ORIGINAL RESEARCH

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

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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.[2] 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.[3,4] Among patients diagnosed with resectable (stages I–III) TNBC who completed tri-modality 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.[5,6] 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.[5,7]

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.[8] There are many ongoing TNBC trails 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.[9]

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.[10,11] 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.[12–15] 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.[16] 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.[17]

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.[18–21] The EGFR autocrine pathway contributes to a number of processes pivotal in breast cancer development and progression, including cell proliferation and migration.[22] 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.[20] The increased EGFR protein and transcript levels correlate with poor prognosis in various epithelial cancers, such as CRC, NSCLC and endometrial cancer.[23–25] In TNBC, EGFR expression is also associated with poor clinical outcome and aggressive metastasis in western population.[19] 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 EFGR 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

Table I Summary of patient samples analyzed in the study

	Numbers	Percentage
Age range of patients	26–89	
Sex Female Male	 58 0	 100 0
Ethnicity Malay Chinese Indian Foreigner	 35 14 6 3	 60.4 24.2 10.4 5.2
Grade distribution Grade1 Grade 2 Grade 3	 4 19 35	 6.9 32.7 60.4
Lymph node Positive Negative Nil	 18 17 23	 31.1 29.3 39.6
Tumor size (cm)	1.5x1.5×1.0–15.0×9.0	
T stage T1 T2 T3 T4	 9 37 11 1	 15.5 63.8 18.9 1.7

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 ≥ of 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).[26] 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 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	44.83
Low trisomy	24	41.4	1+	15	25.86
High trisomy	14	24.1	2+	10	17.24
Low polysomy	5	8.62	3+	7	12.07
High polysomy	6	10.3			
Amplification	3	5.17			
	Total: 58	100		Total: 58	100

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

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 ≥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.[27–29]

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 frequently in breast cancer.[30] The *EGFR* gene amplification has been shown to result in increased protein expression in breast cancer.[31] Immunohistochemical analysis has suggested a strong association of EGFR, CK5/6 and c-KIT protein expression with TNBC cases.[32] 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 over-expression 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.

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.[26] 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.[33–36] The *EGFR* gene copy number (high polysomy and amplification)

Table 3 Correlation of EGFR protein expression and copy number alterations

	EGFR FISH analysis			Total
	Low gene copy number[a] (%)	High polysomy (%)	Gene amplification (%)	
EGFR IHC				
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: [a]Low copy number includes disomy, low trisomy, high trisomy and low polysomy.
Abbreviations: FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.
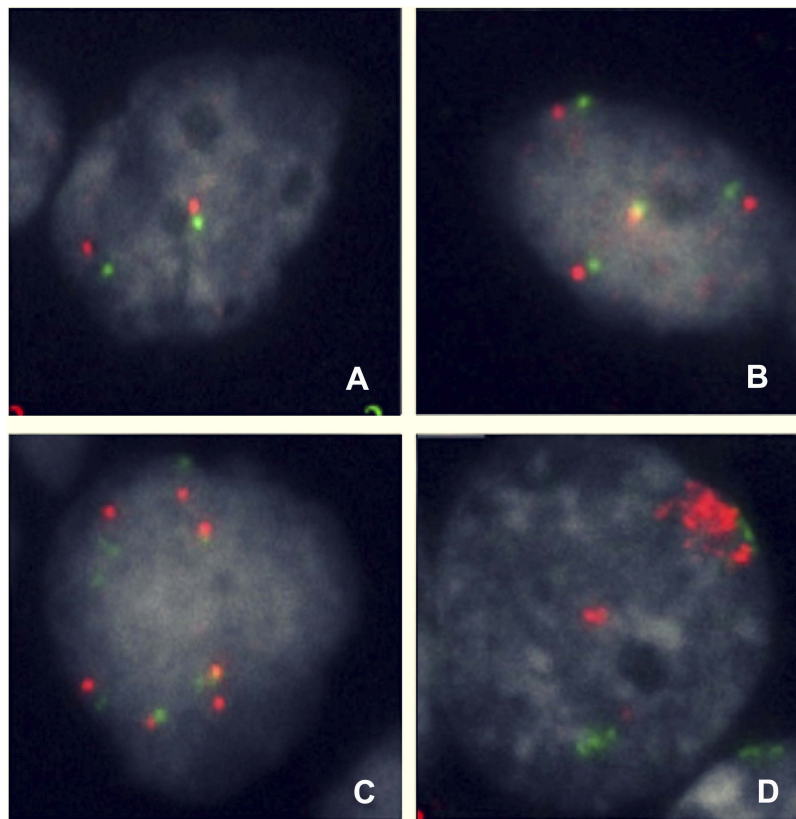
Figure 1 (**A** and **B**) Low gene copy number of *EGFR* gene (green:orange, 2:2 and 3:3), (**C**) high polysomy of *EGFR* gene (green:orange ≥4:4) and (**D**) *EGFR* gene amplification with orange cluster.

Table 4 Immunohistochemistry scoring

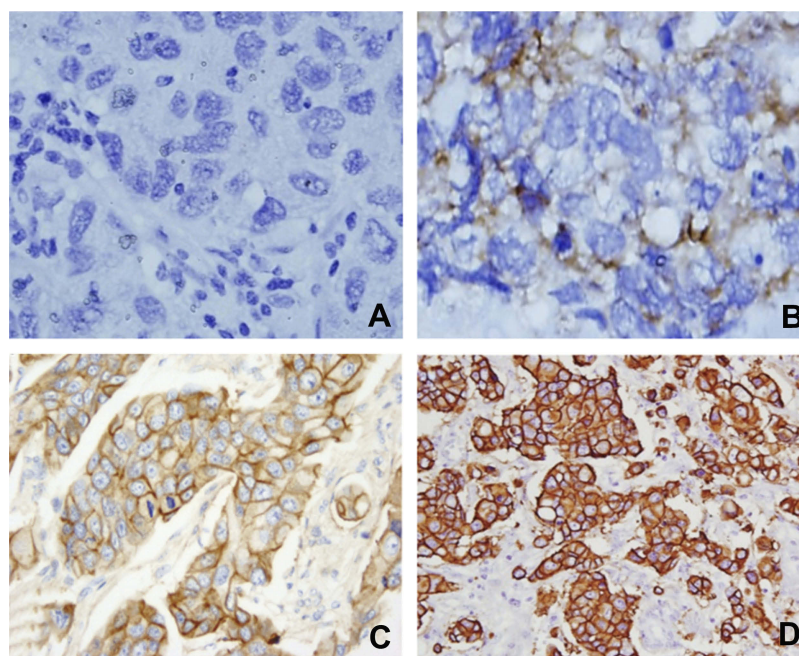| Score number	Observation
0	No staining or weak membranous staining <10% of the tumour cells
1+	Weak membranous staining in ≥10% of the tumour cells
2+	Moderate, membranous staining in ≥10% of the tumour cells
3+	Strong membranous staining in ≥10% of the tumour cells

Figure 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.

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.

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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