https://doi.org/10.33472/AFJBS.6.6.2024.5401-5407

African Journal of Biological Sciences

Research Paper Open Access

Amplification of C-MYC – shedding light on molecular landscape in human breast carcinoma

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Article Info
Volume 6, Issue 6, May 2024
Received: 29 March 2024
Accepted: 30 April 2024
doi: 10.33472/AFJBS.6.6.2024.5401-5407

Abstract

Background: MYC is amplified in approximately 15 % of breast cancers and is associated with poor outcome. c-MYC protein is multi-faceted and involved in many aspects of cell functions like replication, growth metabolism, differentiation and apoptosis and is linked with therapeutic response in breast cancer. The mechanisms of c-MYC action in controlling these different roles remains poorly understood. Materials and methods: A cross sectional study involving 45 cases of breast cancer were studied for amplification of c-Myc gene by FISH. FISH signal images were recorded using a Leica fluorescent microscope equipped with a camera and multiple fluorescence filter sets comprising three color filter sets DAPI, red, and green. Total number of signals were counted in 100 cells. The normal control ratio was 2:2; any increase in the ratio was considered as a gene amplification. The results were reported as the amount of MYC signals divided by the number of centromere signals of chromosome 8(MYC: CEP8). Results: Out of 45 cases of breast carcinoma, amplification of c-Myc gene was noted in 20 cases (44 %). The percentage of cases with gene gains of three copies or higher was 20 % (9 out of 45), with a highest index of 10 to 12 signals per nucleus. Conclusion: MYC gene amplification is critical in the etiology of breast cancer, leading to tumor aggressiveness and treatment resistance. Understanding the molecular mechanisms behind MYC amplification and its clinical implications is critical for developing novel therapeutic methods that target MYC-driven oncogenesis. Identification of MYC target genes is essential in isolating signaling pathways that drive tumor development.

Keywords: Breast cancer, c-Myc amplification, DCIS, invasive carcinoma, fluorescent in situ hybridization.

Introduction:

Breast cancer (BC) is a complicated disease caused by several genetic and epigenetic changes that turn normal breast cells into malignant versions. BC has been classified into five subgroups based on gene expression profiles: Her2/Neu amplified, luminal A, luminal B, basal-like, and normal-like (1-4). It is characterized by variations in histological and biological traits such as invasiveness and metastasis. Protooncogene activation is hypothesized to play a role in tumor formation and progression (5,6). The most generally held theory about human breast cancer progression is that it is a multi-step process that expresses itself as a succession of pathologically defined stages, with ductal carcinoma in situ (DCIS) being the penultimate preinvasive stage before progression to invasive ductal carcinoma (IDC) (7). Breast cancer is well known to be highly variable at both clinical and genetic levels. At the molecular level, breast cancer is characterized by diverse number of genetic abnormalities like unbalanced chromosomal rearrangements, gene amplifications and deletions. Gene amplification is important in the development and progression of cancer and could serve as a potential biomarker for prognosis or as a target for molecular therapy (8). MYC exhibits site-specific DNA-binding activity with its binding factor MYC-associated factor X (MAX). This MYC-MAX binding is rate-limiting for cell cycle progression through the G1 phase and this process is partly regulated by cyclin-dependent kinases in cell proliferation (9). In addition, MYC plays critical role in multiple cellular pathways that promote the survival of cancer cells (10). MYC plays an important role in the pathogenesis of cancer and is particularly important in the survival of cancer cells that are resistant to anti-cancer drugs. Thus, targeting MYC is a logical strategy in drug-resistant breast cancers (11). MYC (also known as c-Myc) is a transcription factor encoded by the MYC gene on chromosome 8 q24.21. The MYC oncoproteins (C-myc, N-myc, and L-myc) regulate almost 15 % of expressed genes, mRNA translation and cell-cycle control, and stress responses have an influence on a wide variety of biological activities like proliferation, differentiation, survival, programmed cell death and immune modulation (12).

Materials and Methods:

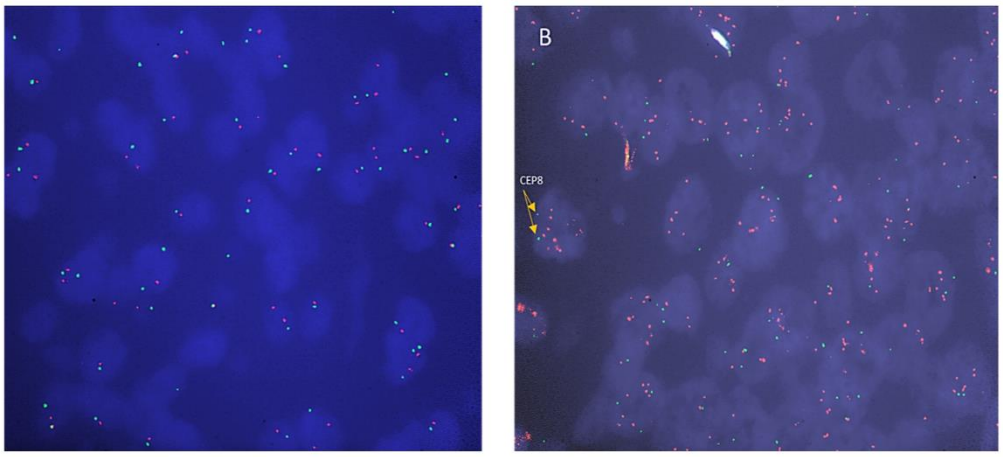
This study was done during the period August 2019 and February 2023 in Division of Human Genetics, Department of Anatomy, Pondicherry Institute of Medical Sciences, Puducherry after obtaining prior approval from the Institutional Ethics Committee (IEC: RC/2020/67). This was a cross-sectional study done in 45 patients of breast carcinoma confirmed by histopathological examination. Cases of pure ductal carcinoma in-situ (DCIS), DCIS with microinvasion and invasive ductal carcinoma were included in the study. Breast carcinoma cases where paraffin blocks could not be retrieved were excluded from the study. A total of 45 formalin fixed paraffin embedded blocks (15 each of DCIS, DCIS with microinvasion and invasive ductal carcinoma) were obtained from the Department of Pathology, Pondicherry Institute of Medical Sciences and Department of Pathology, Basavatarakam Indo American Cancer Hospital, Hyderabad. Sections of 5µ thickness were cut by Leica RM 2245 semiautomated microtome, transferred to glass slides, and stained with routine Hematoxylin and Eosin stain. The representative tumor area was identified by a pathologist and marked by diamond pencil over the under surface of the slide. Another set of sections were taken again from the same blocks on Poly L lysin coated slides and incubated overnight at 60° C in a hot air oven. Clearing was accomplished for 10 min in two changes of xylene and dehydration by two changes of absolute alcohol for 5 min. As part of pre-treatment, the sections were immersed in freshly prepared citrate buffer (2.52 gm of citric acid dissolved in 30 ml of distilled water at a pH of 6.0 by adding NAOH) for 30 min at 98°C. Then the sections were kept in 2X SSC (5 ml of 20X SSC mixed with 45 ml of distilled water) for 5 min. The sections were then digested with Proteinase K buffer (0.5 gm of Sodium lauryl sulphate dissolved in 50 ml of 2X SSC and to 40 ml of this buffer, 80 µl of proteinase K was added) for 2-5 min until the sections were cleared. The sections were then kept in 2 X SSC for 5 min followed by two changes of absolute alcohol for 2 min and then air dried

for 5-10 min at room temperature. Metasystems' DNA XL MYC amplification probe was applied onto slides, covered with cover glass and sealed with rubber cement. Denaturation was carried out at 94° C for 3 min on a thermoBrite followed by hybridization for 20 hr at 37° C. The slides were then taken out from the thermoBrite chamber for post wash. The rubber cement and cover glass were removed and the slides were stringency washed with 0.4x SSC (1ml of 20X SSC in 49 ml of distilled water) at 72°C in a water bath for 2 min. Then the slides were washed with NP 40 buffer (10 µl of NP 40 in 50 ml of 2X SSC) for 30 sec and then thoroughly with distilled water, air dried, counterstained with DAPI, covered with cover glass and finally sealed with nail polish. The signals were viewed and quantified with Leica fluorescent microscope using proper filters and Imaging Cytovision system. The c-Myc amplification was visualized as a red signal, whereas the control probe for the chromosome 8 centromere was visualized as green making them clearly distinguishable for scoring. This approach ensured that only tumor cells were counted, eliminating the normal cells. Two investigators independently scored the nuclei of 100 tumor cells from each FISH-stained segment. The hybridization signals were averaged and the amplification index was calculated as the number of c-Myc signals divided by the number of chromosome 8 centromere signals. The presence of c-Myc gene amplification was identified by a 1.8-fold increase. FISH signal images were taken using a Leica fluorescent microscope equipped with a camera and multiple fluorescence filter sets comprising of three-color filter sets DAPI, red and green. The slides were examined under oil immersion objective (x100) through the DAPI filter and screened for the tumor area as determined earlier by the pathologist. With the TRITC filter, the centromeres were counted as green signals and MYC signals as orange signals. At least 100 tumor cells were counted on a single slide. The signal ratio of the centromere to MYC was calculated in normal cells. The normal control ratio was 2:2 and any increase in the ratio was considered abnormal or exaggerated. The results were reported as the amount of MYC signals.

Results:

Amplification of c-Myc gene was seen in 20 cases (44 %) and the remaining 25 cases (55 %) showed c-Myc signals lower than the cut off value (Table 1). Out of 15 cases of DCIS, c-Myc amplification was seen in 2 cases (13.3 %). In DCIS with microinvasion, amplification was seen in 3 cases (20 %). Amplification of c-Myc was virtually seen in all 15 cases of invasive ductal carcinoma (100 %). The percentage of cases with gene gains of three copies or higher was 20 % (9 out of 45), with a highest index of 10 to 12 signals per nucleus. Figure 1A demonstrates tumor cells with no amplification (1 copy of c-Myc / one copy of CEP8) and 1B demonstrates tumor cells with high amplification (8-12 copies of myc amplification signals / CEP8).

C-*MYC* FLUORESCENCE IN SITU HYBRIDISATION (FISH): IN DUAL PROBE C-MYC SIGNALS - RED, CENTROMERE SIGNALS – GREEN



The nuclei of tumor cells were visualized by DAPI counter-staining. A. 1 : 1 copy ratio of c-myc (c-myc/CEP8), indicating no amplification. (B) Invasive breast tumor showing c-*myc* amplification with 10 to 12 signals per nucleus

1. **Figure 1A and 1B**

C MYC gene analysis by FISH in 3 categories of human breast tumor tissues n=45

C-MYC/ CEP8 C-MYC –no of C MYC signals (red) CEP8 – No of centromeres (green)	DCIS(n=15)	DCIS with Microinvasion (n=15)	Invasion(n=15)	% of samples with FISH ratios in each category
1.0-1.5	11	9	0	20 out of 45 44%
1.6-1.9	2	3	0	5 out of 45 11%
2.0-2.5	1	1	2	4 out of 45 8%
2.6-3	1	2	4	7 out of 45 15%
>3	0	0	9	9 out of 45 20%

	Positive	Negative
FISH	20	25

Table 1.

Discussion:

Breast cancer is well known to be highly variable at both the clinical and genetic levels. Many cases progress rapidly with short survival, whereas others grow indolently with relatively good outcome after treatment. Not surprisingly, at the molecular level, breast cancer is also characterized by a diverse number of genetic abnormalities including unbalanced chromosomal rearrangements, gene amplifications and deletions. The proto-oncogene MYC encodes a nuclear phosphoprotein transcription factor that has an integral role in a variety of cellular processes, such as cell cycle progression, proliferation, metabolism, adhesion, differentiation and apoptosis. In cell culture, the activation of MYC leads to either entry into and progression through the cell cycle, or an increased rate of apoptosis, depending on the cell type and/or context. The breast cancer suppressor gene, BRCA1, has been found to bind to MYC and inhibit its transcriptional and transforming activity(13). MYC gene amplification has been found in about 15% of tumors, whereas 22% to 35% of tumors have reported overexpression at the mRNA level. Protein overexpression has been reported in about 40% of breast tumors, which indicates that mechanisms other than gene amplification, such as transcriptional regulation and mRNA and protein stabilization, are involved in MYC overexpression.

Our study shows a significant prevalence of amplification in patients with invasive ductal carcinoma (IDC). C-Myc amplification was observed in 44 % of all breast cancer cases with a notable difference in prevalence among different subtypes. Interestingly, when we specifically looked at cases of ductal carcinoma in situ (DCIS), we found a lower rate of c-Myc amplification with only 13.3 % of cases exhibiting amplification. A much higher prevalence of amplification was noted in cases of DCIS with microinvasion (20 %) and invasive ductal carcinoma (100 %). This suggests a potential role for c-Myc amplification in the progression from DCIS to invasive carcinoma. This highlights the potential clinical relevance of c-Myc amplification as a biomarker for IDC and suggests its possible implication in progression of breast cancer. These findings align with previous research suggesting the oncogenic role of c-Myc in breast cancer. Several studies have implicated c-Myc amplification in promoting tumor growth, invasion, and metastasis, underscoring its significance as a potential therapeutic target in breast cancer management. However, our study is limited by a relatively small sample size and potential bias in FISH analysis. Therefore, further research with larger cohorts and complementary techniques is warranted to establish the mechanistic role of c-Myc amplification in

breast cancer progression.

In a study by Green, A et al (14) on MYC functions investigated the correlation between c-MYC protein expression and other proteins involved in different cellular functions together with clinicopathological parameters, patients' outcome and treatments in a large early-stage molecularly characterized series of primary invasive BCs (n=1106) using immunohistochemistry. They found the result, High *MYC* and c-MYC expression was significantly associated with poor prognostic factors, including grade and basal-like BCs. In luminal A tumors, c-MYC was associated with ATM (P=0.005), Cyclin B1 (P=0.002), PIK3CA (P=0.009) and Ki67 (P<0.001). In contrast, in basal-like tumors, c-MYC showed positive association with Cyclin E (P=0.003) and p16 (P=0.042) expression only. c-MYC was an independent predictor of a shorter distant metastases-free survival in luminal A LN+ tumors treated with endocrine therapy (ET; P=0.013). In luminal tumors treated with ET, *MYC* mRNA expression was associated with BC-specific survival (P=0.001). In ER-positive tumors, *MYC* was associated with expression of translational genes while in ER-negative tumors it was associated with upregulation of glucose metabolism genes. This study concluded, c-MYC function is associated with specific molecular subtypes of BCs and its overexpression confers resistance to ET. The diverse mechanisms of c-MYC function in the different molecular classes of BCs warrants further investigation particularly as potential therapeutic targets.

The study by Naab, T.J et al (15), to determine the prevalence of MYC amplification and associated markers in BC tumors from African American (AA) women and determine the associations between MYC amplification and clinicopathological characteristics. They analyzed 70 cases of well characterized archival breast ductal carcinoma specimens from AA women for MYC oncogene amplification. This study results were sixty-five (97%) of the tumors showed MYC gene amplification (MYC: CEP8 > 1). Statistically significant associations were found between MYC amplification and HER2-amplified BC, and Luminal B subtypes of BC (p < 0.0001), stage (p < 0.001), metastasis (p < 0.001), and positive lymph node status (p = 0.039). MYC amplification was associated with HER2 status (p = 0.01) and tumor size (p = 0.01). High MYC amplification was seen in grade III carcinomas (MYC: CEP8 = 2.42), pre-menopausal women (MYC: CEP8 = 2.49), PR-negative status (MYC: CEP8 = 2.42), and ER-positive status (MYC: CEP8 = 2.4). This study concluded HER2 positive BC in AA women are likely to exhibit MYC amplification. High amplification ratios suggest that MYC drives HER2 amplification, especially in HER2 positive, Luminal B, and subtypes of BC.

The metanalysis by Deming, S et al (16) conducted with 29 studies reveled that the weighted average frequency of c-Myc amplification in breast tumors was 15.7% (95% CI = 12.5–18.8%), although estimates in individual studies exhibited significant heterogeneity, P<0.0001. C-Myc amplification exhibited significant but weak associations with tumor grade (RR = 1.61), lymph-node metastasis (RR = 1.24), negative progesterone receptor status (RR = 1.27), and postmenopausal status (RR = 0.82). Amplification was significantly associated with risk of relapse and death, with pooled estimates RR = 2.05 (95% CI = 1.51–2.78) and RR = 1.74 (95% CI = 1.27–2.39), respectively. This effect did not appear to be merely a surrogate for other prognostic factors. They suggested that c-Myc amplification is relatively common in breast cancer and may provide independent prognostic information. Katsuta E [17] study investigated the difference between MYC DNA amplification and mRNA high expression in TNBCs utilizing publicly available cohorts. MYC DNA amplified tumors were found to have various mRNA expression levels, suggesting that MYC DNA amplification does not always result in elevated MYC mRNA expression. Compared to other subtypes, both MYC DNA amplification and mRNA high expression were more frequent in the TNBCs. MYC mRNA high expression, but not DNA amplification, was significantly associated with worse overall survival in the TNBCs. The TNBCs with MYC mRNA high expression enriched MYC target genes, cell cycle related genes, and WNT/β-catenin gene sets, whereas none of them were enriched in MYC DNA amplified TNBCs. This study concluded MYC mRNA high expression, but not DNA amplification, reflects not

only its upregulated signaling pathway, but also clinical significance in TNBCs.

In a study by Rodriguez-Pinilla SM et (18) amplification was defined as a MYC: CEP8 ratio >2. Signals for both MYC and CEP8 were assessable in 196/245 (80%) tumors. MYC amplification was found in 19/196 cases (9.7%) and was not associated with tumor size, histological grade, positivity for estrogen receptor, progesterone receptor, HER2, epidermal growth factor, cytokeratin is 14, 5/6 and 17, MIB1 or p53. Only 4% of basal-like carcinomas showed MYC amplification, compared to 8.75% and 10.7% of luminal and HER2 tumors respectively. On univariate analysis, MYC amplification displayed a significant association with shorter metastasis-free and overall survival and proved to be an independent prognostic factor on multivariate survival analysis.

Conclusion:

In conclusion, MYC gene amplification is critical in the etiology of breast cancer, leading to tumor aggressiveness and treatment resistance. Understanding the molecular mechanisms behind MYC amplification and its clinical implications is critical for developing novel therapeutic methods that target MYC-driven oncogenesis. To enhance clinical outcomes for breast cancer patients, more studies into the complex regulatory networks involving MYC, as well as the exploration of novel therapeutic options are required.

Acknowledgement:

I would like to extend my heartfelt gratitude to Mrs. Iswariya lab technician, her invaluable assistance and support throughout the project. Dr. Lakshman Kumar Application Specialist, Meta systems in his expertise, dedication and guidance have been instrumental in achieving concordance results.

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