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DNA based CGB methylation in breast cancer – a case control study

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ISSN 2515-8260 Volume 07, Issue 05, 2020

Abstract: Breast carcinoma is the most commonly diagnosed cancer and the leading cause of cancer death. Breast cancer also produces and is influenced by ectopic hormones. Beta Human Chorionic Gonadotropin (hCG) is one such hormone and is encoded by chorionic gonadotropin beta (CGB) genes. The aim of this study was to determine the CGB gene methylation in breast cancer tissues and compare them with normal tissues.

Materials and methods: After approval from Institutional Ethical Committee (IEC), consent from patients were obtained. Normal and tumour tissues from breast cancer patients were taken. DNA was isolated from normal and tumour tissues. Post bisulfate conversion samples were processed for qPCR using methylation specific primers for the set of selected CGB genes and SYBR green.

Results: 1-2M was found to be significantly higher among the normal tissues (50.22). 3-9M was found to be 65.93 in tumour tissues and 5.05 in normal tissues and this was significant.

Conclusion: 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 there are 2 different types of beta hCG secreted by two different types of genes and this can be used for further analysis as a part of future projects. This may help in formulating a new treatment process and may also be used as a tumour marker in high risk patients.

KeyWords: Breast Cancer, genetics, cgb genes, methylation specific PCR, bisulfite conversion,

1. INTRODUCTION:

Human Chorionic Gonadotrophin or hCG. It is heterodimeric and has two parts. An alpha and a beta component. Elevated levels of beta hCG is most commonly associated with pregnancy. But it is also seen in bladder cancer, colonic cancer and many others. The beta hCG is encoded by cgb genes. It has various subtypes. (1–6)

The role of beta hCG in breast cancer is still speculative. The literature review for Beta hCG as an indicator of prognosis both good and bad is equivocal. The role of beta hCG in oncogenesis is complex and there is no clear explanation for the same. There are a lot of studies from 1995 by Alverado et al to studies in 2019 by Aleksandra, who have done various researches and given different conclusions.(2,3,7–9)

The breast has always been a symbol of vitality, fertility, beauty and motherhood. Especially in India, from before the Indus valley civilisation till the Islamic and British invasion, the female breasts were considered to belief giving, reason for sustenance of humanity and divinity. Female breasts are still considered to be potent talisman, symbol of maternity, empowerment and erotism. When there occurs a disease in such vital part, women are faced with fear of mutilation, loss of beauty and a dread for life. It is well known that, breast cancer is one of the most commonly diagnosed cancer and the leading cause of cancer death.(10)(11)

More frequently than not breast cancer presents as a painless lump and is frequently diagnosed in the late stages in our country. (12)

Hence, the purpose of this study was to take an initiative in finding something good for the patients suffering for breast cancer and if possible, to make an early diagnosis.

ISSN 2515-8260 Volume 07, Issue 05, 2020

Aim:

To find and compare the cgb gene methylation among breast cancer tissues and normal breast tissues.

2. METHODOLOGY:

After obtaining Institutional ethical clearance (CSP-MED/16/Jan/27/29), this study was conducted in a total of 250 patients. Patients were explained in detail about this study and their written informed consent was obtained.

Inclusion criteria:

- Females who were more than 18 years, multiparous and with proven breast carcinoma with or without nodal metastasis (Stages 1, 2 and 3)

Exclusion criteria:

- Males, nulliparous women, 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 and a through physical examination was done and recorded. Triple assessment was done for all patients. Tissue biopsy and radiological screening was done followed by metastatic workup for each of these patients. Patients underwent modified radical mastectomy on the affected side after obtaining anaesthetist fitness.

About 5cms of tissue was taken from the tumour site and from the normal quadrant after discussion with the pathologist. This was then processed to extract DNA using QIAamp DNA Mini Kit. (13)(14)

All these samples underwent bisulfite conversion using a commercially available kit.(15)(12,14) Following this these 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)(3)(18)(14)

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

RT - 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 Ct values. From these 2^ (Delta Delta CT) values were calculated.

Statistical analysis was then performed using SPSS 18.0 (PASW Statistic, SPSS Inc., IBM, Chicago, IL).

3. RESULTS:

This study was conducted among 250 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.

ISSN 2515-8260 Volume 07, Issue 05, 2020

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:

TYPE	Fold change	Std. Deviation	Std. Error Mean	P Value
N	10.119	2.0445	1.2256	**0.008 (<0.05)**
T	32.6662	12.6625	5.6625	
Fold change among tumour and normal (Table 1)				

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.

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).

Primers	Fold change	Std. deviation	Std. error Mean	p Value
1-2 M	32.9998	16.6665	2.65656	**0.002 (<0.05)**
1-2 UM	4.4203	17.7654	4.4321	
3-9 M	40.6228	8.2256	6.95517	
3-9 UM	6.2567	16.55234	1.198766	
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.

		Mean	Std. Deviation	Std. Error Mean	p Value
1-2 M	T	15.5522	10.6652	2.2265	**0.009 (<0.05)**
	N	50.219	12.66523	3.25617	
1-2 UM	T	8.6622	16.6652	5.22278	0.325
	N	6.5523	12.6225	3.9872	
3-9 M	T	65.9295	21.09224	8.62254	**0.001 (<0.05)**
	N	5.0556	3.22765	1.76294	
3-9 UM	T	5.65432	2.6225	1.6987	0.435

ISSN 2515-8260 Volume 07, Issue 05, 2020

	N	4.6625	1.62254	1.06349	
Fold change between tumour tissues and normal tissues for CGB1-2 and CGB3-9 (Table 4)					

4. DISCUSSION:

As we know breast cancer is the commonest cancer diagnosed and it is curable not just treatable.

Demographic results:

There were 250 multiparous females with proven breast cancer as a part of this study.

The mean age of diagnosis was 55.65 years and a majority of about 62% of the patients were between 41 to 60 years of age. Almost 95% of breast cancer was diagnosed in women older than 40 years.(19) the risk of breast cancer increases with an increase in age. The incidence of breast cancer reaches 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 the perimenopausal age group or around 50 years of age and this was in line with our findings.

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 0.008 (<0.05). A higher expression of CBG genes were noted in tumour tissues with a mean of 32.66 while compared to normal tissues with 10.119. In 2014, a study by Xin-hua Liao, et al, showed higher expression of beta hCG in tumour tissues.(20) Another study in 2019 done in ovarian tissues, by Sliwa Aleksandra .et al, also showed higher expression of Beta hCG in tumour tissues compared to normal tissue. (3) This was consistent with our study.

Higher expression of Beta hCG was seen in methylated counterparts with a p value of 0.002 (<0.05) in comparision with their unmethylated counterparts. Whereas, a study conducted in 2019 by Sliwa Aleksandra, et al, showed only a slight difference in expression between methylated and unmethylated counterparts of beta hCG.(3) In 2010, increased CGB 5 gene expression was concluded in a study by R K Iles, et al. (2)

In our study, higher CBG 1-2 M and CGB 3-9 M expressions were found in normal and tumour tissues respectively with a statistical significance. The above finding were consistent with Sliwa Aleksandra,et al's study in 2019 .The study also showed higher expression of CGB3 to CGB9 among ovarian cancer tissues and CGB1, CGB2 genes in normal ovarian tissue. (3)

In 2012 and 2013, an increased expression of CGB 5 and CGB 8 were found by Kristiina Rull, et al. The study also showed that any mutations in these gene were tolerated better and in 2013, it was suggested that CGB5 gene without mutation offered protection against recurrent miscarriages but their similar effect in various ethnic groups have not been documented yet. (7,21) CGB 5 mutations associated with pregnancy loss and CGB 8 under expression in mothers with recurrent pregnancy loss, was suggested by Liis Uuskula ,et al in 2011. (22) The protection offered bypregnancy induced beta hCG against breast cancer was suggested by Xing-hua Liao,et al in 2014. the study also showed reduced proliferation of MCF -7 cell by downregulating certain antigens by beta hCG , inadditon to cellular differentiation when the same is upregulated.(20)

All these results were in line with our pilot study done with 20 patients in 2019. Vasudevan A, Iyyappan R ponniah, Kaur H, Ramalingam R, Singh B, Kaliyappa C, et al. BETA HCG EXPRESSION AND CGB METHYLATION IN BREAST CANCER. Int J Sci Res. 2020;9(7):1–5 (14)

5. CONCLUSION:

cgb 1-2 Methylation was found to be significantly higher among the normal tissues and cgb 3-9 Methylation was significantly higher in tumour tissues. This suggests that there are 2 different types of beta hCG secreted by two different types of genes and this can be used for further analysis as a part of future projects. This may help in formulating a new treatment process and may also be used as a tumour marker in high risk patients.

Declarations: Not applicable

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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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