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BETA HCG EXPRESSION AND CGB METHYLATION IN BREAST CANCER

Clinical Research

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ABSTRACT

Breast carcinoma is the most commonly diagnosed cancer and the leading cause of cancer death. Breast cancer produces ectopic hormones called beta Human Chorionic Gonadotropin [hCG] and is encoded by CGB genes. The aim of this study was to determine the CGB gene methylation in breast cancer. After approval from Institutional Ethical Committee, consent from patients were obtained. Normal and tumour tissues from breast cancer patients were taken. IHC for beta hCG receptors were done. Beta hCG was found to be higher amongst the normal tissues than the tumour tissues. There was a significant relation between beta hCG and ER, Beta hCG and HER 2 neu receptors. DNA was isolated from normal and tumour tissues. Post bisulfate conversion samples were processed for qPCR using methylation specific primers and SYBR green. 1-2M was found to be significantly higher among the normal tissues. 3-9M was found to be significantly higher in tumour tissues. This suggests that beta hCG receptors are higher in normal tissues and the hormone itself is higher in cancer tissues. This is probably due to the lower receptor levels in cancer tissues.

Summary: This study was done on patients with breast cancer, to know the beta hCG receptor status and to further know the CBG gene status of the patients with hCG positive and negative receptor status.

KEYWORDS

Breast Cancer, genetics, cgb genes, methylation, beta hCG

INTRODUCTION:

The breast has always been a symbol of beauty, womanhood and fertility. As a result, both disease and surgery of the breast evoke fear of mutilation and loss of feminity. Cosmetic considerations and false vanity and fear of infertility have hindered early diagnosis and prompt treatment of breast cancer from times of earliest recorded history until today.

Worldwide, breast carcinoma is the most commonly diagnosed cancer and the leading cause of cancer death.(1)(2)

Many early breast carcinomas are asymptomatic; pain or discomfort is not usually a symptom of breast cancer. Breast cancer is often first detected as an abnormality on a mammogram before it is felt by the patient or healthcare provider.

Breast cancer like all other non-trophoblastic cancers are known to produce ectopic hormones (3). One among this is Human Chorionic Gonadotropin (hCG), a glycoprotein with 237 amino acids. It has two subunits α (alpha) and β (beta).

Elevated β -hCG levels are most commonly associated with pregnancy. Recent studies have found elevated β -hCG levels in urinary bladder carcinoma, prostate cancers and breast cancers. The presence of β -hCG receptors in breast cancer tissues is associated with a speculative role. While some studies report the presence of β -hCG receptors is indicative of a good prognosis, (4) there are studies which counter this hypothesis.(5)

The hCG-beta subunit is encoded by a cluster of genes (chorionic gonadotropin beta (CGB)) and contains several glycosylation sites. It consists of one LHB gene, four beta-hCG coding genes (CGB, CGB5, CGB8 and CGB7) and two gene copies with unknown function (CGB1 and CGB2).(6,7)

LH/hCG receptor expressions were reported to be higher in normal breast tissue than in breast cancer, suggesting that the effect of hCG on normal breast tissue might be more pronounced than its effect on breast cancer tissue.(8–11)

Previous reports showed that β -hCG can influence the differentiation of mammary tissue. Moreover, how hCG regulates the balance of proliferation and differentiation is not well understood. Human chorionic gonadotropin also plays a role in cellular differentiation and/or proliferation and may activate apoptosis. (5)

The purpose of this study was to take an initiative in finding the status of beta hCG receptors and to assess its role in patients with breast carcinoma reporting to Sri Ramachandra Institute of Higher Education and Research.

Aim:

To find the difference in methylation pattern of CGB gene in breast cancer tissue and normal tissue samples.

Methodology:

Institutional ethical clearance was obtained (CSP-MED/16/Jan/27/29). Patients were explained regarding the study in detail and their written informed consent was obtained.

Patients who satisfied the inclusion criteria were considered as a part of the study.

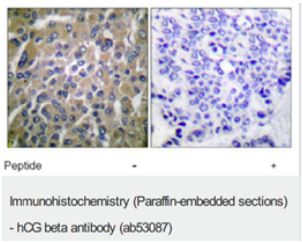
Inclusion criteria:
- Females
- Greater than 18 years
- proven breast carcinoma with or without nodal metastasis (Stages 1, 2 and 3)

Exclusion criteria:
- Males
- Pregnant or nursing mothers
- patients with distant metastasis, stage 4
- Patients with gynaecological or any other carcinoma
- Neoadjuvant chemo or radiotherapy
- patients who have undergone prior surgery for breast cancer
- Patients who did not consent to take part in the study

A detailed history was collected from all the patients and a through physical examination was done. Mammography and biopsy were done to confirm the diagnosis. Patients who were enrolled in the study, all tests including metastatic work up were done. Patients underwent modified radical mastectomy on the affected side after being declared fit by the anaesthetist.

A sliver of tissue was obtained from the tumour itself and a sliver of tissue was taken from the normal quadrant of the same specimen after consultation with the pathologist. These tissues were fixed using neutral buffered formalin and were embedded in paraffin blocks at the department of pathology.

Immunohistochemical analysis was done on each of these paraffin embedded sections. Detection system used was HRP polymer. Antigen retrieval was done by heat induction in a pressure cooker. Immunohistochemistry for beta hCG was done using *ab-53087* at 1:50 dilutions. This anti hCG beta antibody was obtained from rabbit and detects endogenous levels of total hCG beta protein. This is a synthetic peptide derived from human hCG beta. The positive control used was paraffin embedded human breast carcinoma tissue.(12)



Immunohistochemical analysis of paraffin-embedded human breast carcinoma tissue using hCG β antibody, ab53087, at 1/50 dilution.

Picture 1: Immunohistochemical analysis of paraffin embedded human breast cancer tissue using ab53087, at 1/50 dilution.

Following this the stained specimen were assessed using a semi-quantitative score according to Remmele and Stegner, comprising optical staining intensity (graded as 0=no, 1 = weak, 2 = moderate, and 3 = strong staining) and the percentage of positively stained cells (0 = no, 1=< 10%, 2 = 11-50%, 3 = 51-80% and 4 = > 81% cells). (7,13)

According to Miriam Lenhard, et al., the tumour was scored as positive if more than 10% of cells were scored with an immunoreactive score (IRS) higher than 2. The slides were reviewed in a blinded fashion by two independent observers at two separate times with an interval of 2 weeks.(7)

Following this, 40 tissue samples were selected by the pathologist for DNA analysis. Of these, 20 samples were strong positive and 20 samples with strong negativity for beta hCG.

Selection criteria of samples:

Positive samples

Strongly positive among both cancer and normal counterparts Intensity of staining should be 3 or strong staining and percentage of cells stained should be more than 90% Negative samples Strongly negative among both cancer and normal counterparts Intensity of staining should be 0 or no staining and percentage of cells stained should be 0%

Breast tumour and normal tissue (25 mg) were grinded in liquid nitrogen with motor and pestle, and the powdered tissue was treated with tissue lysis buffer and proteinase-K. The mixture was incubated at 56°C for 3 hours and another lysis buffer was added for complete lyses of cells. DNA was precipitated using 100% ethanol and sample was passed through a spin column which was washed with buffer and then eluted in 200 µL of elution buffer. The sample was stored at -20 °C till further processing.(14)

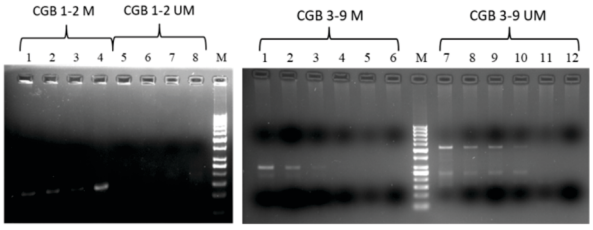
For bisulfate conversion 20µL of DNA samples was mixed with respective amount of bisulfate mix, DNA protect buffer and RNase free water and was placed in thermocycler programmed as per manufacturer protocol. The reaction mixture was then transferred to another vial, to which 560 µL freshly prepared BL buffer containing carrier RNA was added and later was passed through spin column. The column was then treated with BD buffer and washed with buffer following which the sample was eluted using 20 µL of elusion buffer and stored at -20°C.(15)

After bisulfate conversion the samples were processed for qPCR using methylation specific primers and SYBR green with set of primers for 1-2 CGB and 3-9 CGB genes which were obtained from a previous study (16)(17)(18).

Sequence of primers

Primer name	Forward primer	Reverse primer	Annealing	Elongation
CGB1–2_ M	GAAATTAAGTTCGAAGTCGC	CCTATCAACCATAACGATCG	51°C/30 s	72°C/30 s
CGB1–2_ UM	GTAGAAATTAAGTTTGAAGTTGT	CCTATCAACCATAACAATCA	47°C/30 s	72°C/30 s
CGB3–9_ M	TGTTTAGTTTGATGGTATCGC	ATACCCGAAACGATCCCC	58°C/30 s	72°C/30 s
CGB3–9_UM	AATTGTTTAGTTTGATGGTATTGT	AAAATACCCAAAACAATCCCC	55°C/30 s	72°C/30 s

Picture 2: Sequence of primers obtained from previous article.



Picture 3: Gel electrophoresis

Real time PCR was performed on Rotor – gene 10 µL reaction mixture. A methylated DNA sample obtained from the manufacturer was used as a control for calculating the delta Ct values.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (PASW Statistic, SPSS Inc., IBM, Chicago, IL). Correlation analysis of the receptor expression was performed for the histological subtype, tumor stage, grading and clinical data using the non-parametric Kruskal-Wallis rank-sum test and the non-parametric Spearman correlation coefficient. For the comparison of survival times, Kaplan-Meier curves were drawn. The chi-square statistic of the log-rank test was calculated to test differences between survival curves for significance. P values below 0.05 were considered statistically significant.

RESULTS:

This study was conducted among 254 patients. All of these patients were females who were proved to be positive for breast cancer and were taken up for Modified Radical Mastectomy before giving neoadjuvant chemotherapy or radiation.

IHC result analysis:

All patients were between 18 and 80 years of age. 53.9% of the patients were between 41 and 60 years. 18.5% of patients were <40 years and 27.6% of patients were more than 60 years of age. 59% of the patients were post-menopausal. 2% of the patients had bilateral tumour, 50% of patients had left sided tumour and the rest had right sided tumour. 38.6% of patients had tumour in the upper outer quadrant,28.7% of patients had central quadrant tumours, 16.5% of patients had upper inner quadrant tumours, 16% of patients had lower inner and lower outer quadrant tumours. 58.7% patients had tumour stage 2 (T2), 19% and 17% patients had T1 and T3 stage tumours respectively and 5% patients had T4 stage tumour. 45.3% patients had no nodal involvement.25% and 21% patients belonged to N1 and N2 stages respectively. The rest belonged to N3 stage. A vast majority of 48% patients belonged to TNM stage 2, 16% and 36% patients belonged to stage 1 and stage 3 respectively. 55% patients showed ER positivity, 57.5% patients showed PR negativity and 72.4% patients showed HER 2 neu negativity.

Beta hCG was found to be positive 83% of normal tissues and 49% in cancer tissues. On comparing beta hCG receptor status with TNM staging of tumours, there was a significant relation with p value of 0.018, suggesting a beta hCG positivity when T stage increases. On comparing the ER, PR and HER 2 neu status with beta hCG receptor status, there was again a significant relation between ER and beta hCG with a p value of 0.022. there was yet another significant relation between HER 2 neu and beta hCG, the p value was 0.000.

Molecular analysis:
Fold change was calculated with the Ct values obtained from RT-PCR. The mean fold change amongst the study population was 15.09. Highest value being 253.7649 and the lowest recorded was 0.0009.

Fold change among tumour and normal:
The p value for this was calculated to be 0.007 using the Mann-Whitney test. Hence the Fold change values were significantly higher amongst the tumour tissues than the normal tissues.

Table 1: Fold change among tumour and normal

TYPE	Fold change	Std. Deviation	Std. Error Mean	P Value
N	11.1565	21.05682	2.35422	0.007*
T	25.0454	38.13463	4.26358	(<0.05)
Fold change among tumour and normal (Table 1)				

IHC results in relation to fold change:
Fold change among Beta hCG positive patients was 8.560 and among Beta hCG negative patients was 27.64. the p value for this was <0.01, and this is significant. Hence if IHC for beta hCG is positive, the 2^(DD-Ct) values were lower and vice-versa.

Table 2: Beta hCG and Fold change

BETA hCG IHC	Fold change	Std. Deviation	Std. Error Mean	p value
POSITIVE	8.5600	14.10692	1.57720	0.000***
NEGATIVE	27.6419	40.14902	4.48880	(<0.001)
Beta hCG and Fold change (Table 2)				

Fold change among methylated and unmethylated regions:
The fold change in methylated regions were significantly higher than the unmethylated counterparts. The p value was 0.000 (<0.05).

Table 3: Fold change among methylated and unmethylated regions

Primers	Fold change	Std. deviation	Std. error Mean	p Value
1-2 M	23.5187	26.79847	4.23721	0.000***
1-2 UM	8.1303	21.15731	3.34526	(<0.001)
3-9 M	32.8563	46.43433	7.34191	
3-9 UM	7.8984	16.05362	2.53830	
Fold change among methylated and unmethylated regions (Table 3)				

Fold change between tumour tissues and normal tissues for CGB1-2 and CGB3-9:
CGB 1-2M was found to have significantly higher fold change among the normal tissues (30.22). p value was 0.017 which is less than 0.05.

The fold change of CGB 3-9M was 61.93 in tumour tissues and 3.77 in normal tissues. The difference between this was significant as the p value was 0.00 (<0.05). this suggests that 3-9 M is significantly higher in tumour tissues compared to normal tissues.There was no significant difference between the unmethylated primers.

Table 4: Fold change between tumour tissues and normal tissues for CGB1-2 and CGB3-9

		Mean	Std. Deviation	Std. Error Mean	p Value
1-2 M	T	16.8077	14.50535	3.24350	0.017*
	N	30.2298	34.18905	7.64490	(<0.05)
1-2 UM	T	9.1363	28.53280	6.38013	0.110
	N	7.1244	10.12779	2.26464	
3-9 M	T	61.9337	51.14170	11.43563	0.000***
	N	3.7789	5.50605	1.23119	(<0.001)
3-9 UM	T	12.3041	21.57589	4.82452	0.152
	N	3.4928	4.75607	1.06349	
Fold change between tumour tissues and normal tissues for CGB1-2 and CGB3-9 (Table 4)					

DISCUSSION:

Invasive breast carcinoma is one of the common malignancies affecting women worldwide and in India.

Breast cancer is one of the most common malignancies in females that presents with varied behaviours and different response to treatment.(2) The involvement of lymph nodes, distant metastasis, primary tumour size, tumour grade, hormone receptor status and lympho-vascular invasion determine poor prognosis on initial diagnosis.

Demographic results:
There were 254 patients in this study. All of them were females with proven breast cancer.

The mean age of patients in this study was 53 years and about 54% of the patients were between 41 to 60 years of age. It is proven that incidence of breast cancer increases with age to reach a peak of 421.3 cases per 100,000 at 70 to 80 years of age. It is suggested that 95% of new cases occur in women older than 40 years.(19) This was consistent with our study.

A majority of the study subjects were post-menopausal, constituting for about 60%. The other 40% belonged to pre or peri-menopausal group. A study by Chung.Cheng Hsieh, in 1990 suggested that the prevalence of breast cancer is high among peri-menopausal women, who are in their 60's.(20,21)

In our study, 7% of the patients had a positive family history for breast cancer. Breast cancer in the first-degree relatives increases the risk among patients by 2 to 3 times.(9)

There was no difference between tumour favouring a particular side. 50% of the tumours were left sided, 48% of the tumours were right sided and 2% of the patients had bilateral tumours.

Upper outer quadrant had the majority of tumours, 39%, followed by 29% in the central quadrant. Tumours are most commonly found in the upper outer quadrant because of a higher breast tissue content in that area. (22)

In regards to TNM staging, T2 had maximum patients (59%), N0 had 45% of the patients and all patients belonged to M0. This indicates stage 2 to have a majority of patients (48%). In 2002, Lebeau, suggested that maximum patients belonged to stage 2 and stage 3 among developing countries and maximum patients belonged to stage 1 in among the developed countries.(23)

Nottingham's grade 2 had majority 62% of patients followed by grade 1 with 20% of patients and grade 3 with 18% of patients. In 2008, Rakha, et al, have suggested the significance of histological grading in breast cancer. It is both of diagnostic and prognostic value. Lower the grade, better the prognosis.(24) According to a study conducted by Signe Borgquist, in 2015, grade 3 had the maximum number of patients (52%), which is controversial to our study.(25)

In our study, there were 55% patients with ER positivity, 42% with PR positivity and 72% with HER 2 neu negativity. This suggests that luminal type A had a majority of 43% of patients. In 2015, Ajith Vettuparambil, et al, suggested that ER and PR positivity was approximately 56% and 60% respectively. (26) Similarly Eundeok Chang, et al in 2005 stated that patients with ER and PR positivity and HER 2 neu negativity were high (56%, 63% and 60% respectively).(8) Immunohistochemistry for beta hCG in normal tissues showed maximum positivity (83%), whereas in cancer cells the beta hCG showed 51% negativity. A study by Eundeok Chang, et al, in 2005 showed that hCG expression in cancer cells showed a 86% negativity.(8) another study by NJ, Agnantis, et al, in 2005, showed that 55.5% of cancer cells were strongly positive for beta hCG. (27)

Comparative analysis with obtained IHC results:
About 60 patients who were premenopausal showed beta hCG negativity and 81 patients who were postmenopausal showed beta hCG positivity. Comparative analysis between beta hCG and menopausal status of the patients showed no significance as the p value was 0.067 (>0.05)

There were 33 patients in stage T1 who were negative for beta hCG, 68 patients in stage T2 showing beta hCG negativity, 24 patients in stage

T3 and 4 patients in stage T4 who were negative for beta hCG receptors. On comparing T staging with beta hCG receptor status, the p value 0.018 thus indicating a significance. Among patients who were beta hCG positive there were 55 patients in N0 stage, 34 patients in N1 stage, 30 patients in N2 stage and 6 patients in N3 stage. Beta hCG positivity is reducing with increase in nodal staging, but this comparison was not significant as the p value was 0.075 (>0.05). A study by Miriam Lenhard, et al in 2012, suggested that there was no significant correlation between beta hCG expression and tumour or nodal staging or grading. (7)

On comparing nottingham's histological grading with beta hCG receptor status, there were 18 patients with grade 1 tumours, 82 patients with grade 2 tumours and 25 patients with grade 3 tumours who were also beta hCG positive. But there was no significance, as the p value was 0.345 (>0.05) In 2008, Rakha, et al, have suggested the significance of histological grading in breast cancer. It is both of diagnostic and prognostic value.(24) Eundeok Chang, et al in 2005, suggested a significant relation between histological types of tumour and beta hCG receptor status.(8) A study by Miriam Lenhard, et al in 2012, suggested that there was a significant association between tissue expression of beta hCG and tumour grade (p = 0.022).(7)

On comparing beta hCG receptor status and estrogen receptor status: 78 patients or 31% of patients were positive for both receptors and 67 patients or 26% of patients were negative for both receptors. The p-value was 0.022 (<0.05). this suggests that if ER is positive beta hCG also shows positivity and vice- versa. Similarly, between progesterone receptors and beta hCG receptor status, there was no significance and the p value was 0.829 (>0.05). yet again, the comparison between her2neu receptors and beta hCG receptor status was significant. 23 patients were positive for both receptors and 78 patients were negative for both receptors. The p-value was 0.000 (<0.05). this again suggests that if HER2neu was positive for a patient, beta hCG was negative and vice-versa. According to Reimer, et al in 2000, there was a significant correlation between beta hCG receptor status and progesterone receptor status. He stated that "progestins regulate the expression of hCG in breast epithelial cells". (28) Another study by Eundeok Chang, et al in 2005, suggested no correlation between estrogen receptor status or progesterone receptor status or HER 2 neu receptor status.(8) There is also a significant comparison between luminal types and beta hCG receptor status suggesting a higher beta hCG positivity among luminal types A and B, and higher beta hCG negativity among triple negative and HER 2 neu types. The p value for this was 0.000 (<0.05). A study by Miriam Lenhard, et al in 2012, suggested a significant correlation between beta hCG and lutenising hormone receptor in ovarian cancer. (7)

Molecular analysis:

The difference in methylation levels of CGB genes between the mean of tumour and normal tissues were significant with a p Value of 0.007 (<0.05). The mean amongst tumour tissue was 25.04 and normal tissue was 21.05, suggesting a higher expression in tumour tissues. This was supported by Śliwa Aleksandra, et al, in 2019, who also stated that expressions of beta hCG is higher among tumour tissues compared to normal tissues, although the study was done in ovarian tissue. (18) Yet another breast cancer study done by Xing-hua Liao, et al in 2014, also suggests that the beta hCG expressions were higher in tumour tissues compared to normal tissues.(5).

The immunohistochemical results were significantly associated with the expression of beta hCG through RT-PCR (p value = 0.000 which is less than 0.05). this suggests that if beta hCG receptors were positive the expression of beta hCG was low and vice-versa. The higher expression of beta hCG could be compensating the receptor negative status as suggested in a 2010 article by R K Iles, et al.(29) A 2012 article by Miriam Lenhard, suggests that the discrepancy in literature with respect to beta hCG can be explained by the variations in the hCG hormone and the hormone receptor levels.(7)

On comparing the methylated and unmethylated counterparts, the methylated components showed a higher expression of beta hCG. This was significant with a p value of 0.00 (<0.05). According to Śliwa Aleksandra, et al in 2019 there was only a slight difference between beta hCG expression among the methylated and unmethylated.(18)

In our study, the expression of CGB 1-2 M was found to be higher in normal tissues and the expression of CGB 3-9 M was found to be higher among tumour tissues. This was statistically significant with a p value of 0.017 and 0.00 respectively. Both of these are consistent with a study by Śliwa Aleksandra, et al in 2019. The study states that the expression of CGB1 and CGB2 was higher among normal ovarian tissue and CGB3 to CGB9 were higher among ovarian cancer tissues. (18) R K Iles, et al in 2010 have suggested an increase in CGB 5 expression among the cancer tissues. (29)

Kristiina Rull, et al in 2012 and 2013 have suggested that there is an increased expression of CGB 5 and CGB 8 in cancer cells and any mutations in these major CGB genes were tolerated better. They also suggested in 2013 that CGB 5 with no mutations may protect against recurrent miscarriages. They have also suggested that these genes may act differently across various ethnic groups. (30,31) Liis Uusküla, et al in 2011 suggested that mutations in CGB 5 might lead to pregnancy loss and CGB 8 may be under expressed in mothers with recurrent pregnancy loss.(6) Xing-hua Liao, et al in 2014 suggested that beta hCG is responsible for pregnancy induced protection against breast cancer and beta hCG also helps in reducing the proliferation of MCF-7 cells by downregulating certain antigens. They have also suggested that beta hCG when upregulated helps in cellular differentiation.(5)

CONCLUSION:

A 2012 article by Miriam Lenhard, suggests that the discrepancy in literature with respect to beta hCG can be explained by the variations in the hCG hormone and the hormone receptor levels.(7)

Our study suggests that presence of hCG receptors in cancer tissues is lower when compared to normal tissues. 3-9 M is significantly higher in tumour tissues compared to normal tissues and 1-2 M is significantly higher in normal tissues. This suggests that hCG may be elevated in breast cancer because of an upregulation in CGB 3-9 genes, and this is probably because there is a decrease in hCG receptors in cancer tissues. Therefore, future studies can be based on hormone levels and receptor levels separately to validate the same.

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Conflict of Interest: None.

This study was conducted on human breast tissue from patients undergoing surgery for breast cancer. Written informed consent from each patient was obtained prior to initiation of the project. Consent for images and other clinical information was also obtained. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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