Epidermal growth factor receptor (EGFR) gene alteration and protein overexpression in Malaysian triple-negative breast cancer (TNBC) cohort

Zubaidah Zakaria1
Muhammad Farid Zulkifli1–3
Wan Atiqah Najiah Wan Hasain1
Azliah Kamaliah Azhari2,4
Sayyidi Hamzi Abdul Raub2,4
Jeyandry Eswaran5,6
Meera Soundararajan7
Sharifah Noor Akmal
Syed Husain2,4

1Cancer Research Centre (CaRC), Institute for Medical Research (IMR), National Institutes of Health (NIH), Ministry of Health (MOH), Seja Alam, Shah Alam 40170, Selangor Darul Ehsan, Malaysia; 2Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Cheras, Kuala Lumpur 56000, Malaysia; 3Nutrition, Metabolism and Cardiovascular Research Centre (NMCRC), Institute for Medical Research (IMR), National Institutes of Health (NIH), Ministry of Health (MOH), Seja Alam, 40170 Shah Alam, Selangor Darul Ehsan, Malaysia; 4Reference Specialised Laboratory, Pantai Premier Pathology Sdn. Bhd., Kuala Lumpur 59100, Malaysia; 5Northern Institute for Cancer Research, Newcastle University, Newcastle-upon-Tyne, NE1 7RU, UK; 6Newcastle University Medicine Malaysia, Gelang Patah, Johor 79200, Malaysia; 7Department of Applied Sciences, Faculty of Health and Life Sciences, Northumbria University, Newcastle-upon-Tyne NE1 8ST, UK.

Background: Epidermal growth factor receptor (EGFR) is a member of the ErbB family of tyrosine kinase receptor proteins that plays important roles in tumour cell survival and proliferation. EGFR has been reported to be overexpressed in up to 78% of triple-negative breast cancer (TNBC) cases suggesting it as a potential therapeutic target. The clinical trials of anti-EGFR agents in breast cancer showed low response rates. However, a subgroup of patients demonstrated response to EGFR inhibitors highlighting the necessity to stratify patients, who might benefit from effective combination therapy that could include anti EGFR-agents. Population variability in EGFR expression warrants systematic evaluation in specific populations.

Purpose: To study EGFR alterations and expressions in a multi ethnic Malaysian TNBC patient cohort to determine the possibility of using anti-EGFR combinatorial therapy for this population.

Patients and methods: In this study, we evaluated 58 cases of Malaysian TNBC patient samples for EGFR gene copy number alteration and EGFR protein overexpression using fluorescence in-situ hybridization (FISH) and immunohistochemistry (IHC) methods, respectively.

Results: EGFR protein overexpression was observed in about 30% while 15.5% displayed high EGFR copy number including 5.17% gene amplification and over 10% high polysomy. There is a positive correlation between EGFR protein overexpression and gene copy number and over expression of EGFR is observed in ten out of the 48 low copy number cases (20.9%) without gene amplification.

Conclusion: This study provides the first glimpse of EGFR alterations and expressions in a multi ethnic Malaysian TNBC patient cohort emphasising the need for the nationwide large scale EGFR expression evaluation in Malaysia.

Keywords: FISH, fluorescence in situ hybridization, IHC, immunohistochemistry, TNBC biomarker, metastatic breast cancer

Introduction
Human breast cancers represent a heterogeneous group of tumors and can be classified into different molecular subtypes based on histology, cellular origin, mutations, metastatic potential, disease progression, therapeutic response and clinical outcome.1 While significant progress has been made for the treatment and patient outcome of breast cancers, the treatment of triple-negative breast cancer (TNBC) still remains a challenge due to its aggressive characteristics and limited treatment options. TNBC is a group of breast tumors characterized by the absence...
of expression of estrogen receptors (ER), progesterone receptors and human epidermal growth factor receptor 2 (HER2) and lacks the benefit of specific therapy that targets these proteins. This subtype of invasive breast cancer carries a poor disease-free survival and associated with poor clinical outcomes accounting overall for about 17% of all breast cancers. Among patients diagnosed with resectable (stages I–III) TNBC who completed trimodality therapy (surgery ± radiotherapy + adjuvant or neoadjuvant chemotherapy), as many as 50% of patients experience disease recurrence and on an average 37% die in the first 5 years following surgery. When comparing the TNBC of the Asian with the western population the onset age is much lower at 40–50 among Asians compared to western population (average age of occurrence 60–70). The overall survival remains poor due to the common occurrence of early relapse and predominant localization of distal metastases in visceral organs. Currently, no specific targeted therapy approach is available for TNBC outside clinical trials warranting novel targeting therapy discovery.

The commonly used systematic therapeutic options for TNBC are limited to conventional cytotoxic chemotherapy, whereas non-TNBC such as hormone receptor-positive, or HER2-positive breast cancer benefit from anti-hormonal or HER2-targeted therapy. Current treatment strategies for patients with TNBC include targeting angiogenesis through vascular endothelial growth factor and proliferation signaling focusing on agents for DNA repair, either directly through (ADP-ribose) polymerase inhibitors or indirectly through DNA-binding or DNA-damage potentiation. There are many ongoing TNBC trials for combination therapy that include anti-EGFR agents due to its central role in TNBC oncogenesis, metastasis and treatment outcome. Moreover, anti-EGFR agents (gefitinib) inhibit phosphorylation in an in vitro setting on TNBC cell lines (BT20, HCC1937 and MDA-MB-231) resulting in cell cycle arrest indicating the possibility of using recently developed anti-EGFR agents in TNBC treatment.

The *EGFR* gene is located on chromosome 7p11.2, which belongs to the ErbB family of receptor tyrosine kinases that includes a ligand-binding extracellular domain, transmembrane domain, and a cytoplasmic catalytic kinase domain. The kinase was implicated in various cancers owing to their signaling function in Ras-MEK-ERK, PI3K-AKT-mTOR and Src-STAT3 pathways. The EGFR pathway contains well-established oncogenes including *EGFR, KRAS, BRAF* and *PIK3CA* genes that modulate gene activations in solid tumors including lung, colorectal cancer (CRC) and pancreatic ductal adenocarcinoma. The EGFR and EGFR-like peptides are often over-expressed in human carcinomas, and in vivo and in vitro studies have shown these proteins induce cell transformation. Moreover, anti-EGFR therapies correlated with longer survival of patients with colon carcinoma, non-small cell lung cancer (NSCLC) and squamous-cell of head and neck carcinoma.

Several clinical studies analyzing EGFR protein expression in various populations showed a wide variety of results ranging from 13% positive EGFR expression detected through immunohistochemistry (IHC) in Korea and Italy to a very high positive expression of 76% in Switzerland and 72% in France. The EGFR autocrine pathway contributes to a number of processes pivotal in breast cancer development and progression, including cell proliferation and migration. Again gene amplification analyses from various countries showed a very high variation between 2% in German population to about 24% in Switzerland. Atypical EGFR activation in tumor cells can result from increased transcriptional expression, mutation and/or gene amplification. The increased EGFR protein and transcript levels correlate with poor prognosis in various epithelial cancers, such as CRC, NSCLC and endometrial cancer. In TNBC, EGFR expression is also associated with poor clinical outcome and aggressive metastasis in western population. It is therefore essential to profile for EGFR expression and variations in specific population to ascertain the possibilities of using EGFR as an independent prognosticator and anti-EGFR therapies.

Although the EGF expression and gene alterations are extensively studied in the western population, the EGFR status in Malaysian TNBC cohort is completely lacking. Here, we report the EGFR copy number alteration and protein overexpression studied using fluorescence in situ hybridization (FISH) and IHC, respectively, in the representative Malaysian TNBC cohort to review the possibility of stratifying patients using EGFR status and using anti-EGFR therapy (Table 1).

### Materials and methods

**Patients and tissue samples**

Fifty-eight formalin-fixed paraffin-embedded (FFPE) satisfactory tissues of patients with TNBC from 2005 to 2013 were obtained from Universiti Kebangsaan Malaysia Medical Centre (UKMMC). The cases included in this
study are the ones that are classified as invasive ductal TNBC based on the expression of standard biomarkers (ER, progesterone and HER-2), immunohistochemical data and those cases with complete clinical data.

**Immunohistochemistry (IHC) analysis**

Tissue sections with 4 µm thickness were cut from the FFPE blocks, dried, deparaffinized and rehydrated using standard operating procedures. The EGFR expression was detected using monoclonal mouse anti-human EGFR, wild-type (DAK-H1-WT) (Dako, Santa Clara, CA, USA) antibody and EnVision™ FLEX+ High pH kit (Dako). EGFR antibody was applied to a representative section of the TNBC cases as manufacturer’s protocols, with Proteinase K proteolytic epitope retrieval for 5 mins, followed by incubation with the primary EGFR antibody at room temperature for 30 mins. DakoCytomation Mouse IgG1 (Dako) was used as negative control and colon cancer was used as positive control. Both positive and negative controls were run simultaneously with the test samples. The slides were stained lightly with hematoxylin prior to scanning and viewing.

EGFR expression was scored by pathologist as following: 0, no staining or weak membranous staining in <10% of the tumor cells; 1+, weak membranous staining in ≥10% of the tumor cells; 2+, moderate membranous staining in ≥10% of the tumor cells; 3+, strong membranous staining in ≥10% of the tumor cells. Complete and incomplete membranous staining were both accepted and a score of 2+ onwards was considered to be EGFR overexpression (Table 2).

**FISH assay for the detection of EGFR gene alterations**

FISH detection of EGFR gene alteration was performed by hybridizing slides with fluorescent-labeled dual-colored probes, XL EGFR amp (MetaSystem, Altlussheim, Germany) according to the manufacturer’s instructions. The probe mixture consists of an orange labeled probe which hybridizes to the EGFR locus at 7p11 and a green labeled probe which hybridizes to the 7cen region. The fluorescence hybridization signal was observed using fluorescence microscope and analyzed using CytoVision (Leica Biosystems, Nussloch, Germany) software.

For each sample, 50 non-overlapping tumor cells were evaluated. EGFR gene copy number was classified into normal disomy (≤ 2 copies in >90% of cells); low trisomy (≤ 2 copies in ≥ 40% of cells, three copies in 10–40% of cells and ≥ 4 copies in <10% of cells); high trisomy (≤ 2 copies in ≥ 40% of cells, 3 copies in ≥ 40% of cells and ≥ 4 copies in <10% of cells); low polysomy (≥ 4 copies in 10–40% of cells); high polysomy (≥ 4 copies in ≥ 40% of cells) and EGFR gene amplification (presence of high EGFR gene clusters and a ratio of the EGFR gene to chromosome 7 of ≥ 2 or >15 copies of EGFR per cell in ≥10% of cells). The cases were then classified into two major groups which were low EGFR copy number (disomy, low trisomy, high trisomy and low polysomy) and high EGFR copy number (high polysomy and gene amplification).

**Statistical analysis**

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) software, version 10 (SPSS, Chicago, IL, USA). The Chi-square test was used to determine the relationship between EGFR gene alterations and EGFR protein expression. The result was considered to be statistically significant at a P<0.05.

**Table 1** Summary of patient samples analyzed in the study

| Age range of patients | Numbers | Percentage |
|-----------------------|---------|------------|
|                       | 26–89   |            |

| Sex                     | Numbers | Percentage |
|-------------------------|---------|------------|
| Female                  | 58      | 100        |
| Male                    | 0       | 0          |

| Ethnicity               | Numbers | Percentage |
|-------------------------|---------|------------|
| Malay                   | 35      | 60.4       |
| Chinese                 | 14      | 24.2       |
| Indian                  | 6       | 10.4       |
| Foreigner               | 3       | 5.2        |

| Grade distribution      | Numbers | Percentage |
|-------------------------|---------|------------|
| Grade 1                 | 4       | 6.9        |
| Grade 2                 | 19      | 32.7       |
| Grade 3                 | 35      | 60.4       |

| Lymph node              | Numbers | Percentage |
|-------------------------|---------|------------|
| Positive                | 18      | 31.1       |
| Negative                | 17      | 29.3       |
| Nil                     | 23      | 39.6       |

| Tumor size (cm)         | Numbers | Percentage |
|-------------------------|---------|------------|
| 1.5x1.5x1.0–15.0x9.0    |         |            |

| T stage                 | Numbers | Percentage |
|-------------------------|---------|------------|
| T1                      | 9       | 15.5       |
| T2                      | 37      | 63.8       |
| T3                      | 11      | 18.9       |
| T4                      | 1       | 1.7        |

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Results

EGFR copy number alteration
In this study, FISH results were divided into low gene copy number and high gene copy number. Of 58 TNBC cases, gene amplification was shown in three (5.2%) cases while high polysomy in six (10.3%) cases. The 49 remaining cases showing disomy were detected in six cases (10.3%), low trisomy in 24 cases (41.4%), high trisomy in 14 cases (24.1%) and low polysomy in five cases (8.6%). The percentage of cells with EGFR gene amplification was evaluated in 50 non-overlapping cells showing abnormal signal patterns in $\geq 10\%$ cells (Tables 2 and 3). Representative cases of low copy number and high copy number of EGFR gene are shown in Figure 1.

EGFR protein overexpression evaluation
IHC study on 58 cases of TNBC showed 17 (29.3%) cases with high immunoreactivity (overexpression) with score value of 2+ and more. Of these 17 cases, 10 cases (17.2%) were scored as 2+ and 7 cases (12.1%) were scored as 3+ (annotation of the scoring described in Table 4). The remaining cases were scored as 0 (44.8%) and 1+ (25.9%). The classification for IHC results (0, 1+, 2+ and 3+) were based on the morphological protein expression observed after staining (Figure 2). For many decades, invasive breast carcinomas were only classified according to histological type, grade and expression of hormone receptors but separating the EGFR overexpressing subset in TNBC will allow us to explore the possibility of using anti-EGFR inhibitors in TNBC treatments.

Table 2 Summary of FISH and IHC scores for the patient samples analyzed

| FISH result   | Sample number | Percentage | IHC scoring | Patient number | Percentage |
|---------------|---------------|------------|-------------|----------------|------------|
| Disomy        | 6             | 10.3       | 0           | 26             | 44.8       |
| Low trisomy   | 24            | 41.4       | 1+          | 15             | 25.8       |
| High trisomy  | 14            | 24.1       | 2+          | 10             | 17.2       |
| Low polysomy  | 5             | 8.62       | 3+          | 7              | 12.0       |
| High polysomy | 6             | 10.3       |             |                |            |
| Amplification | 3             | 5.17       |             |                |            |
| Total: 58     | 100           |            |             | Total: 58      | 100        |

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

Discussion

The protein expression analysis by IHC coupled with gene copy number evaluation by FISH is one of the most effective methods in selecting patients for anti-EGFR therapy. In our study, the EGFR copy number was evaluated based on Capuzzo et al (2005) criteria which were derived from the University of Colorado Cancer Center. In the small Malaysian cohort used in this study, the EGFR gene was amplified in 5.2% cases of the mixed (i.e., Malay, Chinese, Indian, and foreign) race TNBC population. The EGFR gene amplification observed is low, but it is in concordance with amplification studies reported from the Turkish and Korean population where the amplification rate was 1.62% and 2%, respectively. The EGFR gene copy number (high polysomy and amplification) frequently in breast cancer. The EGFR gene amplification has been shown to result in increased protein expression in breast cancer. Immunohistochemical analysis has suggested a strong association of EGFR, CK5/6 and c-KIT protein expression with TNBC cases. Therefore, in this study, we evaluated the EGFR protein expression levels and gene copy number alteration and investigated the correlation between these parameters in Malaysian women population with TNBC.

The association between EGFR protein expression pattern and EGFR gene copy number is listed in Table 2. There was a positive correlation between EGFR protein expression and gene copy number with $P<0.015$. EGFR protein overexpression (IHC score of 2+ and 3+) was observed in all (100%) of the samples with gene amplification. In seven cases with high polysomy, EGFR overexpression was shown in four (57.2%) of the cases, while the remaining two cases showed low immunoreactivity. Ten (20.9%) of the 48 low copy number cases demonstrated EGFR protein overexpression.

Correlation between EGFR protein expression and gene copy number alteration
Gene amplification is an important mechanism for onco-gene overexpression in malignant tumors and occurs
Table 3 Correlation of EGFR protein expression and copy number alterations

| EGFR IHC | EGFR FISH analysis | Low gene copy number% (%) | High polysomy (%) | Gene amplification (%) | Total |
|----------|---------------------|---------------------------|------------------|-----------------------|-------|
| 0        | 25 (51.0)           | 1 (16.7)                  | 0                | 26                    |
| 1+       | 14 (28.6)           | 1 (16.7)                  | 0                | 15                    |
| 2+       | 7 (14.3)            | 1 (16.7)                  | 2 (66.7)         | 10                    |
| 3+       | 3 (6.1)             | 3 (50.0)                  | 1 (33.3)         | 7                     |
| Total    | 49                  | 6                          | 3                | 58                    |

Note: *Low copy number includes disomy, low trisomy, high trisomy and low polysomy.

Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

Table 4 Immunohistochemistry scoring

| Score number | Observation                                               |
|--------------|----------------------------------------------------------|
| 0            | No staining or weak membranous staining <10% of the tumour cells |
| 1+           | Weak membranous staining in ≥10% of the tumour cells      |
| 2+           | Moderate, membranous staining in ≥10% of the tumour cells  |
| 3+           | Strong membranous staining in ≥10% of the tumour cells     |

Figure 1 (A and B) Low gene copy number of EGFR gene (green:orange, 2:2 and 3:3). (C) high polysomy of EGFR gene (green:orange ≥4:4) and (D) EGFR gene amplification with orange cluster.

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varies from 2.0% to 24.0% in other TNBC investigations from Germany and Switzerland, respectively. 20,34 Similar to the gene amplification variation, EGFR mutational analyses revealed no EGFR mutations in European population but about 0% and 11% mutation frequency in French and Chinese population, respectively. 35 Further, in the same vein, the correlation observed between gene amplification and protein expression in many studies also show positive as well as negative correlations. 20,36,37 Our results from the Malaysian cohort show that EGFR protein overexpression was generally positively associated with high EGFR gene copy number. Moreover, overexpression of EGFR is also observed in 10 of the 48 TNBC patients who have low EGFR gene copy number suggesting a significant role for EGFR mediated signaling in Malaysian TNBC cohort. The variabilities observed in EGFR status in various TNBC cohorts could be attributed to differences in TNBC biology, cellular behavior of EGFR and the highly complex nature of oncogenesis, but it is essential to note the diversity in criteria used for determining cut-off EGFR copy number, techniques used for expression detection, and methods of interpretations. 38 For example, a single centric Japanese study by Nakajima et al reported EGFR gene amplification and EGFR gene-activating mutations not correlating to TNBC outcome indicating the complex role of EGFR in TNBC development. 38

In TNBC, the anti-EGFR antibodies, such as cetuximab or EGFR tyrosine kinase inhibitors (EGFR-TKI) like gefitinib and erlotinib are used to suppress the EGFR signaling pathway. The major breakthrough in EGFR-targeted therapy against metastatic TNBC came from the European Society of Medical Oncology which reported a 20% response in patients with metastatic TNBC receiving Cetuximab in combination with Cisplatin opposed to another patient cohort receiving Cisplatin alone where the response rate was only 10%. 39 The anti-EGFR antibody therapy in patients with TNBC who lack EGFR mutations but display EGFR amplification show benefits in few clinical trials. 39,40 In our study, we found EGFR protein overexpression in 29.3% of the TNBC cohort, where only 15.5% showed high EGFR copy number. Moreover, the clinical significance of EGFR amplification and EGFR overexpression could not be evaluated in our current study due to the small number of informative cases. The EGFR status in large cohort of Malaysian TNBC patient samples needs to be studied to check whether EGFR-targeted therapy can be useful in Malaysian TNBC patients. In conclusion, our study from a small informative cohort shows EGFR overexpression and positive correlation between high copy number and protein expression in TNBC highlighting the need to perform a nationwide systematic EGFR status with clinical outcome in Malaysian TNBC patients. This will enable us to determine the possibility of using anti-EGFR combinatorial therapy for this population.

Figure 2 Representation of stained cells based on the criteria shown in Table 4: (A) 0, (B) 1+, (C) 2+ and (D) 3+. The basis of scoring is given in detail in Table 4.
Ethics approval
This study received approval from the Medical Research Ethics Committee (MREC) of the Ministry of Health (MOH) Malaysia (approval reference number: KKM/NIHSEC/P14-876), with a waiver of informed consent for the use of archived tissue samples. All procedures performed in studies involving archived tissue sample were conducted according to the Declaration of Helsinki and handled with strict data confidentiality.

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Disclosure
The authors declare no conflicts of interest in this work.

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