Abstract

Objective: Triple negative breast cancer (TNBC), despite being the uncommon subtype, contributes a major portion to mortality and associated with poor prognosis. The purpose of this study was to evaluate the cytological criteria for the diagnosis of TNBC through fine-needle aspiration cytology (FNAC). Material and Method: Clinical, cytological, histological, and immunohistochemical (IHC) data of 256 patients were evaluated, and patient were classified as TNBC and non-TNBC phenotype by IHC. All cytological specimens were reviewed for 12 criteria: cellularity, tubule/gland formation, syncytial clusters, large bare nuclei, nuclear atypia, chromatin pattern, cell borders, nucleolus, cytoplasm, lymphocytic infiltrate, calcification, and necrosis. The Fischer’s exact test was used to show test association. Result: Out of 256 patients, 82 patients were TNBC, and 174 patients were non-TNBC. TNBC phenotype showed statistically significant association to cellularity, tubule/gland formation, syncytial cluster formation, bare nuclei, nuclear atypia, cell borders, lymphocyte infiltration, and necrosis. Conclusion: FNAC can be helpful in making diagnosis of TNBC and along with ER, PR, HER2 characterization, helpful in planning treatment strategy, saving time, manpower, and resources in the patient management.

Keywords: Breast carcinoma, estrogen receptor, fine needle aspiration cytology, progesterone receptor, triple negative breast cancer

Introduction

Breast cancer is a heterogeneous disease and consists of several distinct histopathological, immunohistochemical (IHC), and gene expression based subtypes.[1] Gene expression based studies using DNA microarray have superseded the traditional histological classification as the genetic/molecular classification not only classify the breast cancer but also have prognostic and therapeutic implications also.[3]

DNA microarray profiling has classified breast carcinoma into 5 subtypes. Luminal A: ER+, PR+, and HER2-; Luminal B: ER+, PR+, and HER2+; Basal like: ER-, PR-, and HER2-; HER2 enriched: ER-, PR-, and HER2+, and normal breast like.[3,4]

Although gene expression DNA microarray studies are the gold standard to identify and classify breast carcinoma, major disadvantage being sophisticated, expensive, and need expertise.[5] Contrary to it, the IHC method is cheaper, easy to reproduce, and can be easily performed as a routine procedure in laboratories; hence, many researchers attempted to use IHC result interchangeable to gene expression profiling.[6]

The term basal-like and triple negative breast cancer (TNBC) most often used interchangeably as there is overlap of clinical and biological behavior of these two tumor, but they are not synonymous.[7] Approximately, 75% of basal-like are triple negative, but 25% of them may express HER2 or hormone receptor.[3] TNBC are characterized by lack of expression of ER, PR, and HER2 with or without high Ki-67 index, whereas basal-like cancers are positive for basal markers such as 5/6 and 17.[3,4] TNBC accounts for 10%–25% of all breast cancer and are characterized by more aggressive behavior and poor prognosis; hence, an accurate diagnosis is must before any therapeutic intervention started.[9]
Fine-needle aspiration cytology (FNAC) is widely used as a screening procedure to detect breast cancer at an early stage. FNAC is a safe, fast, inexpensive, easy, and reproducible test that can accurately diagnose carcinoma thus can guide clinician for the treatment protocol including breast conservation therapy. A well-fixed cytological specimen adjacent with cell block can be an important diagnostic tool for ICH and other molecular assay tool, and cytomorphological analysis may many times help in predicting the molecular phenotype of breast cancer.[10-14]

There are several studies describing the clinical and histological features of the TNBC, but only few studies describe the cytological features of TNBC.[14-17] The study was conducted with an aim to evaluate the cytomorphological characters of TNBC and to compare with those of non-TNBC types.

**Materials and Methods**

The current study was a retrospective study conducted on 256 patients of breast carcinoma from a period of July 2009 to July 2017 operated for breast malignancy. The data of patients operated for breast cancer were retrieved. The tumors in which the IHC markers were available were included in the study. FNA specimen, histological, and IHC sections (ER, PR expression, HER2 over expression, and Ki-67 index) of all cases were analyzed.

Hematoxylin and eosin and Papanicolaou (PAP) stained cytological smears were reviewed again by two pathologists who were blinded to the hormone receptor and HER2 status. Following cytological features were included for analysis: cellularity (scant, +; moderate, ++; or cellular, +++), tubule/gland formation (defined as group of polarized cells forming tubule or gland-like structures: absent, 0; few, +; or many, ++), syncytial clusters (defined as groups of cells with inconspicuous, intercellular membranes, and nuclear overlapping: absent, 0; few, +; or many, ++), large bare nuclei (defined as large single high grade malignant nuclei without cytoplasm: absent, 0; few, +; or many, ++), nuclear atypia (mild, +; moderate, ++; or marked, +++), chromatin pattern (fine or hyperchromatic), cell borders (well-defined or ill-defined), nucleolus (absent, 0; few, +; or many, ++), cytoplasm (scant or abundant), lymphocytic infiltrate (absent, 0; few, +; or many, ++), calcification (present or absent), and necrosis (absent, 0; few, +; or many, ++).

Histological findings were analyzed for the following parameter: histological type and modified Bloom Richardson grade, lymph-node status, margins, necrosis, and stromal lymphoid aggregates.

For the study, TNBC were defined as those that were immuno-histochemically ER, PR, and HER2 negative. Non-TNBC were defined as those that were positive for any of these markers.

Hormone receptor analysis was performed on specimen. Formalin fixed paraffin embedded blocks were recut at a thickness of 4 micron on Poly –L lysine coated slide followed by deparaffinization and rehydration into the descending grades of alcohol into water. Antigen retrieval was done using citrate buffer using pressure cooker heating method. Sections were stained for ER, PR, HER-2, and Ki-67 according to the protocol provided by the manufacturer. The clones used were as follow, ER- RBT11, PR- RBT22, HER2- RBT-HER2, and Ki-67-EP5, bio SB, Santa Barbara, CA 93110 U.S.A.

ER and PR results were screened manually. The percentage of malignant cells with positive nuclear staining and intensity of staining (0, negative staining; +, weak staining; +++, intermediate staining; or +++ strong staining) were recorded for each case and interpreted as positive when >1% tumor cells show positive nuclear staining. All cases with negative staining were regarded as negative. HER2 staining results were scored as follows: 0, no membrane staining; +, partial membrane staining in >10% of tumor cells with no complete circumferential staining; ++, circumferential membrane staining in >10% of tumor cells, and ++++, circumferential membrane staining in >30% of tumor cells. For statistical analysis, cases with +++ staining were considered positive.

The Ki-67 index is a non-histone nuclear protein present in increased number in proliferating cells. A minimum of 500 malignant cells were examined, percentage of positive stained cells were recorded, and a cut-off of 20% was considered as high Ki-67 index. Analysis of all the data of each case was done, the Fischer’s exact test was used to compare TNBC and non-TNBC for each cytological variable, and a $P < 0.05$ was considered statistically significant.

**Results**

Out of total 256 cases, 82 cases (32%) tumors were identified triple negative after IHC examination. The mean age of patient recruited was 46.2 years. The mean age of menarche was 12 years in cases of TNBC group as compared to 13 years in non-TNBC group. The mean age of birth was 20.3 years as compared to 20.9 years in non-TNBC group. Majority of the patient belong to the 40–60 years age group (51.2% in TNBC vs. 47.15% in non-TNBC group). On comparing the clinical data of the groups for risk factors such as age of the patient, age of menarche, age at first childbirth, and history of oral contraceptive pill (OCP) intake, there were no statistically significant differences between the two groups [Table 1]. Menopausal status evaluation revealed more premenopausal patients in the TNBC group compared to non–TNBC, and the difference was statistically significant. Similarly, tumor size more than 5 cm was observed in TNBC (75.6%) as compared with non-TNBC (56.3%), and the finding was statistically significant. Positive axillary lymph node was observed in 80.5% cases in TNBC as compared to 63.2% in non-TNBC group, and the data was significant statistically. On modified Bloom Richardson grading of the tumor, the tumors in TNBC group (31.7% cases) were of higher than grade 3 as compared to non TNBC (16.1%), and the difference was statistically significant. Local recurrence was significantly more common in...
TNBC group (9.7%) than the non-TNBC group (4.6%), but this was statistically not significant. The histological classification of TNBC and non-TNBC was shown in Table 2. Majority cases from both categories were diagnosed as infiltrating duct carcinoma not otherwise specified (NOS). Comparison of cytological characters of TNBC and non-TNBC showed statistically significant difference on Fisher’s exact test \((P < 0.05)\) [Table 3]. TNBC group had more cellular smears, more syncytial cell cluster, scant tubule/gland formation as compared to non-TNBC \((P < 0.05)\). Similarly, large bare nuclei, nuclear atypia, ill-defined borders, necrosis, and lymphocytic cell infiltrate were more common in TNBC group as compared to non-TNBC group [Figure 1], and these were significant statistically. Although multiple nucleoli were present in TNBC group, the data were not significant statistically. Similarly, no significant difference was observed for chromatin pattern, cytoplasm character, and calcification.

**Discussion**

TNBC contributes a major portion in mortality despite being its small proportion among all breast cancers. Although a lot has been studied about the clinical characteristics, histological, and IHC features of TNBC, only a few studies have been done in relation to cytomorphological features of the TNBC.\[14\] In the current study, we analyze the clinical as well as cytomorphological features of TNBC.

The prevalence of TNBC ranges between 27%–35% with an average of 31% in different previous studies.\[8,19\] Current study also had a prevalence rate of 32% for TNBC category. The mean age of presentation in current study was 46 years, which was slightly younger than the one described in western data but similar to studies on Indian subcontinent patients.\[8,19\]

Axillary lymph node involvement (80.5%), tumor size more than 5 cm at the time of presentation (75.6%), increase relapse rate (9.7%), higher visceral metastasis (65.9%), and high modified Bloom Richardson’s histological grade (grade 3) (31.7%) in cases of TNBC and all the data were comparable with other studies.\[20-22\]

Current study analyzed the TNBC and non-TNBC, on the basis of the following criteria’s such as cellularity, tubules/gland formation, syncytial clusters, large bare nuclei, nuclear atypia, chromatin pattern, cell borders, nucleoli, cytoplasm characteristics, lymphocytic cell infiltrate, calcification, and necrosis. According to study of Duflot et al., necrosis, prominent nucleoli, and abundant cellularity are the criteria more frequently associated with basal phenotype of breast carcinoma.\[14\] Similarly, other studies observed that nuclear findings such as large or naked nuclei, irregular nuclear margins, multiple nucleoli along with lymphocytic infiltrate, and squamous metaplasia are significantly associated with basal-like tumor than non-basal subtypes.\[22-24\]

In the present study, we demonstrate that increased cellularity, less or absent tubules formation, syncytial clusters formation,
Table 3: Comparison of cytological characters of TNBC and non-TNBC

| Parameter | Status       | TNBC (n=82) | Non-TNBC (n=174) | P         |
|-----------|--------------|-------------|------------------|-----------|
| Cellularity | Scant+       | 04 (4.9%)   | 32 (18.4%)       | 0.01486   |
|           | Moderate++   | 24 (29.2%)  | 44 (25.3%)       |           |
|           | Cellular+++  | 54 (65.8%)  | 98 (56.3%)       |           |
| Tubules/gland formation | Absent | 72 (87.8%) | 76 (43.6%) | <0.00001 |
|           | Few          | 08 (9.7%)   | 36 (20.6%)       |           |
|           | Many         | 02 (2.4%)   | 62 (35.0%)       |           |
| Syncytial clusters | Absent | 06 (7.3%)   | 110 (62.3%)      | <0.00001 |
|           | Few          | 32 (39.0%)  | 34 (19.5%)       |           |
|           | Many         | 44 (53.7%)  | 30 (17.2%)       |           |
| Large bare nuclei | Absent | 16 (19.5%)  | 62 (35.6%)       | 0.000912  |
|           | Few          | 28 (34.1%)  | 58 (33.3%)       |           |
|           | Many         | 48 (58.5%)  | 54 (31.0%)       |           |
| Nuclear atypia | Mild      | 12 (14.6%)  | 72 (41.3%)       | <0.00001  |
|           | Moderate     | 24 (29.3%)  | 78 (44.9%)       |           |
|           | Marked       | 46 (56.1%)  | 24 (13.8%)       |           |
| Chromatin | Fine         | 52 (63.4%)  | 122 (69.5%)      | 0.3159    |
|           | Hyperchromatic | 30 (36.6%) | 52 (29.3%) |           |
| Cell borders | Well-defined | 21 (25.6%) | 124 (71.3%) | <0.0001   |
|           | Ill-defined  | 61 (74.4%)  | 50 (28.7%)       |           |
| Nucleoli | Absent       | 13 (15.8%)  | 24 (14.4%)       | 0.65059   |
|           | Few          | 34 (41.5%)  | 65 (37.3%)       |           |
|           | Many         | 35 (42.7%)  | 85 (48.8%)       |           |
| Cytoplasm | Scant        | 24 (29.3%)  | 58 (33.3%)       | 0.5674    |
|           | Abundant     | 58 (70.7%)  | 116 (66.7%)      |           |
| Lymphocyte | Absent      | 12 (14.6%)  | 48 (27.6%)       | <0.00001  |
|           | Few          | 25 (30.5%)  | 121 (69.5%)      |           |
|           | Many         | 45 (54.9%)  | 15 (8.6%)        |           |
| Calcification | Absent     | 77 (93.9%)  | 158 (90.8%)      | 0.4724    |
|           | Present      | 05 (6.1%)   | 16 (9.2%)        |           |
| Necrosis | Absent       | 24 (29.2%)  | 105 (60.3%)      | <0.00001  |
|           | Few          | 28 (34.1%)  | 48 (27.6%)       |           |
|           | Many         | 30 (36.6%)  | 21 (12.1%)       |           |

In conclusion, FNAC can be helpful in making diagnosis of TNBC and along with ER, PR, and HER2 characterization, helpful in planning treatment strategy, saving time, manpower, and resources in the patient.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES
1. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406:747-52.
2. Sorlie T. Molecular portraits of breast cancer: Tumour subtypes as distinct disease entities. Eur J Cancer 2004;40:2667-75.
3. Krishnamurthy S, Poornima R, Challra VR, Goud YG. Triple negative breast cancer - our experience and review. Indian J Surg Oncol 2012;3:12-6.
4. Geyer FC, Rodrigues DN, Weigelt B, Reis-Filho JS. Molecular classification of estrogen receptor-positive/luminal breast cancers. Adv Pathol 2012;19:39-53.
5. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: A critical review. J Clin Oncol 2008;26:2568-81.
6. Dogra A, Doval DC, Sardana M, Chedi SK, Mehta A. Clinicopathological characteristics of triple negative breast cancer at a tertiary care hospital in India. Asian Pac J Cancer Prev 2014;15:10577-83.
7. Yamamoto Y, Ibusuki M, Nakano M, Kawase T, Hiki R, Iwase H. Clinical significance of basal-like subtype in triple-negative breast cancer. Breast Cancer 2009;16:260-7.
8. Saha A, Chattopadhyay S, Azam M, Sur PK. Clinical outcome and pattern of recurrence in patients with triple negative breast cancer as compared with non-triple negative breast cancer group. Clin Cancer Invest J 2012;1:201-5.
9. Gupta A, Jain J, Kumar A, Kumar S, Wadhwa N. Triple negative breast cancer - An overview and review of literature. Asian J Med Sci 2012;3:16-20.
10. Collins BT, Garcia TC, Hudson JB. Effective clinical practices for improved FNA biopsy cell block outcomes. Cancer Cytopathol 2015;123:540-7.
11. Billgren AM, Tani E, Liedberg A, Skoog L, Rutqvist LE. Prognostic significance of tumor cell proliferation analyzed in fine needle aspirates from primary breast cancer. Breast Cancer Res Treat 2002;71:161-70.
12. LoFgren L, Skoog L, Von Schoultz E, Tani E, Isaksson E, Fernstad R, et al. Hormone receptor status in breast cancer-a comparison between surgical specimens and fine needle aspiration biopsies. Cytopathology 2003;14:136-42.
13. Marinšek ZP, Nolde N, Kardum-Skelin I, Nizzoli R, Onal B, Rezanko T, et al. Multinational study of oestrogen and progesterone receptor immunocytochemistry on breast carcinoma fine needle aspirates. Cytopathology 2013;24:7-20.
14. Dufloth RM, Alves JM, Martins D, Vieira DS, Chikota H, Zeferino LC, et al. Prognostic significance of tumor cell proliferation analyzed in fine needle aspirates from primary breast cancer. Breast Cancer 2009;16:260-7.
15. Breuer J, Lochmuller H, Horninger W, Niederle B, Klotz L, Hiebl H, et al. Clinical significance of basal-like subtype in triple-negative breast cancer. Breast Cancer Res Treat 2002;71:161-70.
16. LoFgren L, Skoog L, Von Schoultz E, Tani E, Isaksson E, Fernstad R, et al. Molecular portraits of human breast tumours. Nature 2000;40:747-52.
17. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. Lancet 2003;14:136-42.
18. Marinkovic Z, Vukotic M, Popovic M, Jankovic V, Jovanovic B, Petrovic B, et al. Clinicopathological characteristics of triple negative breast cancer in people of North East India: Asian Pac J Cancer Prev 2014;15:10577-83.
19. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA,
et al. Triple-negative breast cancer: Clinical features and patterns of recurrence. Clin Cancer Res 2007;13:4429-34.
20. Nabi MG, Abangar A, Wahid MA, Kuchay S. Clinicopathological comparison of triple negative breast cancers with non-triple negative breast cancers in a hospital in North India. Niger J Clin Pract 2015;18:381-6.
21. Sen S, Gayen R, Das S, Maitra S, Jha A, Mahata M. A clinical and pathological study of triple negative breast carcinoma: Experience of a tertiary care centre in eastern India. J Indian Med Assoc 2012;110:686-9.
22. Ambroise M, Ghosh M, Mallikarjuna VS, Kurian A. Immunohistochemical profile of breast cancer patients at a tertiary care hospital in South India. Asian Pac J Cancer Prev 2011;12:625-9.
23. Akashi S, Kuwabara H, Kurisu Y, Takahashi Y, Yasuda E, Takeshita A, et al. Fine-needle aspiration cytology of triple-negative basal-like breast cancer. Diagn Cytopathol 2013;41:283-7.
24. Ishihara A, Tsuda H, Kitagawa K, Yoneda M, Shiraishi T. Morphological characteristics of basal-like subtype of breast carcinoma with special reference to cytopathological features. Breast Cancer 2009;16:179-85.
25. Bonzanini M, Morelli L, Bonandini EM, Leonardi E, Pertile R, Palma PD. Cytological features of triple negative breast Carcinoma. Cancer Cytopathol 2012;120:401-9.
26. Wakasa T, Nakamura M, Kagiya T, Taniuchi E, Sakurai T, Kakudo K. Loss of cellular cohesion in cytology composes a special subgroup of breast tumors – analyses of 37 cases. Acta Cytol 2014;58:89-95.