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Amplification of c-myc Locus is Independently Associated with the Deletions of Chromosome 8p in Breast Carcinoma

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ABSTRACT Attempts have been made in this study is to find out the mechanism of c-myc gene activation in breast carcinoma (CaBr) by analyzing alterations (rearrangement/amplification) in the ~580 Kb surroundings of this gene. The alteration in the c-myc locus was correlated with the deletions in chromosome (chr.) 8p to find if there is any association between the two phenomenons. The c-myc locus alteration was analyzed by Southern hybridization using the pal-1/ c-myc/ mlvi-4 probes. Overall, amplification in the c-myc locus was seen in 26% of the samples with 22% in the pal-1 region, 19% in the c-myc gene and 7% in the mlvi-4 region. This indicates that the c-myc gene activation may occur due to the amplification in the pal-1 region located 550 Kb 5' and mlvi-4 region located 20 Kb 3' of c-myc. About 42% of the samples showed loss of heterozygosity (LOH) in ≥ 40% of the microsatellite markers tested. Atleast 21% of the samples showed co-alterations in both arms of chr.8. No significant association was observed between the amplification in the c-myc locus and deletions in chr.8p. Thus the deletions in chr.8p and the amplification in the c-myc locus are independently associated with the development of CaBr.

INTRODUCTION

CaBr is one of the most common cancers diagnosed in women worldwide and is a leading cause of cancer related deaths (Greenlee et al. 2000). In India, it is the second most common cancer among women, with 75,000 new cases being reported each year (Chopra et al. 2001). Etiological and epidemiological studies have identified both genetic and environmental factors like increasing age, the dose and duration of estrogen exposure, early menarche, late menopause, nulliparity etc to be responsible for the development of CaBr (Ang et al. 2001).

Molecular cytogenetic studies have identified deletions in the chr.8p and ampli-fications in the chr.8q24 to be one of the most common chromosomal abnormalities in the development of ductal carcinoma insitu (DCIS) from normal epithelium (Ingvarsson 1999; Polyak 2002). But there are no data to suggest whether the alterations in chr.8 are independent events or there is any cooperativity among the alterations.

The amplification of the c-myc gene, located in chr.8q24.1, is found in 1-50% of the CaBr samples by Southern blot analysis (Deming et al. 2000). The variation in frequency of c-myc gene amplification among the investigators might be due to the differences in etiology, ethnicity and sample preparation. In other studies, it is evident that the c-myc gene can be activated by rearrangement/amplification/proviral integration in the long distance 5' and 3' region of this gene (Boxer LM et al. 2001). An HPV integration site is located at 550 Kb upstream of c-myc gene, named as pal-1 locus (Lazo et al. 1989; Ferber et al. 2003). Whereas, the homologous region of mlvi-4 (Moloney murine leukemia virus integration site-4) is located about 20 Kb 3' of human c-myc (Tsichlis et al. 1990). In different human tumor cell lines for e.g. Colo320, HL60 etc, various sizes of the c-myc amplicon has been noted extending to at least 550 Kb 5' of the c-myc gene reaching the pal-1 locus and to about 300 Kb downstream reaching beyond the pvt-1 locus (Joos et al. 1992).

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But the importance of the pal-1 and mlvi-4 regions in CaBr has not yet been studied.

The allelotyping studies have identified about 41-74% deletions in the chr.8p region in CaBr (Kerangueven et al. 1995; Yaremko et al. 1995; Yaremko et al. 1996; Seitz et al. 1997; Wang et al. 1999; Yokota et al. 1999; Sigbjornsdottir et al. 2000). But there is ambiguity in localizing the common consensus deletion in these studies. We have identified 3 discrete areas of high LOH in chr.8p23.1-23.2, chr.8p23.1 and chr.8p 21.3- 22 regions in CaBr of Indian patients (Bhattacharya et al. 2004). But it is not still clear if there is any association with the alterations (amplification/ rearrangement) in the c-myc locus and deletions in chr.8p.

In the present study we would like to know if the c-myc gene activation occurs due to alterations (amplification/rearrangement) in the long distance region of the gene. Thus we have analyzed the alterations in the c-myc locus by Southern hybridization using the pal-1, c-myc, mlvi-4 probes in primary breast lesions of Indian patients. The alteration in the c-myc locus was then correlated with the deletions in chr.8p (Bhattacharya et al. 2004) to see if there is any association between the two phenomenons.

MATERIALS AND METHODS

Sample Collection and Clinical Data: Seventy primary breast lesions and their corresponding normal tissues or peripheral blood leukocytes (PBL) were obtained from 67 previously untreated patients with breast lesions from Chittaranjan National Cancer Institute, Kolkata. The samples were frozen immediately after collection and stored at -80°C until further use. All the samples were collected after obtaining prior informed consent from the patients and clearance from the ethical committee of the hospital. The detailed patient history is presented in Table 1. The tumors were graded and staged according to UICC TNM classification (Harmer 1978). The mean age of the patients was 46.9 years with 65 female cases and 2 male cases. Among the patients, 59 were Hindus and 8 were Muslims. Among the 65 female patients, 4- cases were premenopausal cases and the rest were postmenopausal (Table 1).

Of the three bilateral cases used in our study, sample no. 2972 and 5287 were found to be synchronous carcinoma and operated on the same day. However, in case of 2972, both the left and right carcinoma were of stage III at the time of diagnosis but for case 5287, the right tumor was of stage III and the left carcinoma was of stage IV at the time of diagnosis. In the case of 3924, the patient reported a stage III carcinoma in the right breast at the time of diagnosis while fibroademoma in the left breast with palpable lumps was detected about 25 years earlier. The cases of 2972 and 5287 did not have a family history of breast cancer while the case of 3924 had a positive family history.

Microdissection and DNA Isolation: The normal cells present as contaminant in the specimens were removed by microdissection procedure as described by Dasgupta et al (2002). Serial 10-20μm sections for microdissection and representative 5μm sections for histologic examination were cut out from the specimens using cryostat (Leica CM 1800, Germany). The 5μm sections were stained with hematoxyline and eosin for diagnosis as well as marking of the tumor rich regions. The normal cells were removed from the marked regions of the 10-20μm sections by microdissection procedure leaving the tumor rich regions. The sections containing >60% tumor cells were taken for DNA extraction.

DNA was extracted from the microdissected tissue sections and their corresponding normal tissue or PBL by proteinase-K digestion followed by phenol: chloroform extraction (Sambrook et al. 1989). When the adjoining normal tissue of the lesion was contaminated with infiltrating tumor cells, then PBL was taken for normal DNA extraction.

Probe Information: The c-myc genomic probe is a 1.3 Kb Cla1- EcoR1 fragment encompassing part of exon 3 of human c-myc gene (Lazo 1988). The mlvi-4 probe is a 1.4 Kb BamHI- Eco RI fragment (Tsichlis 1990). The pal-1 probe is a 1.4 Kb Hind III- Bgl II fragment (Lazo 1989). The CD3γ probe is a 0.8 Kb c-DNA probe (Krissansen 1986) located at chr.11q23.3 and used as control gene due to very low incidence of LOH/MA (Micro satellite size alteration) in this region as detected by allelotyping study using the flanking microsatellite markers (D11S1340 and D11S924) of the CD3γ gene (unpublished data). The CD3γ probe was used as an internal control in southern blot hybridization to standardize the amount of DNA load-ed and to asses the relative gene copy number of the c-myc / mlvi-4/ pal-1 gene. The probes were labeled with [α ^{32}P] dCTP by random

Table 1: Clinicopathological and molecular features of the breast lesions

Lesions	*Age / Sex / Religion*	*Clinical Stage*	*Lymph node status*	*Tumor Grade*	*Menopausal Status Pre / Post*	*pal-1 status*	*c-myc status*	*mlvi-4 status*	*% LOH*
3970	40/F/H	benign phyllodes	na	na	Pre	NA	NA	NA	ND
5393	25/F/H	Fibroadenosis	na	na	Pre	NA	NA	NA	6.7
4616	42/F/H	Fibroadenoma	na	na	Pre	NA	NA	NA	0
6038	64/F/H	benign phyllodes	na	na	Post	ND	ND	ND	0
1770	40/F/H	malignant phyllodes	na	na	Pre	ND	ND	ND	0
5892	35/F/H	malignant phyllodes	na	na	Pre	ND	ND	ND	0
553	36/F/H	I	-	ND	Pre	ND	ND	ND	6.7
1183	58/F/H	II	+	iii	Post	ND	ND	ND	26.7
1186	42/F/H	II	+	ii	Pre	ND	ND	ND	53.3
2434	55/F/H	II	-	ii	Post	NA	NA	NA	6.7
2548	61/F/H	II	+	i	Post	NA	NA	NA	13.3
2737	35/F/H	II	+	ii	Pre	ND	ND	ND	20
3756	44/F/H	II	-	ii	Post	NA	NA	NA	ND
3069	48/F/H	II	+	i	Post	ND	ND	ND	0
4800	50/F/H	II	+	ii	Post	ND	ND	ND	6.7
4832	59/F/H	II	+	ii	Post	NA	NA	NA	0
5234	43/F/H	II	+	ii	Pre	NA	A, 2↑	NA	ND
5364	38/F/H	II	+	iii	Pre	NA	NA	NA	53.3
5432	52/F/H	II	+	iii	Post	ND	ND	ND	40
6061	23/F/H	II	-	ii	Pre	ND	ND	ND	13.3
76	45/F/H	III	+	iii	Post	NA	NA	NA	20
107	38/F/H	III	+	ii	Pre	ND	ND	ND	6.7
113	40/F/H	III	-	iii	Pre	A, 2↑	A, 3↑	A, 5↑	26.7
170	55/F/H	III	+	ND	Post	ND	ND	ND	46.7
324	35/F/H	III	-	ii	Post	NA	NA	NA	73.3
366	25/F/H	III	-	ND	Pre	ND	ND	ND	13.3
588	28/F/H	III	-	iii	Pre	ND	ND	ND	26.7
1818	45/F/H	III	+	iii	Pre	ND	ND	ND	0
1865	42/F/H	III	-	iii	Pre	ND	ND	ND	20
2033	40/F/H	III	+	iii	Pre	A, 2↑	NA	NA	40
2490	65/F/H	III	-	iii	Post	ND	ND	ND	13.3
2766	52/F/H	III	+	ii	Post	ND	ND	ND	46.7
2972R	30/F/H	III	+	ii	Pre	ND	ND	ND	40
2972L		III	+	ii	Pre	ND	ND	ND	46.7
3156	35/F/H	III	+	ii	Pre	ND	ND	ND	53.3
3158	36/F/H	III	-	iii	Pre	ND	ND	ND	6.7
3184	40/F/H	III	-	iii	Pre	ND	ND	ND	66.6
3266	32/F/H	III	+	ii	Pre	ND	ND	ND	26.7
3365	20/F/H	III	-	i	Pre	ND	ND	ND	6.7
3025	62/F/H	III	+	iii	Post	ND	ND	ND	60
3098	60/F/H	III	+	ii	Post	ND	ND	ND	40
3379	46/F/H	III	-	ND	Post	NA	NA	NA	ND
3615	48/F/H	III	-	ND	Post	NA	NA	NA	ND
3924R	52/F/H	III	+	ii	Post	ND	ND	ND	26.7
3924L		Fibroadenoma	na	na	Post	ND	ND	ND	33.3
4010	52/F/H	III	+	i	Post	ND	ND	ND	60
4124	35/F/H	III	-	ii	Pre	ND	ND	ND	46.7
4131	63/F/H	III	+	ii	Post	ND	ND	ND	53.3
4272	53/F/H	III	+	ii	Post	NA	NA	NA	53.3
4370	60/F/H	III	+	ii	Post	A, 5↑	NA	NA	53.3
4447	45/F/H	III	+	ii	Post	A, 2↑	A, 2↑	NA	6.7
4482	29/F/H	III	+	i	Pre	NA	NA	NA	46.7
4604	40/F/H	III	+	iii	Pre	NA	NA	NA	20
4671	45/F/H	III	+	iii	Pre	ND	ND	ND	13.3
4734	35/F/H	III	+	i	Pre	ND	ND	ND	26.7
4953	25/F/H	III	+	iii	Pre	NA	NA	NA	ND
5036	40/F/H	III	-	ii	Pre	ND	ND	ND	0
5051	50/F/H	III	+	ii	Post	A, 2↑	A, 2↑	NA	ND
5164	60/M/H	III	+	ii	na	NA	NA	NA	46.7
5287R	32/F/H	III	-	ii	Pre	ND	ND	ND	60

Table 1 contd.....

Lesions	Age / Sex / Religion	Clinical Stage	Lymph node status	Tumor Grade	Menopausal Status Pre / Post	pal-1 status	c-myc status	mlvi-4 status	% LOH
5287L		IV	-	ii	Pre	ND	ND	ND	60
5337	52/F/H	III	+	ii	Post	ND	ND	ND	40
5375	60/F/H	III	-	ii	Post	NA	NA	NA	40
5451	45/F/H	III	+	ii	Pre	ND	ND	ND	66.6
5580	30/F/H	III	+	ii	Pre	A, 2↑	A, 4↑	A, 2↑	26.7
5585	38/F/H	III	-	i	Pre	NA	NA	NA	ND
5596	50/M/H	III	+	ii	na	ND	ND	ND	6.7
6189	43/F/H	III	+	i	Pre	ND	ND	ND	53.3
5557	38/F/M	IV	+	ii	Pre	NA	NA	NA	13.3
5700	36/F/H	IV	+	iii	Pre	ND	ND	ND	13.3

Sex: F, Female; M, Male; Religion: H, hindu; M, muslim;
Menopausal status: Pre, premenopausal,Post, postmenopausal, na: not applicable
A: Amplification, NA: not amplified, ND: not done;
The upwards arrow and numbers in the gene status column indicate the increment in copy number of the gene.
Tumor Grade : i=Well Differentiated, ii=Moderately Differentiated, iii=Poorly Differentiated Squamous Cell Carcinoma

priming method and used for analysis (Feinberg 1983).

Southern Blot Analysis: 10 µg of genomic DNA from each sample was digested with Hind III for over night at 37ºC. The digested samples were then electrophoresed in 0.8% agarose gel for over night at 30V. The DNA in the gel was transferred to Genescreen nylon membrane (NEN, USA) by capillary transfer method. The pre-hybridization of the membranes were done in a solution containing 2 X SSC, 1 % SDS, 10 % dextran sulphate, 50% deionized formamide and 5X Denhardht's solution for overnight at 42ºC. The purified labeled probe with specific activity 10^8 - 10^9 CPM / µg of DNA was added to the pre-hybridisation solution and hybridized for overnight at 42ºC. After hybridization, the membranes were washed as follows: 10 min at room temperature in 2X SSC followed by washing at 42ºC in 2X SSC, 1% SDS for 2X 20 min and finally in 0.2 X SSC, 1% SDS at 42ºC for 2X 20 min. The membranes were then exposed to Kodak X-Omat film at -80ºC for 7-8 days with intensifying screen. The intensity of the hybridized bands on the autoradiographs was determined by densitometric scanner (Shimadzu CS-1900). The intensity of c-myc / mlvi-4/ pal-1 band in each sample was normalized with respect to the intensity of the CD3γ band of the corresponding sample. The copy number of c-myc / mlvi-4 / pal-1 loci in the breast lesions was calculated from the ratio of the normalized intensities of the bands in breast lesions and normal sample. The c-myc / mlvi-4 / pal-1 locus was considered to be amplified when the ratio was ≥ 2 (Deming et al. 2000).

Allelotyping Study: We used 15 highly polymorphic microsatellite markers located on chr.8p to study the allelic alterations (Bhattacharya et al, 2004). The detailed experimental procedure used for the allelotyping analysis is given in our previous study (Bhattacharya et al. 2004). The samples showing alteration in ≥ 40% of the microsatellite markers tested can be considered as highly unstable (Boland 1998).

Statistical Analysis of the Clinical Data: To determine the association of the different clinicopathological features (age of onset, menopausal grade, tumor grade and lymph node status) of the breast lesions with c-myc locus alterations as well as the inter relationship between the alterations of chr.8, the Chi- square analysis was performed. A probability value of P ≤ 0.05 was considered as statistically significant.

RESULTS

c-myc Locus Alteration in the Breast Lesions: The c-myc locus was found to be amplified in 26% (7/27) cases for at least one of the three probes analyzed (Table 1, Fig. 1). The c-myc gene, pal-1 region and mlvi-4 regions were amplified in about 19% (5/27), 22% (6/27) and 7% (2/27) samples respectively. The co-amplification of the pal-1 – c-myc - mlvi-4 locus was seen in 2 cases (sample # 113 and 5580). The c-myc was seen to be co-amplified with pal-1 in 2 cases (samples # 4447 and 5051). No significant clinicopathological association was found between the amplification in c-myc locus with the different clinical parameters like age of onset, menopausal status, tumor grade and lymph node involvment (Table 2).

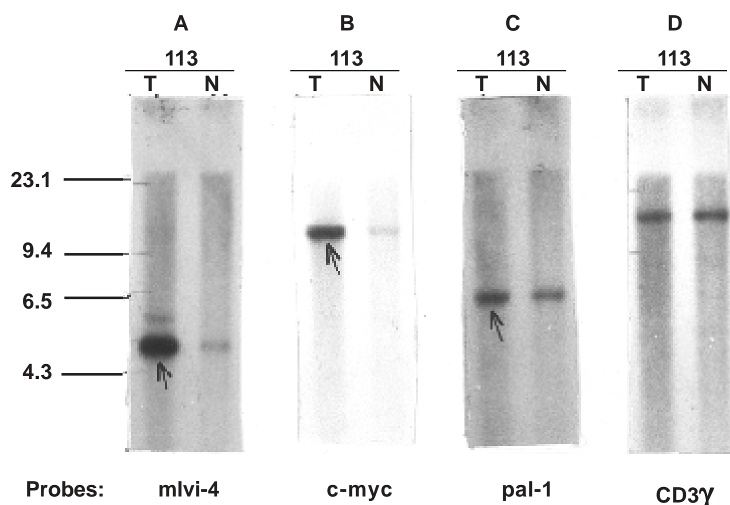

Fig. 1. Representative photograph showing amplification of the c-myc locus.

Panel A: Hybridization of the samples with the mlvi-4 probe
Panel B: Hybridization of the samples with the c-myc probe.
Panel C: Hybridization of the samples with the pal-1 probe.
Panel D: Hybridization of the samples with the CD3ã probe.
T: DNA of the primary tumor cells after microdissection; **N**: corresponding PBL.
↑ indicates rearrangement. Lambda DNA digested with Hind III was used as marker shown on the left side of Panel A.

Inter-relationship of the Amplification in the c-myc Locus with the Deletions in chr.8p: In the deletion mapping of chr.8p using 15 microsatellite markers, about 42% (26/62) of the samples showed deletions in ≥ 40% of the markers (Table 1). In chr.8p, we have detected 3 discrete highly deleted areas in the chromosomal 8p23.1-23.2, 8p23.1, 8p21.3-22 regions (Bhattacharya et al, 2004). About 21% (4/19) samples showed ampli-fication in the c-myc locus and LOH for at least one of the markers (Table 1). No significant association was found between the samples showing deletion in ≥ 40% of the microsatellite marker in chr.8p and amplification in the c-myc locus (Table 3).

Table 2: Clinicopathological correlation of amplification in the c-myc locus with the breast lesions.

Clinicopathological parameters	*Amplification of the c-myc locus*		*P-value*
	Locus +	*Locus -*	
Age at Diagnosis			
≤ 40	5	14	≤ 1
≥ 40	2	6	
Menopausal Status			
Premenopausal	4	9	≤ 0.2
Postmenopausal	3	1	
*Lymph node Status**			
Positive	6	10	≤ 1
Negative	1	7	
*Tumor Grade**			
Grade I	0	4	
Grade II	5	7	≤ 1
Grade III	2	4	

* For breast carcinoma samples

Table 3: Inter relationship of the deletions in chr. 8p with amplification in c-myclocus

Samples showing LOH at chr. 8p	*c-myc locus*		*P-value*
	amplification +	*amplification -*	
≤ 40%	2	6	≤ 1
≥ 40%	2	9	

DISCUSSION

Attempts have been made in this study to investigate whether the c-myc gene activation occurs due to distant alteration in the 5' and/or 3' region of this gene in primary CaBr. Also, whether the alterations of the c-myc locus have any association with the deletions in chr.8p has also been studied. It is evident that the pal-1 region, c-myc gene and the mlvi-4 region are amplified in some breast lesions. This indicates that in CaBr, c-myc can be activated by distant

alterations of this gene like that have been seen in some lyphomas, colon carcinoma etc (Joos et al. 1992; Boxer et al. 2001). The amplification seen in the proviral insertion site either in the 5' or 3' region of c-myc gene i.e. pal-1 and mlvi-4 region may activate the c-myc gene by cis-acting mechanism operating over long distances of genomic DNA. In Hela cell, HPV18 integration at the pal-1 site is found to enhance the c-myc expression (Lazo et al. 1989). On the other hand the mlvi-4 region is altered in some human neoplasias (Tsichlis et al. 1990). Like Colo320 and HL60 cell lines, the co-amplification seen in the pal-1-c-myc-mlvi-4 region in some CaBr samples indicates that similar type of c-myc activation occurs in the different tumor types. But unlike us, no one has seen the amplification in the pal-1 region (22%) and mlvi-4 region (7%) in CaBr. However, similar to our result of c-myc gene amplification (19%), other investigators (Cline et al. 1987; Varley et al. 1987; Adnane et al. 1989; Garcia et al. 1989; Tavassoli et al. 1989; Brouillet et al. 1990; Berns et al. 1992; Roux-Dosseto et al. 1992; Beiche et al, 1994) have observed 15-25% amplification of this gene in their studies. On the other hand, another group of investigators (Escot et al. 1986; Bonilla et al. 1988; Munzel et al.1991; Kreipe et al. 1993; Scorilas et al. 1993; Champeme et al. 1994; Harada et al. 1994; Scorilas et al. 1995) have observed 27 – 50% c-myc gene amplification in their studies. This might be due to the differences in ethnicity, etiology and sample preparation.

The high incidence of deletion of chr.8p in ≥ 40% of the markers indicates that the deletion in this region is associated with the development of CaBr. In our previous study we have detected 3 discrete areas of high LOH in the chr.8p i.e. chr.8p23.1-23.2, chr.8p23.1 and chr.8p 21.3-22 regions associated with CaBr (Bhattacharya et al. 2004). But we did not find any association between the c-myc locus amplification and deletions in chr.8p. This indicates that the deletion and the c-myc locus amplification are two independent phenomenon associated with the development of CaBr. Though these two phenomenons are independent, yet they may impose selective pressure for growth advantage of the tumor.

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