

## ORIGINAL ARTICLE

### Expression and mutational analysis of GATA3 in Malaysian breast carcinomas

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#### Abstract

The GATA3 gene is a potential tumour marker and putative tumour suppressor gene in breast cancer. Its expression is associated with better prognosis and disease free survival in breast cancer patients. We aimed to evaluate GATA3 transcriptome expression and mutation in breast carcinomas and correlate its expression with oestrogen receptor (ER), progesterone receptor (PR), lymph node (LN) status, tumour grade and c-erbB-2 expression. Twenty-two breast infiltrating ductal carcinomas and paired normal tissues were used in Branch DNA assay to detect GATA3 mRNA expression. Normalized data for GATA3 mRNA expression were grouped according to the ER, PR and LN status, tumour grade and c-erbB-2 expression of the tumours. Statistical significance was tested using *t*-test and ANOVA at 95% confidence interval level. Mutational analysis of GATA3 was performed by direct sequencing of the coding regions of GATA3 mRNA. Our findings showed that GATA3 gene were over-expressed and under-expressed by >2 fold change in 12 and 4 tested samples, respectively. Eighty per cent of ER positive breast carcinomas were GATA3 positive. There was a statistically significant correlation between GATA3 expression and ER at 95% confidence interval level between the study groups. On the contrary, GATA3 expression was not statistically significant with PR, LN, tumour grade and c-erbB-2 expression in our study. In addition, we observed that there was no mutation in mRNA coding region in 16 breast carcinomas that showed GATA3 differential gene expression. Our preliminary results suggested that GATA3 is linked to the ER. This scenario suggests that GATA3 may play a crucial role in oestrogen receptor positive breast cancer patients. Whether GATA3 expression is involved in regulating tumour cell growth in oestrogen responsive breast cancer is a key question that remains to be answered.

**Keywords:** breast cancer, differential expression, oestrogen receptor, mutation

#### INTRODUCTION

The GATA3 is a highly conserved gene, which absolute expression level is important in placental development, hematopoiesis, adipogenesis, cell-fate specification, regulation of differentiation, and control of cell proliferation and movement.<sup>1-4</sup> Gene expression studies had implicated GATA3 as a good prognostic biomarker in breast cancer and it is expressed in association with estrogen receptor gene (ER).<sup>5-8</sup> The ER positive breast tumours had been classified into luminal A and luminal B subtypes and ER negative tumours

into basal-like, Her-2+ and normal breast-like subtypes.<sup>6</sup> These subtypes were significantly correlated with tumour recurrence and patient survival.<sup>9</sup> High expression of GATA3 and ER is seen in tumours of the luminal A subtype, which is a subtype that is associated with improved prognosis.<sup>9</sup> Lower expression of GATA3 however is related to disease recurrence. There is also tentative data showing that different polymorphisms of the GATA3 gene may associate with differential susceptibility to breast cancer.<sup>10</sup>

The proteins of the GATA family are zinc-finger transcription factors involved in the development and differentiation of cell types in vertebrates.<sup>11-14</sup> The GATA3 gene is mapped to human chromosome 10p and consisted of 6 exons. Mutations of GATA3 were detected in various breast tumours and MCF-7 cell line, suggesting that the protein might act as a tumour suppressor.<sup>15-16</sup> Sequence alteration in the second zinc-finger region of GATA3 or the region immediately 3' of this region is believed to inhibit GATA3 function.<sup>16</sup>

Progression of an epithelium derived tumour into an invasive phenotype involves fibroblast-like transformation known as epithelial-mesenchymal transition (EMT).<sup>17-18</sup> Recent studies postulated that GATA3 could induce the cancerous cells to undergo an epithelium-like differentiation, which is the reversal of EMT to suppress tumour metastasis. This happens by turning on the E-cadherin tumour suppressor gene expression.<sup>18</sup> The metastasis capability of GATA3 levels is also observed in human breast cancer cell lines. The non-metastatic cell line, MCF7 has high GATA3 levels whereas the metastatic cell line, MDA-MB-231 has low GATA3 levels.<sup>19</sup>

Subsequent study of animal model of GATA3 showed that cancer cells progress rapidly from adenoma to carcinoma when knocking down GATA3 gene. By restoration of GATA3 gene in late carcinoma could actually promote cell differentiation and suppression of tumour dissemination.<sup>19</sup> This result indicated that GATA3 is involved in the mammary epithelial cell differentiation.<sup>3</sup>

We studied genetic aberrations of GATA3, in terms of transcriptome expression and mutation of the gene in Malaysian breast carcinomas, and investigated the association of GATA3 expression with breast cancer pathological features, including the ER, PR and LN status, and c-erbB-2 expression.

## MATERIALS AND METHODS

### *Patients and biopsy specimens*

Fresh samples of infiltrating ductal carcinoma (IDC) and matched normal breast tissues were collected from mastectomies performed at Hospital Kuala Lumpur and Hospital UKM in Malaysia from April 2006 - April 2007. According to the histopathology reports of the patients, the tumour sizes ranged between 0.44 cm<sup>3</sup> - 90.75 cm<sup>3</sup>. The mean and median

tumour sizes were 28.72 cm<sup>3</sup> and 16.00 cm<sup>3</sup>, respectively. Patients' ages ranged from 36 to 75 years old with a median age of 52 years at the time of diagnosis. Most of the samples were from post-menopausal patients. All samples were snap-frozen and stored in liquid nitrogen. Before and after cutting sections for RNA isolation, poly-L-Lysine coated slides were prepared for Diff Quick Staining (Imeb Inc.) to select only samples with 70% or more tumour cells.

A total of 22 breast carcinomas and their paired normal tissues were used in this study. The tumours were labeled in numbers that were non-consecutive in this study. Some of the tumours were used up for other studies. Therefore, only those samples with higher RNAs concentration were selected for this study.

Tumour grade was evaluated using the Bloom-Richardson grading system. The ER, PR and c-erbB-2 expression status for all samples were determined by immunohistochemistry (IHC) and the results were as evaluated by the reporting pathologists. Tumours were regarded as ER or PR positive if >10% of tumour cells expressed nuclear positivity. Pathologists used 30% tumour cell positivity as the cut-off value for c-erbB-2 over-expression. The positivity was exclusively membranous. The clones for ER, PR and c-erbB-2 were 1DS (DAKO), PgR 636 (DAKO) and SP3 (Neomarker), respectively. The dilutions for ER and PR were in a ratio of 1:75 whereas the dilution for c-erbB-2 was 1:350. The antigen retrieval methods were carried out according to manufacturer's recommendation. Briefly, for ER, Tris/EDTA (pH9) was used at 98°C for 40 mins. For PR and c-erbB-2, citrate buffer (pH6) was used at 98°C for 40 mins. Breast carcinomas with known ER, PR and c-erbB-2 status were used as control. The lymph node status of the patients was taken from the data in the histopathology reports of the patients. Table 1 showed the breast cancer patient characteristics.

Approval was obtained from Medical Research & Ethics Committee, Ministry of Health Malaysia to carry out this study.

### *RNA isolation*

Minced tissues were homogenized in Trizol Reagent (Invitrogen), and total RNAs were isolated and purified through RNeasy columns (QIAGEN Mini Kit) according to manufacturer's instructions. The integrity of the purified total RNA was assessed by visualization of the 28S / 18S ribosomal RNA ratio on 1% agarose denaturing gel. The purity and quantity of the

**TABLE 1: Histopathological characteristics of breast cancer patients**

Tumour	Grade	ER	PR	LN status Malignant/Total assessed	c-erbB-2
125	3	+	+	0/10	-
136	2	-	-	8+/19	+
142	3	+	+	2+/18	-
151	2	+	-	1+/1	+
152	1	-	+	0/8	+
158	3	-	+	2+/7	-
159	1	+	+	0/11	-
163	2	+	+	0/11	-
165	2	+	+	6+/24	+
172	2	+	+	0/10	-
173	NA	-	-	0/8	-
174	2	-	-	4+/29	+
177	2	+	+	0/9	-
180	3	+	+	9+/18	-
181	3	+	+	3+/16	+
184	3	+	+	5+/17	-
186	3	-	-	0/23	+
188	1	+	+	2+/22	-
191	2	+	+	0/12	-
192	3	+	-	9+/28	+
194	1	-	-	1+/12	+
195	1	+	+	0/16	NA

ER: oestrogen receptor; PR: progesterone receptor; LN: lymph node.

-: negative ; + : positive; NA: not available

total RNAs were determined using Nanodrop based on absorbance at 260 nm and 280 nm.

### GATA3 mRNA expression study

#### Branch DNA assay

The branch DNA assay was performed according to manufacturer's instructions (Quantigene® 2.0 reagent system, Panomics). Briefly, 20  $\mu$ L of total RNAs (100 ng) from breast carcinomas and normal tissues were mixed with 33.3  $\mu$ L of Lysis Mixture, 1  $\mu$ L of Blocking Reagent, and 0.1  $\mu$ L of target gene-specific probe set which were GATA3 and housekeeping gene, GAPDH [CE (capture extender), LE (label extender), BL (blocker) ]. Each sample was then dispensed into an individual well of the Capture Plate. The Capture Plate was sealed with foil tape and incubated at 55 °C for 16 - 20 h. Followed by incubation, the Capture Plate was washed with 200  $\mu$ L of wash buffer and then two more times with 300  $\mu$ L of wash buffer. Residual wash buffer was removed by centrifuging the

inverted Capture Plate at 240 x g. Signals for the bound target mRNAs were developed by sequential hybridization with branched DNA pre-amplifier and amplifier at 55 °C for 1 h and alkaline phosphatase-conjugated label probe at 50 °C for 1 h. Three washes with wash buffer were used to remove unbound materials after each hybridization step. Substrate diioxetane was added to the wells and incubated at room temperature for 5 min. Luminescence signals from each well were measured using Microplate Luminometer. The luminescent signal is linearly proportional to the number of RNA molecules present in the sample.

#### Data analysis and statistics

In this study, each sample was performed in replicate. For all samples, background signals were determined in the absence of total RNAs and subtracted from signals obtained in the presence of RNA samples. Samples were then filtered via limit of detection (LOD) of the assay, where  $LOD = \text{background signal} + 3 \times$

Standard Deviation (SD) of background signal. Assay signals below LOD were regarded as “absent” in our study. The average signals for replicate samples were calculated. The SD and the percentage of Coefficient of Variation (CV) were then determined for each sample. The percentage of CV for all samples in this study was < 20 %. To determine gene expression changes of GATA3 in tumour (T) relative to the corresponding normal tissue (N), the fold change (FC) was calculated using the formula  $FC = \log_2 (GATA3_T / GAPDH_T) / (GATA3_N / GAPDH_N)$ . Statistical significance was tested using *t*-test and ANOVA where appropriate ( $p < 0.05$ ).

### Mutational analysis of GATA3

#### *cDNA synthesis*

Total RNAs were reversed transcribed to cDNAs using AMV reverse transcriptase (First strand cDNA synthesis kit for RT-PCR (AMV), Roche Diagnostics). The cDNAs synthesis reactions were performed in 20  $\mu$ L volumes containing 100 ng of total RNAs, 1X reaction buffer, 5 mM MgCl<sub>2</sub>, 1 mM dNTPs, 3.2  $\mu$ g of random primer p(dN)6, 50 units of RNase inhibitor and  $\geq$  20 units of AMV reverse transcriptase. Total RNAs and appropriate volume of nuclease free water were first incubated at 65 °C for 15 min. The master mix was then added to the tube and incubated at 25 °C for 10 min, and then at 42 °C for 1 h. The reaction was then incubated at 99 °C for 5 min to denature the enzymes. Finally, the reaction was cooled to 4 °C for 5 min.

#### *Polymerase Chain Reaction (PCR)*

Exonic primer pairs spanning the entire GATA3 coding region (NM\_002051 nucleotides 558 – 1889) were designed using Primer3 input v.0.4.0. Two sets of primers were used to amplify GATA3 gene. The primer sequences were GATA3a 5'-CAC CGA AAG CAA ATC ATT CA-3' (forward strand) and 5'-AGT GGG ACG ACT CCA GCT T-3' (reverse strand); GATA3b 5'-GGA CGA GAA AGA GTG CCT CA-3' (forward strand) and 5'-CTG CAA AAA TGC AAG TCG AA-3' (reverse strand). The cDNAs amplification was carried out in 25  $\mu$ L volumes containing 1-3  $\mu$ g of cDNA, 1X i-PCR Master Mix (i-DNA Biotechnology Pte Ltd) and 0.4 pmol/  $\mu$ L of each primer. An initial denaturation step at 94 °C for 5 min was followed by 35 cycles of amplification (30 s at 94 °C, 1 min at 64 °C (GATA3a) or 65 °C (GATA3b) and 1 min at 72 °C), and a final extension at

72 °C for 10 min in a thermal cycler. All the PCR products were visualized using GelRed stained 1.5 % agarose gel. The DNA bands were excised from the gels and eluted with 30  $\mu$ L of water according to manufacturer's instructions (Qiagen gel extraction kit). The purified PCR products were subjected to cycle sequencing reaction in forward and reverse directions and the results were blasted with NCBI database.

## RESULTS

#### *GATA3 expression study*

GATA3 were expressed in all 22 samples in our study. Among 22 paired samples, 16 of the breast tumours showed differential expression by >2 fold change after normalized with the paired normal controls. Among 16 breast carcinomas, GATA3 was over-expressed and under-expressed in 12 (75 %) and 4 (25%) tumours, respectively (Figure 1).

Our findings indicated a statistically significant correlation between ER and GATA3 expression ( $p < 0.05$ ). High GATA3 expression was detected in ER positive breast tumours. Nevertheless, GATA3 expression was not significantly associated with tumour grades, PR status, LN status and c-erbB-2 overexpression in our study.

#### *GATA3 mutation study*

Mutational analysis of GATA3 coding regions were performed for 16 breast carcinomas, which expressed GATA3 transcripts by >2 fold change. Our results revealed that there was no mutation in all breast carcinomas studied.

## DISCUSSION

According to the molecular genetic pathway of GATA3 in mammary gland as described by Assein-Labat *et al.* and Kouros-Mehr *et al.*, GATA3 binds to the promoter region of FoxA1 gene to activate its expression. The ER will then bind to the promoter region, which is downstream of promoter FoxA1 to turn on the transcription of down-stream target genes. Transcriptional activation of ER target genes will direct progenitor cells to differentiate into luminal epithelial cells in mammary gland.<sup>19-20</sup>

Herein, we report for the first time that GATA3 expression is significantly correlated with ER in our cohort of Malaysian breast carcinoma cases ( $p < 0.05$ ). A total of 12 out of 15 GATA3 positive cases (80 %) were ER positive. Our finding is consistent with Mehra *et al.*, Voduc

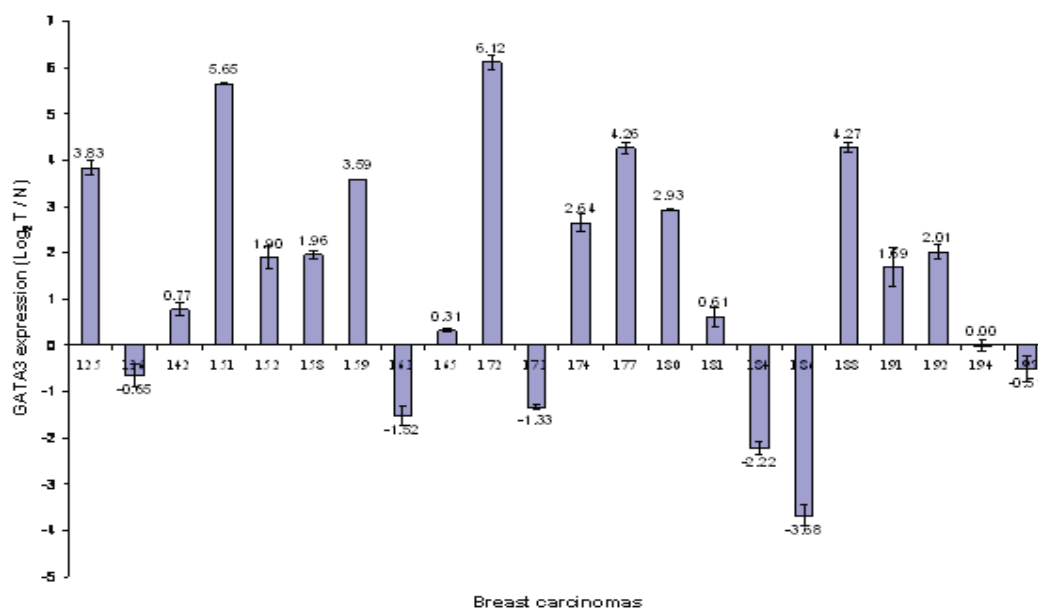


FIG. 1: Log<sub>2</sub> T / N of GATA3 expression in 22 breast carcinomas after normalization with GAPDH. The error bars indicate variation between duplicate samples. T: tumour; N: normal.

*et al.* and Parikh *et al.*<sup>21-22</sup> Of the 12 cases, nine were up-regulated by >2 fold change. Sample 172 showed the highest expression of GATA3 mRNA, which is up-regulated by 69.4 folds in the tumour relative to the normal control. Similarly, among ER positive cases, 80% were GATA3 positive. This percentage is higher than the findings reported by Mehra *et al.* and Voduc *et al.* who found that 39% and 46%, respectively of ER positive cases were GATA3 positive.<sup>21-22</sup> We identified three ER positive breast carcinomas (163, 184 and 195) and three ER negative breast carcinomas (136, 173 and 186) that showed low GATA3 levels. The lowest GATA3 expression was observed in patient 186, which was under-expressed by 12.8 fold in the tumour. Sample 186 was a grade 3 tumour on the date of the diagnosis. On the other hand, three down-regulated ER positive samples were from a mixture of grade 1, 2 and 3 tumours suggesting that there is no obvious relationship between low GATA3 expression in ER positive and higher tumour grades in this study. Our statistical results revealed that there is no statistically significant correlation between GATA3 levels and tumour grade, PR, LN and c-erbB2 expression ( $p < 0.05$ ). Our findings are contradictory with the results of Mehra *et al.* and Voduc *et al.*, which reported that GATA3 is related with lower tumour grade, lymph node (LN) negative, progesterone receptor (PR) positivity, and the absence of c-erbB-2

over-expression<sup>21-22</sup>

Mutation status of GATA3 coding regions in 16 breast tumours that differentially expressed GATA3 transcripts by >2 fold change in this study was analyzed. No mutation was identified in GATA3 mRNA coding region in this study. Our preliminary findings suggested that the differential expression of GATA3 mRNA in breast cancer is unlikely to be caused by mutation in the gene itself. Other genes might trigger the aberration in GATA3 transcriptional activity in breast cancer patients.

There is statistically significant correlation between GATA3 levels and ER status in Malaysian breast cancer patients. Higher GATA3 expression is closely related to the ER positive breast cancer patients. Our present study found that 80% of ER positive breast cancers are GATA3 positive. This means that most of the ER positive breast cancers express GATA3 mRNA. However, there is a limitation in this study since the ER, PR and c-erbB-2 status were evaluated and reported by several pathologists. Interobserver variability occurs due to the varying experience of the reporting pathologists, different techniques and markers used in different laboratories. Therefore, a larger sample size is needed to confirm the relationship between GATA3 expression and ER status of breast cancer patients. We suggest standardization of the IHC techniques and the determination

of the IHC result by a single pathologist to reduce variation that might affect the reliability of the study findings. GATA3 might be one of the important breast tumour biomarkers. It is important to clarify the role of GATA3 in the controlling of tumour cell growth in estrogen responsive breast cancer.

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