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ARTICLE

Prognostic Value of Chromosome 1 and 8 Copy Number in Invasive Ductal Breast Carcinoma among Iranian Women: An Interphase FISH Analysis

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Breast cancer is amongst the leading causes of death in women worldwide and the most common cancer amongst Iranian women. Unfortunately, the current clinical and histological criteria can only help 60 percent of women with breast cancer in diagnosis and long-term treatment. Therefore, genetic markers both at single gene and chromosomal level can play an important role in improving the diagnosis and prognosis of breast cancer patients. The aim of this retrospective study was to investigate the role of chromosome 1 and 8 copy number assessed by interphase fluorescence in situ hybridization (FISH), as prognostic parameters in 50 Iranian women, aged 35 to 64 years, with sporadic invasive ductal breast carcinoma. Chromosome 1 and 8 copy numbers were evaluated in relation to established clinicopathological parameters, the immunohistochemical markers ER, PR, P53 and cathepsin D, DNA index by flow cytometry, age and survival status of the patients. FISH using centromeric probes for chromosomes 1 and 8 was applied to interphase cell suspensions prepared from archived, Carnoy-fixed tumor cells and selected paraffin-embedded tumor sections. Aneusomy for chromosomes 1 and 8 was present in all 50 patients to different levels. The total abnormality rate for chromosome 1 was 33.92 percent (4.24 percent monosomy and 29.68 percent polysomy), whereas for chromosome 8 this rate was 28.30 percent (6.48 percent monosomy and 21.82 percent polysomy). Statistically significant association ($p<0.05$) was demonstrated between monosomy 1 and patients' age below 50 years, and between monosomy 1 and poor survival, respectively. Disomy 8 was significantly associated with P53 expression. A borderline significant correlation was demonstrated between polysomy 8 and diploid DNA content, as well as between disomy 1 and disease-free status of the patients. Chromosome 1 and 8 copy numbers may be considered as useful prognostic markers in invasive ductal carcinoma of the breast. (Pathology Oncology Research Vol 11, No 3, 157–163)

Key words: breast cancer, Iranian women, interphase FISH, clinicopathological parameters, chromosome 1 and 8 aneusomy, prognosis

Introduction

Breast cancer with prevalence of 8-12 percent is the second most common cancer in females amongst American women and the second cause of death of women living in the Western countries.[13,20,32] In the Middle East, breast cancer is the most common malignancy among women.[19] In Iran, it is also the number one female cancer, comprising 21.4 percent of all malignancies in women.[11,34] More than 70 percent of Iranian women with breast cancer expire due to advanced stage of the disease.[26]

The present clinical-histological parameters, however, can only help 60 percent of patients with breast cancer to achieve long-term disease-free status.[3] The genetic markers both at the level of single genes, such as oncogenes and tumor suppressor genes, as well as that of chromo-

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somes can, therefore, be of much value in improving the diagnosis and prognosis of breast cancer patients.

Different non-random chromosome abnormalities at both structural (chromosomes 1, 3, 6, 11, 13, 16 and 17) and numerical (chromosomes 7, 8, 12 and 20) level have been reported in breast cancer.[30] Gain of long arm of chromosome 1, loss of short arm of chromosome 8, and aneusomy of chromosomes 1 and 8 have been recorded in breast cancer.[23,31,39] Interphase fluorescence in situ hybridization (FISH) technique has proved to be of great value in identifying chromosomal abnormalities in malignancies, particularly in solid tumors.[9,30]

However, a limited number of reports have shown a correlation between chromosomal abnormalities and clinicopathological (stage, grade, tumor size, metastasis) or immunohistochemical parameters (P53, ER, PR, cathepsin D), DNA index, and survival as prognostic criteria.[24,27,33]

Chromosomes 1 and 8 are of particular interest as both harbor oncogenes and tumor suppressor genes. For example, chromosome 1 contains different oncogenes such as NRAS, LMYC, BLYM, FGR, SKI, ABL, and chromosome 8 harbors the oncogenes CMYC and HKR4, as well as DBC2 tumor suppressor gene.

In order to investigate the prognostic importance of chromosome 1 and 8 copy number in breast cancer, in a retrospective study, we have used chromosome-specific centromeric region DNA probes and FISH in primary tumor cells from archived, Carnoy-fixed cultured cell suspensions and selected paraffin-embedded tumor sections from 50 Iranian women, aged 35-64 years, with sporadic invasive ductal carcinoma. The relationship between chromosome 1 and 8 copy numbers and various established clinicopathological and immunohistochemical parameters, DNA index, age and the survival status of the patients was evaluated.

Materials and Methods

Tissue samples

The patients were all examined by one surgeon, assessed by comprehensive clinical investigation, and suspected cases underwent biopsy and/or partial or total mastectomy. All patients received adequate treatments in accordance with the standard protocols. None of the patients had any family history of breast cancer or any other malignancies at the time of diagnosis.

Cells were obtained from Carnoy (3:1 methanol:acetic acid) fixed cultured tumor cells and selected formalin-fixed, paraffin-embedded breast tumor blocks, retrieved from the archival materials.

Specimens were taken from 50 female patients with invasive ductal carcinoma. The age of patients ranged from 35 to 64 years (mean age of 48.1 years) with 30 patients below 50 years and 20 above 50. None of them had received any preoperative treatment.

Fluorescence in situ hybridization

Cell suspensions from cultured tumor cells. Following direct culturing of tumor cells in RPMI-1640 complete medium, the cells were harvested using 0.075 M KCl hypotonic solution and fixed with 3:1 methanol:acetic acid. The cell suspensions were spread on standard cytological slides. Some slides were pretreated in RNase (0.1 mg/ml), when necessary.

Cell suspensions from paraffin-embedded tissues. Sections of 4-5 μm thickness were cut onto silanized slides and heated at 65°C overnight. The sections were then dewaxed by two successive 10-min washes in xylene at room temperature, followed by 2x5-min washes in 100% ethanol. Pretreatment with 30% sodium bisulfite in 2x standard saline citrate, pH 7.0 for 15 min at 45°C was followed by a brief rinse in 2xSSC. Treatment with proteinase K (400 μl of a stock solution, 25 mg/ml) in 40 ml of 2xSSC, pH 7.0 at 45°C for 15 min was followed by a brief rinse in 2xSSC and 2 min dehydration in a series of graded alcohols.[28]

FISH procedure. The probes were obtained from Cytocell (www.cytocell.com) and Qbiogene (www.qbiogene.com). Probes specific for repetitive alphoid sequences at the centromeric region of chromosome 8 and classic satellite of 1q12 for chromosome 1 were used. The probes were directly labeled with FITC, Texas Red or Rhodamine fluorochromes.

Chromosomal DNA and probe DNA were denatured simultaneously by placing the slides in a 75 ± 1°C heating place for 5 min. Hybridization was carried out at 37°C in a humidified chamber for 48-72 hours. Post-hybridization washes were carried out with 0.5x SSC/0.1% SDS at 65°C for 3-5 minutes. 15 μl DAPI/Antifade (final concentration 0.02 μg/ml) was applied to the slides as counterstain.

Slides were examined using Leica fluorescent microscope (CW4000) equipped with appropriate filter combination for DAPI/FITC/Texas Red/Rhodamine fluorochromes (430 ex/468em and 532 ex/625 em) and an x100 objective.

The evaluation of the slides was carried out according to accepted criteria.[14] The categories of numbers of signals per nucleus were 1, 2, and 3 or more, resulting in monosomy, disomy and polysomy respectively. In each case, the number of clear, distinct signals in 50-200 (depending on cell density) non-overlapping nuclei was counted. The number of cells with different number of FISH signals was expressed as the mean of the percentage of cells counted. Aneusomy was regarded as the sum of cells with monosomy and polysomy.

Normal control samples for FISH experiment. Three normal chorionic villi and three normal peripheral blood samples were used as normal controls. According to the

conventional cytogenetics analysis, they all had a normal karyotype. Two negative controls without any FISH probes were also used.

Clinicopathological evaluation

Routine histological examination was performed with hematoxylin-eosin staining. Conventional histological classification of the World Health Organization[38] was applied. The combined histological grade (1, 2 or 3) of invasive ductal carcinoma was determined according to Elston.[7] Tumor staging was performed according to the tumor/nodes/metastasis system of the International Union Against Cancer.[37] Tumor size (<2 cm, 2-5 cm and >5 cm) and lymph node status were evaluated separately. The mean follow-up time was 48.51 months (range: 0.24–122 months).

Immunohistochemistry

Immunostaining for ER, PR, P53, and cathepsin D was performed by avidin-biotin immunoperoxidase method as previously reported.[15] All antibodies were purchased from Dako (Glostrup, Denmark).

Flow cytometry

The flow cytometer FACScan (Becton Dickinson, San Jose, CA) was used for DNA analysis and cell cycle determination according to standard protocols.

Statistical analysis

Data were analyzed with programs in SPSS 11.5 for Windows release. To assess the correlation between chromosome 1 and 8 copy numbers and clinicopathological status, we used cross tabs statistics, Pearson chi-square and Fisher's exact tests. To compare the mean numbers, independent t-test and one-way Anova were used. Differences were considered significant when p value was less than 0.05 with 95 percent confidence limit.

Results

Patients

Fifty patients with sporadic invasive ductal carcinoma were investigated for chromosome 1 and 8 copy number using interphase FISH technique. Patients' age distribution (older or younger than 50 years) and clinicopathological parameters (stage, grade, site, axillary lymph node involvement, tumor size, number of lymph nodes involved in metastasis, survival status, DNA index, ER, PR, P53, and cathepsin D expression) analyzed in this study are presented in *Table 1*.

Tumors of the majority of the patients were of stage II (72.3%), while the remaining ones were at stage III (17%), stage I (8.5%) and stage IV (2.1%). Three patients had no available information for the stage. Of patients for whom grade was available, 4.4% had grade 1, 37.8% grade 2 and 57.8% grade 3 tumors. No grade information was available for 5 patients. 46.8% of the carcinomas were node-negative, and 54.2% had nodal

Table 1. **Clinicopathological data**

	No.	*%*
Age: < 50 years	30	60
≥ 50 years	20	40
Stage I	4	8.5
Stage II	34	72.3
Stage III	8	17.0
Stage IV	1	2.1
Grade 1	2	4.4
Grade 2	17	37.8
Grade 3	26	57.8
Site: right	24	48.0
left	26	52.0
ALN met.: positive	26	54.2
negative	22	45.8
Tumor size : < 2 cm	6	12.2
2-5 cm	41	83.7
> 5 cm	2	4.1
No. of LN with met.: 0	22	46.8
1-3	12	25.5
4-9	6	12.8
≥ 10	7	14.9
Disease-free	31	77.5
Distant met.	6	15.0
Death	3	7.5
DNA content: diploid	18	52.9
aneuploid	6	17.6
diploid + S↑	10	29.4
ER: positive	28	60.9
negative	18	39.1
PR: positive	27	64.3
negative	15	35.7
P53: positive	14	41.2
negative	20	58.8
Cathepsin D: increased	32	86.5
normal	5	13.5

ALN: axillary lymph node, met.: metastasis diploid + S↑: diploid DNA content with increased S phase

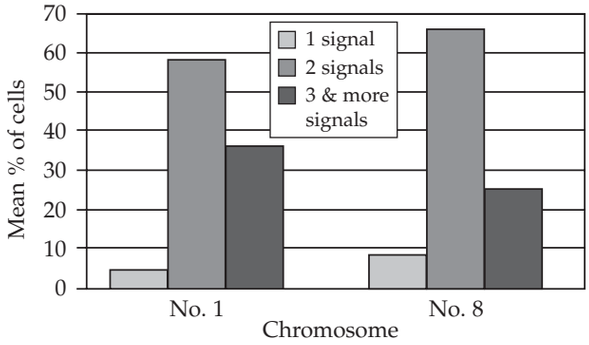

Figure 1. *Distribution of chromosome 1 and 8 FISH signal numbers in breast cancer samples (n=50)*

involvement. Nodal sampling was not performed in 2 patients. The mean age was 48.1 years, ranging from 35 to 64 years. Sixty percent of the patients were below 50 and 40% were more than 50 years old. Data on estrogen receptor (ER) status was available for 46 patients, of whom 60.9% were positive and 39.1% were negative. For progesterone receptor (PR) status, 64.3% were positive, and 35.7% were negative. The PR data for 8 patients was missing. Only 34 patients had available data for P53 protein expression; 41.2% had positive expression, while 58.8% were negative. For cathepsin D level, 13.5% had normal level, and 86.5% had increased level, while 13 patients had no information.

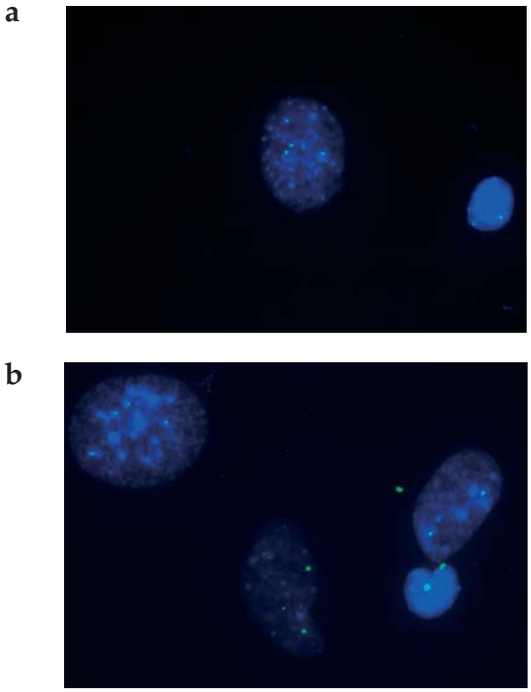

Figure 2. *Typical chromosome 8 interphase FISH signals showing disomy* ***(a)*** *and polysomy* ***(b)*** *in breast tumor cells*

Table 2. **Distribution of chromosome 1 interphase FISH signal number in the 50 patients**

	Min	*Max*	*Mean*	*SD*
1 signal	0	24	4.24	4.87
2 signals	13	103	43.52	17.60
≥3 signals	6	70	29.68	17.49
Total abn.	13	85	33.92	16.52
Total cells	46	148	77.44	27.42

Data are expressed as percentage of cells. SD: standard deviation. Total abn.: mean percentage of cells with 1 or ≥3 signals. Total cells: mean percentage of cells with 1, 2, or ≥3 signals

Table 3. **Distribution of chromosome 8 interphase FISH signal number in the 50 patients**

	Min	*Max*	*Mean*	*SD*
1 signal	0	21	6.48	4.85
2 signals	22	180	57.10	38.22
≥3 signals	3	129	21.82	19.51
Total abn.	10	65	28.30	11.52
Total cells	45	210	85.40	47.73

Data are expressed as percentage of cells. SD: standard deviation. Total abn.: mean percentage of cells with 1 or ≥3 signals. Total cells: mean percentage of cells with 1, 2, or ≥3 signals

The follow-up time was available for 43 patients, with a mean of 48.51 months, ranging from 0.24 to 122 months. Patients were divided into three groups according to the survival status. Group 1 (77.5% of the patients) were disease-free, with a mean follow-up time of 51.94 months. Group 2 (15%) had developed distant metastasis with a mean follow-up time of 41.37 months. Group 3 (7.5% of patients) died with a mean follow-up time of 27.33 months. One of group 3 patients had developed bone metastasis before death.

It was found that 48% of the patients had developed tumor in the right breast, whereas 52% had involvement of the left breast. No patient had bilateral breast carcinoma. According to tumor size, the cases were grouped into three categories: <2 cm; 2 to 5 cm, and >5 cm. The majority of patients belonged to group 2 (83.7%), followed by group 1 (12.2%), and only 4.1% patients belonged to group 3. The data was not available for one patient. According to the number of lymph nodes involved in metastasis, patients were categorized into four groups: group 1 with no lymph node involvement, group 2 with 1-3, group 3 with 4-9, and group 4 with 10 or more lymph nodes involved. In group 1 there were 46.8% patients, 25.5% were in group 2, 12.8% were in group 3, and 14.9% belonged to group 4. There was no available data for 3 patients. DNA content was organized into three categories: group 1 with normal diploid DNA content, group 2 with aneuploidy, and group

3 with diploid DNA content but increased S phase. Only 34 patients had information for the DNA content. 52.9% of the patients had diploid DNA content, 17.6% belonged to group 2 and 29.4% were in group 3.

FISH analysis

Interphase FISH was carried out successfully on all of the 50 breast tumor samples and 6 controls. They demonstrated a heterogeneous copy number pattern, consisting of monosomic, disomic, and polysomic cell populations (*Figures. 1,2*).

For chromosome 1, the mean percentage of disomic cells was 43.52%, for monosomy it was 4.24%, while for polysomy 29.68% (*Table 2*). The above figures for chromosome 8 were 57.10%, 6.48%, and 21.82% respectively (*Table 3*).

In control samples (peripheral blood and chorionic villus samples), the mean percentage of monosomic, disomic and polysomic cells for chromosome 1 was 2.25%, 96.25% and 1.5%, respectively. For chromosome 8, the mean percentage of cells was 2% for monosomy, 97% for disomy, and 1% for polysomy in control samples.

The cut-off point for percentage of cells with abnormal copy number (monosomy and polysomy) was considered as 3 for both chromosomes 1 and 8 in all the patients.

Association and correlation studies

In order to compare the mean values of the percentage of cells with monosomy, disomy and polysomy between groups with different status for each variable, the independent t-test and one-way Anova test were used. Monosomy for chromosome 1 was associated with age less than 50 years (*Table 4*), and with poor survival status (death) of the patients (*Table 5*). Increased number of cells with monosomy for chromosome 1 was more prevalent in patients below the age of 50 years (p=0.018). Patients who had died had significantly increased cell number with chromosome 1 monosomy (p=0.022). For chromosome 8, tumors expressing P53 protein had higher number of cells with disomy than P53-negative ones (p=0.046) (*Table 6*). No other significant association was observed.

To assess the correlation between chromosome copy numbers and the various clinicopathological parameters, Pearson chi-square and Fisher's exact tests were used. Samples were divided into two groups for each status of chromosome copy number (number of FISH signals), according to the mean percentage of cells (less than 20% or more than 20%). No significant correlation with any of the clinicopathological variables was observed. However, borderline significant correlation was found between disease-free status and chromosome 1 disomy (p=0.065) (*Table 7*), and between diploid DNA content and chromosome 8 polysomy (p=0.059) (*Table 8*). DNA content did not demonstrate any significant association with polysomy for chromosome 1.

Table 4. **Relationship between chromosome 1 monosomy and age**

Age (years)	*Patients no. (%)*	*Mean % of cells**	*p value*
< 50	19 (63.3)	7.79	0.018
≥ 50	11 (36.7)	3.91	

*Mean percentage of cells with chromosome 1 monosomy

Table 5. **Relationship between chromosome 1 monosomy and survival status**

Survival status	*Patients no. (%)*	*Mean % of cells**	*p value*
Disease-free	18 (72.0)	5.94	
Distant met.	5 (20.0)	5.20	0.022
Death	2 (8.0)	16.50	

*Mean percentage of cells with chromosome 1 monosomy; met.: metastasis

Table 6. **Relationship between chromosome 8 disomy and P53 expression**

P53 expression	*Patients no. (%)*	*Mean % of cells**	*p value*
Positive	14 (41.2)	74.43	0.046
Negative	20 (58.8)	46.55	

*Mean percentage of cells with chromosome 8 disomy

Table 7. **Relationship between chromosome 1 disomy and survival status**

DNA content	*No. (%) of patients with mean % of cells**		*p value*
	<20	≥20	
Disease-free	3 (27.3)	15 (65.2)	
Distant met.	4 (36.4)	2 (8.7)	0.065
Death	4 (36.4)	6 (26.1)	

*Mean percentage of cells with chromosome 1 disomy; met.: metastasis

Discussion

Using interphase FISH, all 50 breast cancer samples involved in the present study demonstrated aneusomy for chromosomes 1 and 8. The aneusomy rate was 43.80% for

Table 8. **Relationship between chromosome 8 polysomy and DNA content**

DNA content	*No. (%) of patients with mean % of cells**		*p value*
	<20	≥20	
Diploid	3 (27.3)	15 (65.2)	
Aneuploid	4 (36.4)	2 (8.7)	0.059
Diploid +S↑	4 (36.4)	6 (26.1)	

*Mean percentage of cells with chromosome 8 polysomy
Diploid + S↑: diploid DNA content with increased S phase

chromosome 1 and 33.14% for chromosome 8. These figures are similar to other reported cases,[4,8,22,23,31] suggesting that Iranian women with invasive ductal carcinoma encounter some degree of genetic instability in their tumor cells, which is comparable to that of their counterparts in other countries. A marked intratumoral cytogenetic heterogeneity for chromosomes 1 and 8 was observed in all cases, which reflects the heterogeneous genetic nature of breast tumor cells.

Nevertheless, the use of satellite probes for the pericentromeric regions of chromosomes 1 and 8 does not provide information on structural changes not involving the centromere. However, it is recognized that the numbers of hybridization domains in the interphase FISH experiments for a particular chromosome-specific repeat sequence could be considered as the direct measure for the number of the target chromosomes.[17,29]

The age range of our patients was 35 to 64 years with mean age of 48.1 years. The majority of younger patients (less than 50 years) had significantly increased number of cells with monosomy 1 compared to the older patients. High rate of monosomy 1 in younger patients, due to the loss of potential tumor suppressor genes, can cause worsening of the patients' outcome. However, various reports from Iran have shown that Iranian breast cancer patients are relatively young compared to other countries,[2,26] and their tumors are characterized by a more aggressive biology.[31] Mehdipour et al[25] in a large series of Iranian breast cancer patients reported a similar mean age of 49 years for all the patients and for those with no family history of breast cancer, respectively, and our study showed a similar mean age.

In assessing the relationship between chromosome 1 and 8 copy number (number of interphase FISH signals) and different survival status, the only significant association was found between chromosome 1 monosomy and death of the patients. However, the level of chromosome 1 monosomy was similar in disease-free patients and those with distant metastasis. In other words, loss of one chromosome 1 may be related to poor outcome for the patients. Chromosome 1 potentially harbors several tumor suppressor genes,[21] and it has been shown that allelic losses at 1p region of chromosome 1 and loss of p73 at 1p36.3, a putative tumor suppressor gene resembling P53, led to poor survival in breast cancer patients.[6,41]

Various reports have associated P53 expression with poor prognosis in breast cancer patients.[10,16,18] In our study, high mean percentage of cells with disomy 8 was significantly associated with P53-positivity. P53, however, did not show any significant association with other chromosome copy number. The findings could suggest an indirect prognostic role for disomy 8 in relation to P53 expression amongst Iranian women.

Other clinicopathological parameters did not show any statistically significant correlation with chromosome 1 and 8 copy numbers. However, two borderline significant associations were indicated. Disease-free status showed an almost significant correlation with disomy for chromosome 1. This could suggest that normal copy number for chromosome 1 favors a better survival outcome for the patients, which is in agreement with other reports.[1,27,35] Furthermore, chromosome 8 polysomy was almost significantly associated with diploid DNA status. However, polysomy 1 did not show any significant association with DNA content. Literature data has indicated the importance of FISH studies as compared to DNA index measurements with flow cytometry. Harrison et al[12] demonstrated that 75% of their breast cancer patients with disomic chromosome 1 had an aneuploid DNA content. Truong et al[36] also demonstrated chromosome abnormalities using FISH studies in breast cancer patients with diploid DNA content. A diploid status does not reflect gains or losses of different chromosomes, which warrants cytogenetic and FISH studies. These findings could reflect that interphase FISH is more sensitive than DNA flow cytometry for detecting chromosome abnormalities.

In conclusion, all 50 cases of sporadic invasive ductal carcinoma, involved in this study, demonstrated aneusomy for chromosomes 1 and 8. Both poor survival and younger age (<50 years) were significantly associated with chromosome 1 monosomy. P53 expression was significantly associated with chromosome 8 disomy. The above findings could suggest some prognostic roles for chromosome 1 and 8 copy number. However, caution should be observed in interpretation of these data as the number of patients is small, and evaluation of data on a larger set of patients would be necessary for more conclusive results.

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