative group, but this difference was not statistically significant and it was consistent with the two previous studies conducted by Rusminan et al. and Riddi et al.[17,24] However, Santosa et al. reported a significant age difference between CK5/6 positive and negative groups (p = 0.026).[20]

Several studies have evaluated risk factors associated with basal-like TNBC, such as a higher parity, lack of breastfeeding, obesity, and young age at first childbirth.[22,25,26] Although all of these risk factors were not evaluated, but all basal-like subtype carcinomas in this study were in reproductive age group.

Mitosis is the only clinicopathological feature that was found to be associated with CK5 expression (p = 0.032) in this study and this is contrary to Rusminan et al. study.24 All other clinicopathologic parameters did not significantly associate with CK5 expression, which is in line with most previous studies.[17,19,20,24,27] However, other studies found a significant association between CK5/6 expression with histological grade and tumor size (p = 0.007 and 0.033, respectively).[16,18]

Although there is a strong relationship between basal-like subtype based on gene expression profiling (GEP) and basal cell markers, not all tumors identified as basal-like subtype express basal CKs

and vice versa. Nonetheless, the classification of breast carcinoma based on GEP is difficult to apply widely in clinical practice because of its extremely expensive cost. Consequently, the classification of basal-like subtypes is often based on immunohistochemical techniques to assess the expression of biomarkers.[15] Nielsen et al. first proposed the definition of basal-like subtype as a tumor exhibiting negative expression of ER, PR, HER2 but positive against CK5/6 and/or EGFR.28 This panel has been widely used and showed a sensitivity of 76% and a specificity of 100% for basal-like subtypes.29 Some authors believe the addition of CK14 to the panel may provide a better definition of basal-like subtypes.[15]

Basal CK expression is sometimes difficult to assess because of its weak and focal reactivity. In addition, different clones of basal CK used in various studies and variety of expression patterns also add to the complexity in translating the gene expression profile results into immunohistochemical panels to identify basal-like subtypes.[15] According to Abdelrahman et al., the use of a single basal cell marker might lose about half the cases of basal-like subtypes.18 This indicates that CK5 negativity does not necessarily rule out the basal-like subtype. Therefore, we recommend the use of other basal markers to help ensure basal-like subtypes.

In this study, 56.9% of TNBC cases showed TNBC negative CK5 expression. These may also be part of other proposed TNBC subtypes, including molecular apocrine and claudin low.[30] In addition, recent molecular analysis studies have shown that TNBC is a very heterogeneous tumor group. Lehmann et al., Burstein et al., and Jézéquel et al. have reported various molecular subclassifications of TNBC and their association with prognostic indicators.[31-33]

CK8/18 is less commonly used than CK5 in TNBC. Generally, several studies have reported an association between the lack or low expression of CK8/18 and TNBC subtype, breast carcinoma with BRCA1 mutation, and worse prognosis.1[1,12,34] Of 58 TNBC cases in this study, 8 cases (13.8%) showed negative CK8/18 expression and 3 cases of which showed positive CK5 expression. According to Mulligan et al., negative CK8/18 expression along with basal-like phenotype and family history may improve the ability to identify tumors with BRCA1 germline mutations, which may help selection of breast carcinoma patients requiring genetic testing.[34]

There was no significant association between CK8/18 expression and all observed clinicopathological features. This is in line with Yadav et al. and Hashmi et al.35,36 In contrast to Woelfle et al., low CK8/18 expression was found to be associated with larger tumor size ($p = 0.008$), higher grade and mitotic count ($p = 0.0001$).11 Aiad et al. also reported a significant association of loss of CK8/18 expression with higher grade ($p = 0.05$) and mitotic count ($p = 0.033$).[12] The discrepancies between these studies are most likely due to the different interpretation of CK8/18 expression. Furthermore, Cimpean et al. and Aiad et al. have described several patterns of CK8/18 expression in breast carcinoma, i.e. diffuse cytoplasmic and membrano-cytoplasmic pattern.[12,37]

CK8/18 expression was observed in the majority (88%) of basal-like carcinoma identified by CK5 expression. This indicates a mixed basal and luminal phenotype. Livasy et al. who developed Nielsen et al. panel by adding CK8/18 also reported similar results.38 Co-expression of basal and luminal cell CKs suggests that these tumors may derived from stem cells that undergo various degrees of basal and luminal differentiation. Immunofluorescence study has clearly demonstrated that progenitor cells (CK5/14+) will differentiate into luminal cells (CK8/18+) and myoepithelial cells (SMA+) through luminal intermediate/progenitor cells (CK5/14+, CK8/18+) and myoepithelial intermediate/progenitor cells (CK5/14+, SMA+).[39] Based on this differentiation lineage, it can be concluded that the origin of most basal-like TNBC, especially in this study, is luminal intermediate/progenitor cell. Supporting this conclusion, GEP for basal-like carcinoma more closely resembles that of normal luminal progenitor cells. This suggests that these cells are the target population of basal-like subtype carcinomas, although this is still debatable to date.40 Hence further studies of the stem cells and proliferative lesions as a precursor of breast carcinoma might be important to be evaluated to prove this allegation.

## CONCLUSION

CK5 and CK8/18 expressions showed no significant association with observed clinicopathological parameters in TNBC. However, CK5 was significantly associated with mitosis.

## COMPETING INTERESTS:

The authors have no relevant financial interest in the products or companies described in this article.

## AUTHORS' CONTRIBUTIONS:

ALW, main author of manuscript, contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. D involved in drafting the manuscript or revising it critically for important intellectual content. B involved in drafting the manuscript and contributed to sample collection, reagents, materials, and analysis tools. All authors read and approved the final manuscript.

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## ETHICAL APPROVAL:

Health Research Ethical Committee, University of Sumatera Utara, Medan, Indonesia approved this study.

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