RESEARCH ARTICLE

Increased Tumour Infiltration of CD4+ and CD8+ T-Lymphocytes in Patients with Triple Negative Breast Cancer Suggests Susceptibility to Immune Therapy

Bushra Sikandar[1], Muhammad Asif Qureshi[1,2]*, Saima Naseem[1], Saeed Khan[1], Talat Mirza[1]

Abstract

Background: Patients with triple negative breast cancer (TNBC) have limited therapeutic options, largely because the complex tumour environment is not well-characterized. These patients are potential, but largely un-fathomed, candidates for immunotherapy. It is therefore highly relevant to characterize leukocyte complexity in TNBCs. **Objective:** To investigate leukocyte complexity in tumour environment of patients with TNBCs. **Materials and methods:** A total of 104 consecutive breast cancer patients undergoing mastectomy were recruited in the study after ethical approval. Clinico-pathological parameters were recorded and H and E staining was performed to investigate tumour morphology. Receptor status was investigated using antibodies against ER, PgR and Her-2, and patients were classified as having TNBC or non-TNBC tumours (including Luminal A, Luminal B and Her2 overexpressing tumours). Immune-cell infiltration was investigated using special stains and antibodies: α-CD3 (T-lymphocytes), α-CD20 (B-lymphocytes), α-CD4 (helper T-lymphocytes) and α-CD8 (cytotoxic T-lymphocytes). Immune cell densities were quantified as cell/mm^2 using the CAP guidelines. **Results:** Of the 104 breast cancer patients investigated, a total of 27 (26%) had TNBC and 77(74%) non-TNBC. Patients with TNBC showed significantly increased tumour infiltration of lymphocytes (T and B-lymphocytes) compared to the patients with non-TNBC, while myelocytic infiltration was not significantly different in the two groups. Within the TNBC group, infiltration of T-lymphocytes (equal densities of CD4+ and CD8+ T-lymphocytes) was significantly higher compared to B-lymphocytes. **Conclusion:** Patients with TNBC show increased lymphocytic infiltration (more T-lymphocytes compared to B-lymphocytes). This suggests higher immunogenicity of TNBCs and may indicate a higher responsiveness of these cancers to immunotherapy.

Keywords: Breast cancer- immune cell densities- immune infiltrates- triple negative- immunotherapy

Asian Pac J Cancer Prev, **18 (7)**, 1827-1832

Introduction

Breast cancer is the most common malignancy reported in women across the globe (Ferlay et al., 2015; Globocan., 2012). Pakistan has the highest incidence of breast cancer amongst all Asian countries (Menhas et al., 2015; Qureshi et al., 2016). In the year 2012, estimated total of 34,038 (40% of all cancers in women) new breast cancer patients were reported in Pakistan with a mortality of 16,232 (30.8% of cancer associated death in women) patients (Globocan., 2012). More alarmingly, prevalence of young age breast cancer is dramatically increasing in Pakistan, (Bhurgri et al., 2000; Hashmi et al., 2014), suggesting a potentially distinct tumour-predisposing genetic and socio-demographic profile in this region.

Histologically, breast cancers have been classified into more than 15 different subtypes by the world health organization, indicating a high heterogeneity of disease morphology (Zardavas et al., 2015). While many of the histological subtypes are not clinically relevant, classification of breast cancers into (a) luminal A, (b) luminal B, (c) Her2 over-expressing and (d) triple negative breast cancers, are considered to better reflect therapeutic and prognostic outcomes (Zardavas et al., 2015; Goldhirsch et al., 2013).

Triple negative breast cancers (TNBCs) are a distinct sub-type of breast cancers which do not express estrogen receptor (ER), progesterone receptor (PgR) and Her2 (Hudis et al., 2011; Dietze et al., 2015). Of the total breast cancer patients, approximately 15% are reported to have TNBC (Tan et al., 2009; Faheem et al., 2012), however they are more common in females with African descent (Dietze et al., 2015). In Pakistan, at least one of the functional cancer registries reported that a total of 30.5%

*[1]Department of Pathology, Dow International Medical College, Dow Diagnostic Research and Reference Laboratory, Dow University of Health Sciences Karachi, Pakistan, [2]Institute of Translational Immunology and Research Centre for Immunotherapy, Johannes Gutenberg University of Mainz, Germany. *For Correspondence: asif@asifqureshi.com. Bushra Sikandar and Muhammad Asif Qureshi have equal contribution in this study.*

of the breast cancers are TNBCs and that more than 50% of these TNBCs are diagnosed in females younger than 40 years of age (Bhatti et al., 2014).

Due to limited therapeutic options, TNBCs are high-mortality tumours with increased metastatic potential to liver, brain and lung (Haffty et al., 2006). Importantly, TNBCs exhibit a distinct racial and social distribution with increased prevalence in Afro-American and Asian women (Morris et al., 2007; Kakarala et al., 2010; Cheng et al., 2013). While exact mechanistic events responsible for this racial variation are not completely known, it is plausible to envisage that a distinct genetic and/or socio-ethnic tumour pre-disposing profile plays a role in a differential prevalence of these tumours.

Patients with TNBCs are potential, but largely un-fathomed, candidates for immunotherapy. While a possible role of tumour infiltrating lymphocytes (and other immune cells) has been widely investigated in pathogenesis and outcomes of conventional breast cancer phenotypes (including infiltrating ductal carcinoma) (DeNardo et al., 2001; Demeria et al., 2010; Ruffell et al., 2012; DeNardo et al., 2007), leukocyte complexity of TNBCs are largely un-explored. There are some preliminary data (based only on H and E staining) to demonstrate lymphocytic infiltration in tumour environment of TNBCs and that the tumour-infiltrating lymphocytes potentially affect patients' prognosis (Hashmi et al., 2014; Adams et al., 2015; Pal et al., 2011). However, detailed characterization of TNBC environment for tumour infiltrating immune cells is largely un-addressed. In the study described herein, we have gone onto characterizing/(semi)quantifying the leukocyte complexity of tumour environment of patients with TNBCs. Our data highlight novel avenues for future research to treat a highly fatal breast cancer subtype.

Materials and Methods

Patient recruitment and ethical approval

The study was conducted at the Dow University of Health Sciences Karachi after obtaining ethical approval from the Institutional Review Board, Ref # IRB-460/DUHS/14. A total of 104 subsequent breast cancer patients who were planned to undergo mastectomy were recruited in the study after taking informed consent. Subsequent to surgery, breast tissues with lymph node dissection were transferred to the Histology section in 10% neutral buffer formalin. Samples were examined for gross features (such as tumour size) and paraffin blocks were prepared for subsequent staining and microscopy.

Fixation, tissue processing and microscopy

Tissues were fixed in 10% neutral buffered formalin. Gross examination was performed to record morphological parameters. Specimens were subsequently routinely processed for 12 hours in an automated "Medite TPC 15" tissue processor. The processing steps are as follows: 2 changes of 10% neutral buffered formalin each for 1 hour, 70% alcohol for 1 hour, 80% alcohol for 1 hour, 2 changes of 95% alcohol for 1 hour each, 2 changes of absolute alcohol for 1 hour each, 2 changes of xylene for 1 hour each and finally 2 changes of paraffin wax 1 hour each. Step for blocking was finally performed on TES 99 Medite, automated paraffin embedding station.

After fixation, tissue sections (3 to 4μm in thickness) were cut from the paraffin blocks and processed for H and E staining. Based on microscopic examination of the H and E stained slides, clinic-pathological parameters were recorded including tumor type, tumor grade, percentage of ductal carcinoma in-situ (DCIS) component (either <5%, 5-10% or >10%), lymph node status (as 1-3 positive lymph node or > 3 positive lymph node) and lympho-vascular invasion. Grading of tumor was performed using the Modified Scraff-Bloom Richerdson grading system.

Special Stains (Giemsa and Toluidine blue)

Dissected sections were transferred into water bath (46-48°C). Sections were mounted on a glass slide, marked with representative number. The slides were kept in oven at 60°C for 30 minutes. Sections were de-waxed in two changes of xylene for 5 minutes each. Sections were rehydrated in 100% alcohol, 95% alcohol, 80% alcohol and 70% alcohol, for 2 minutes each and then the slides were plunged in running tap water for 1 minute. Sections were stained with giemsa and toluidine blue for 3 minutes (MERCK, USA). Excess stain was rinsed off by washing the sections in running tap water for 1minute. Sections were then mounted in distrenedibutylphthalatexylene (D.P.X) (BDH, USA) for subsequent microscopy.

Immunohistochemistry quantification of leukocyte densities

In order to identify receptor status of the samples under study, tissue sections were stained using monoclonal antibodies against ER, PgR and Her2 (Cell Marque, USA). 3-4 μm thick paraffin-embedded tissue sections were cut and mounted on histogrip (Zymed Laboratories Inc) (Appendix IX) coated slides, dried at 56ºC for 30 minutes. Specimens were deparaffinized with Xylene, rehydrated in serial graded (100%, 90%, 70%, and 50%) water-ethanol/alcohol solutions and rinsed in deionized water. Target retrieval were performed with target retrieval solution (Cell Marque, USA) in preheated (90-95ºC) water bath for 20 minutes.

For scoring of ER and PgR, Allred scoring criteria was used, whereas Her2 were scored using WHO guidelines (Surgical Case Summary., 2012). Based on the ER, PgR and Her2 staining patterns, samples were either labelled as triple negative (negative for ER, PgR and Her2) or non-triple negative cases.

In order to quantify tumour infiltrating leukocytes, tissue sections were stained using special stains and/or antibodies – including wright Geimsa (for macrophages) (Merck, USA), toluidine blue (for mast cells) (Merck, USA), α-CD 3 antibody (for T-lymphocytes), α-CD4 antibody (for helper T-cells), α-CD8 antibody (for cytotoxic T-cell), α-CD20 (for B-lymphocytes), and H and E (for neutrophils). All antibodies were purchased from Cell Marque, USA. Immune cell densities (cells/mm^2) were calculated using whole slide scanning approach at ×400 magnification using an Olympus microscope as described by the College of American Pathologists (CAP) protocol as previously described (Sikandar et

al., 2015). Photomicrographs of representative tumor sections at×400 were taken for digital annotation using Image J and Irfanview softwares. Data were entered in SPSS version 16 and correlation of immune cell densities were investigated with receptor status using T-test and ANOVA as required. A p-value of <0.05 was considered as statistically significant.

Results

Clinico-pathological parameters of breast cancer patients

Mean age of the patients recruited in our study was 47 years. The youngest patient was 20 years old while the oldest patient was 72 years old. Of the 104 patients that we investigated, a total of 9 had tumor size ≤2cm, 56 had tumour size >2-5cm and 39 had tumour size >5cm. A total of 74 patients had IDC-II, 21 had IDC-III, 5 had IDC-I, 1 had mucinous carcinoma (grade-I), 1 had invasive papillary carcinoma (grade-II), 1 had invasive lobular carcinoma (grade-II) and 1 had metaplastic carcinoma (grade-III). 14 patients showed presences of <5% DCIS component, 13 patients showed 5-10% DCIS component and 4 patients showed >10% DCIS component. 32 patients had 1-3 lymph node positive and 43 of the patients had >3 lymph node positive, whereas 38 patients showed negative lymph node status for tumour metastasis. 42 patients had positive lympho-vascular invasion of the tumour cells (Table 1).

Table 1. Clinico-Pathological Parameters of Patients Included in the Study

Clinico-pathological parameter	Type of breast cancer	
	TNBC (n=27)	non-TNBC (n=77)
Patients' age		
20-29	0	2
30-39	4	17
40-49	10	26
50-59	7	16
60-69	4	13
70-79	2	3
Tumour size		
<2cm	0	9
2-5cm	14	42
>5cm	13	26
Tumour grade		
Grade I	0	6
Grade II	16	60
Grade III	11	11
Lymph node status		
0	10	28
1-3	4	19
>3	13	30
Lympho vascular invasion		
Present	10	32
Absent	17	45
DCIS component		
<5%	1	13
5-10%	5	8
>10%	1	3
Absent	20	53

TNBC, triple negative breast cancer

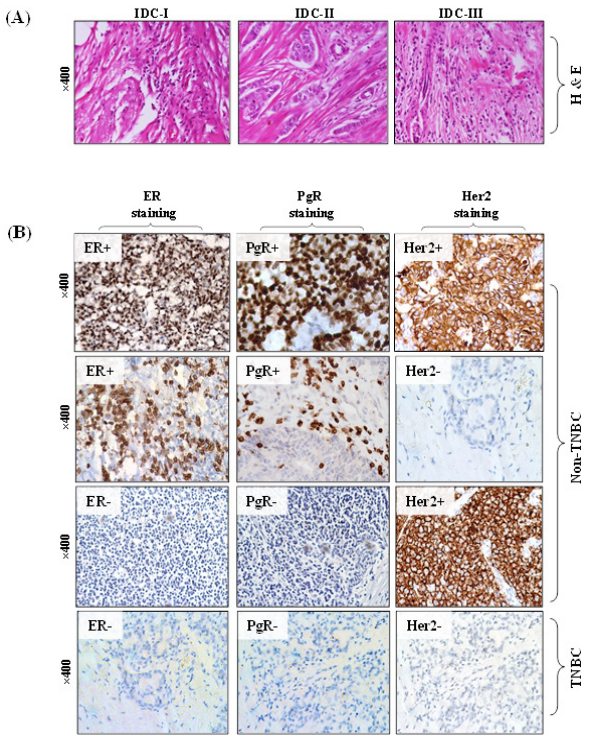

Figure 1. H and E, ER, PgR and Her2 staining. (A), Representative micrographs of original magnification ×400 showing H and E staining of breast cancer tissues; (B), Representative micrographs of original magnification ×400 showing ER, PgR and Her2 staining and classification of breast cancer tissues into TNBC and non-TNBC tissues.

Receptor expression profiling

In order to investigate receptor expression patterns, four categories were defined as (a) tissues that are positive only for Her2, (b) tissues that are positive for both ER and PgR, (c) tissues that are positive for ER, PgR and Her2 and (d) tissues that are triple negative, i.e, ER-, PgR- and Her2- (Figure 1). Of the 104 patients that we investigated, 16 (15%) were only Her2 positive (category a), 37 (35.5%) were positive for ER and PgR expression (category b), 24 (23%) were positive for ER, PgR and Her2 expression (category c) and 27(25%) were triple negative (category d). For subsequent analyses, we further categorized breast cancer patients into two groups, (1) triple negative (category d) and (2) non-triple negative (category a+b+c). In summary, a total of 27 (25.9%) patients had triple negative tumours while 77 (74%) patients had non-triple negative tumours.

Immune cell densities in triple negative and non-triple negative breast cancer tissues

Our data demonstrate significantly higher densities

Table 2. Immune Cell Densities in TNBC and Non-TNBC Tissues

Tumour type	T-cells		B-cells		Neutrophils		Macrophages		Mast cells	
	Cells/mm^2	p	Cells/mm^2	p	Cells/mm^2	p	Cells/mm2	p	Cells/mm^2	p
TNBC	462.5	<0.0001	264.9	<0.001	6.7	0.6	2.7	0.3	6.6	0.7
Non-TNBC	251.8		150.8		6.1		3.9		6.1	

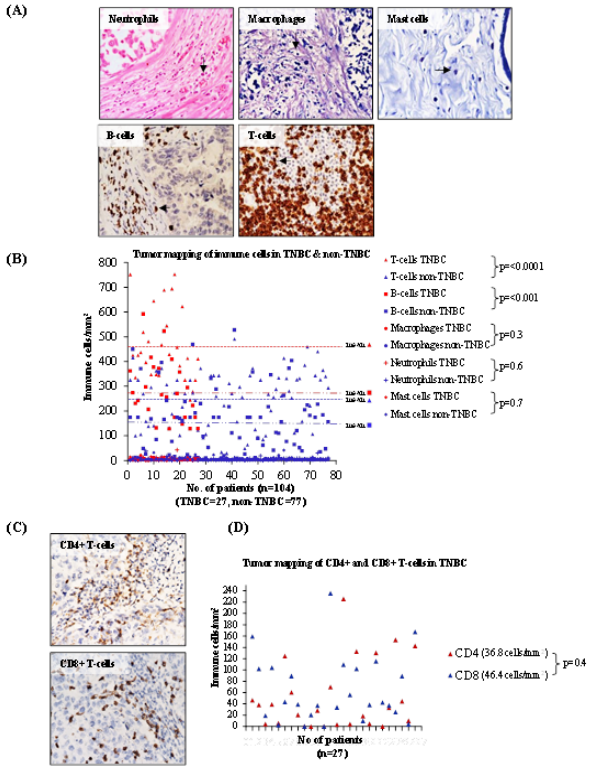

Figure 2. Immune Cell Densities in TNBC and Non-TNBC Tissues. (A), Representative micrographs of original magnification ×400 showing neutrophils, macrophages, mast cells, B-cells and T-cells. (B), Tumour mapping of immune cells/mm² (neutrophils, macrophages, mast cells, B-cells and T-cells) in TNBC and non-TNBC tissues. Y-axis=immune cells/mm², x-axis=no. of patients, each data point indicates one of the 104 patients. (C), Representative microphotographs of original magnification x400 showing CD4+ and CD8+ T-cells in TNBC tissues. (D), Tumour mapping of CD4+ and CD8+ T-cells/mm² in TNBC

of lymphocytes (both B- and T-lymphocytes) in TNBC tissues compared to the non-TNBC group (Table 2). However, infiltration of myeloid cells (neutrophils, macrophages and mast cells) was not significantly different in the two groups (Table 2) (Figure 2). In the triple negative tissues, there was increased infiltration of T-lymphocytes compared to B-lymphocytes. Further analysis of infiltrating T-lymphocyte revealed that there was no significant difference in CD4+ and CD8+ T-lymphocytes infiltration within the TNBC group.

Discussion

In the study described herein, we show increased infiltration lymphocytes in tumour microenvironment of triple negative breast cancers (TNBCs). Interestingly, there was increased infiltration of CD4+ and CD8+ T lymphocytes compared to the B-lymphocytes, suggesting their potential role in tumour progression and/or their utilization as immunotherapeutic modulators.

TNBCs have limited therapeutic options and therefore a major therapeutic challenge in the field of oncology (Bianchini et al., 2016). Due to lack of expression of ER, PgR and Her2, TNBCs are not susceptible to receptor targeted therapies such as Herceptin. While chemotherapy is widely used for TNBC patients, it's response is not long lived and accompanied with a range of cytotoxic side-effects (Gucalp et al., 2011; Carey et al., 2012; Rouzier et al., 2005; Rouzier et al., 2005, Harris et al., 2006). There are preliminary data that patients with lymphocytic infiltration (largely reported only on H and E staining) in TNBCs may benefit from targeted immunotherapy (Stagg et al., 2013). However, lack of detailed knowledge regarding leukocyte complexity (based on immune-specific markers) in TNBCs is the major conundrum in this regard. More recently, targeted therapy using "Pembrolizumab" (programmed cell death protein 1 inhibitor) has been reported (Nanda et al., 2016). However, it's wider use in patients in different geographic areas remains to be investigated. In this context, and in order to design effective targeted therapies for TNBCs, it is highly relevant to dissect the tumour environment and identify cellular and stromal players of therapeutic significance in TNBCs.

In our study, a total of 25% of the enrolled breast cancer patients had TNBCs. Prevalence of patients with TNBCs in Pakistan is not clearly known. However, various registries have reported frequency of TNBCs in a range from 18% to 30% of all breast cancer patients (Hashmi et al., 2014). While the prevalence of TNBCs is highly variable in different parts of the world, it's high prevalence in Asian and Afro-American women is well known (Brewster et al., 2014). However, exact reasons for such geographical variation are not entirely known. It is possible that various genetic and/or sociodemographic factors play differential roles in TNBC pathogenesis in different populations. For example, high prevalence of BRCA1 germline mutations have been reported in Pakistani women (Rashid et al., 2016). It is therefore important to dissect tumour biology of TNBCs in defined ethnic/geographical populations so that targeted therapies could be designed.

Our data demonstrate increased tumour infiltration of T and B lymphocytes in TNBCs as compared to non-TNBC tissues, suggesting a high immunogenicity of these tumours and a possible susceptibility to immunotherapy. Moreover, we did not find differences in CD4+ and CD8+ T-lymphocytes in TNBCs. While descriptive data detailing leukocyte complexity in TNBCs are lacking, CD8+ T-lymphocyte infiltration has been associated with

a better prognosis in TNBC patients (Liu-Shuzhen et al., 2012). However, it is important to note that in this study, CD4+ T-lymphocytes (and other immune cells) were not looked at. Moreover, another study also demonstrated increased infiltration of tumour infiltrating lymphocytes (based on H and E) in TNBCs (Garcia-Teijido et al., 2016; Pruneri G et al., 2015).

Major strengths of our study are at least twofold, (a) we went onto characterize lymphocytes using specific antibodies against B cells and T-cell subtypes and (b) we also investigated myelocyte infiltration in TNBCs and our data show that tumour infiltration of neutrophils, mast cells and macrophages is not different in TNBCs compared to non-TNBC tissues. Contrasting to our findings, increased macrophage infiltration in TNBCs have been previously reported. However, the role of tumour infiltrating macrophages is debatable with available data favouring good as well as bad prognostic outcomes (DeNardo et al., 2007; Qian et al., 2010; Yuan ZY et al., 2014).

Our data identifies novel/relevant information to guide future research to design appropriate targeted immunotherapy for patients with TNBCs. We have identified differential lymphocyte densities in TNBCs compared to the non-TNBC tissues. It is well known that tumour behavior, receptor expression and survival of patients with breast cancer are affected by the subtype, densities and inflammatory mediators released by the tumour infiltrating leukocytes (DeNardo et al., 2007; Loi et al., 2014). Moreover, several targeted immunotherapies for breast cancer patients have been attempted in recent past (Domschke et al., 2016). However, such immune based therapies are in very early stages for TNBC patients, largely because the complex tumour environment is not well-characterized. It would therefore be interesting to further characterize leukocyte subtypes (such as Tregs, innate lymphoid cells, NKT-cells) in TNBC tissues to better understand the pathogenesis and tumour behavior. Taken together, characterization of tumour environment of TNBCs is highly relevant and could unravel novel targets of therapeutic significance.

References

Adams S, Goldstein LJ, Sparano JA, et al (2015). Tumor infiltrating lymphocytes (TILs) improve prognosis in patients with triple negative breast cancer (TNBC). *Oncoimmunology*, **4**, e985930.

Bhatti AB, Khan AI, Siddiqui N, et al (2014). Outcomes of triple-negative versus non-triple-negative breast cancers managed with breast-conserving therapy. *Asian Pac J Cancer Prev*, **15**, 2577–81.

Bhurgri Y, Bhurgri A, Hassan SH, et al (2000). Cancer incidence in Karachi, Pakistan: first results from Karachi cancer registry. *Int J Cancer*, **85**, 325–9.

Bianchini G, Balko JM, Mayer IA, et al (2016). Triple negative breast cancer: challenges and opportunities of a heterogenous disease. *Nat Rev Clin Oncol*, **13**, 674-90.

Brewster AM, Chavez-MacGregor, Brown P (2014). Epidemiology, biology, and treatment of triple-negative breast cancer in women of African ancestry. *Lancet Oncol*, **15**, 625-34.

Carey LA, Rugo HS, Marcom PK, et al (2012). TBCRC 001: Randomized phase II study of cetuximab in combination with carboplatin in stage IV triple-negative breast cancer. *J Clin Oncol*, **30**, 2615–23.

Cheng HT, Huang T, Wang W, et al (2013). Clinicopathological features of breast cancer with different molecular subtypes in Chinese women. *J Huazhong Univ Sci Technolog Med Sci*, **33**, 117–21.

Demaria S, Pikarsky E, Karin M, et al (2010). Cancer and Inflammation: Promise for biological therapy. *J Immunother*, **33**, 335-51.

DeNardo DG, Brennan DJ, Rexhepaj E, et al (2001). Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Can Res*, doi: 10.1158/2159-8274.CD-10-0028

DeNardo D, G.Coussens LM (2007). Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Res*, **9**, 212.

Dietze EC, Sistrun C, Miranda-Carboni G, et al (2015). Triple-negative breast cancer in African-American women: disparities versus biology. *Nat Rev Cancer*, doi: 10.1038/nrc3896.

Domschke C, Schneeweiss A, Stefanovic S, et al (2016). Cellular immune responses and immune escape mechanisms in breast cancer: determinants of immunotherapy. *Breast Care (Basel)*, **11**, 102-7.

Faheem M, Mahmeed H, Khurram M, et al (2012). Estrogen receptor, progesterone receptor, and Her 2 Neu positivity and its association with tumour characteristics and menopausal status in a breast cancer cohort from northern Pakistan. *Ecancer*, **6**, 283.

Ferlay J, Soerjomataram, Dikshit R, et al (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*, **136**, 359-86.

Garcia – Teijido P, Cabal ML, Fernandez IP, et al (2016). Tumour-infiltrating lymphocytes in triple negative breast cancer: future of immune targeting. *Clin Med Insights Oncol*, **10**, 31-9.

Globocan (2012). Cancer fact sheets, 2010 [cited 2016 Feb 11]. Available from http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.

Goldhirsch A, Wood WC, Coates AS, et al (2013). Strategies for subtypes: Dealing with the diversity of breast cancer-Highlights of the St. Gallen international expert consensus on the primary therapy of early breast cancer 2013. *Ann Oncol*, **22**, 1736-47.

Gucalp A, Traina TA (2011). Triple-negative breast cancer: Adjuvant therapeutic options. *Chemother Res Pract*, ID, 696208. doi.org/10.1155/2011/696208.

Haffty BG, Yang Q, Reiss M, et al (2006). Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. *J Clin Oncol*, **24**, 5652–7.

Harris LN, Broadwater G, Lin NU, et al (2006). Molecular subtypes of breast cancer in relation to paclitaxel response and outcomes in women with metastatic disease: Results from CALGB 9342. *Breast Cancer Res*, **8**, R66.

Hashmi AA, Edhi MM, Naqvi H et al (2014) Molecular subtypes of breast cancer in South Asian population by immunohistochemical profile and Her2neu gene amplification by FISH technique: association with other clinicopathologic parameters. *Breast J*, **20**, 578-85.

Hashmi AH, Edhi MM, Naqvi H, et al (2014). Clinicopathologic features of triple negative breast cancers: an experience from Pakistan. *Diagn Pathol*, **9**, 43.

Hudis CA, Gianni L (2011). Triple-negative breast cancer: an unmet medical need. *Oncologist*, **16**, 1-11.

Kakarala M, Rozek L, Cote M, et al (2010). Breast cancer

histology and receptor status characterization in Asian Indian and Pakistani women in the U.S.–aSEER analysis. *BMC Cancer*, **10**, 191.

Liu Shuzhen L, Lachapelle Jonathan L, Leung Samuel L, et al (2012). CD8+ lymphocyte infiltration is an independent favorable prognostic indicator in basal-like breast cancer. *Breast Cancer Res Treat*, **14**, R48.

Loi S (2014). Host antitumor immunity plays a role in the survival of patients with newly diagnosed triple-negative breast cancer. *J Clin Oncol*, **32**, 2935-7.

Menhas R, Umer S (2015). Breast cancer among Pakistani women. *Iran J Public Health*, **44**, 586-7.

Morris GJ, Naidu S, Topham AK, et al (2007). Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's surveillance, epidemiology, and end results database. *Cancer*, **110**, 874-6.

Nanda R, Chow LQM, Dees EC, et al (2016). Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib KEYNOTE-012 study. *Oncology*, **34**, 2460-7.

Pal SK, Childs BH, Pegram M (2011). Triple negative breast cancer: unmet medical needs. *Breast Cancer Res Treat*, **125**, 627-36.

Pruneri G, Vingiani A, Bagnardi V, et al (2015). Clinical validity of tumour-infiltrating lymphocytes analysis in patients with triple negative breast cancer. *Ann Oncol*, **27**, 249-56.

Qian BZ, Pollard JW (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell*, **141**, 39-51.

Qureshi MA, Mirza T, Khan S, et al (2016). Cancer patterns in Karachi (all districts), Pakistan: First results (2010-2015) from a Pathology based cancer registry of the largest government-run diagnostic and reference center of Karachi. *Cancer Epidemiol*, **44**, 114-22.

Rashid MU, Muhammad N, Bajwa S, et al (2016). High prevalence and predominance of BRCA1 germline mutations in Pakistani triple-negative breast cancer patients. *BMC Cancer*, **16**, 673.

Rouzier R, Perou CM, Symmans WF, et al (2005). Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res*, **11**, 5678–85.

Ruffell B, Au A, Rugo HS, et al (2012). Leukocyte composition of human breast cancer. *PNAS*, **109**, 2796-801.

Sikandar B, Qureshi MA, Mirza R, et al (2015). Differential immune cell densities in ductal carcinoma in situ and invasive breast cancer: Possible role of leukocytes in early stages of carcinogenesis. *Pak J Med Sci*, **31**, 274-279.

Stagg J, Allard B (2013). Immunotherapeutic approaches in triple-negative breast cancer: latest research and clinical prospects. *Ther Adv Med Oncol*, **5**, 169–81.

Surgical pathology cancer case summary [Internet] 2012. Available at: http://www.cap.org/apps/docs/committees/cancer/cancer_protocols/2012/BreastInvasive_12protocol_3100.pdf . Accessed 03 November 2016.

Tan GH, Taib NA, Choo WY, et al (2009). Clinical characteristics of triple-negative breast cancer: experience in an Asian developing country. *Asian Pac J Cancer Prev*, **10**, 395-8.

Yuan ZY, Luo RZ, Peng RJ, et al (2014). High infiltration of tumor-associated macrophagesin triple-negative breast cancer is associated with higher risk of distant metastasis. *Onco Targets Ther*, **7**, 1475-80.

Zardavas D, Irrthum A, Swanton C, et al (2015). Clinical management of breast cancer heterogeneity. *Nat Rev Clin Oncol*, **12**, 381-94.