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Hormone Sensitivity in Breast Cancer: Influence of Heterogeneity of Oestrogen Receptor Expression and Cell Proliferation

R.I. Nicholson, N. Bouzubar, K.J. Walker, R. McClelland, A.R. Dixon, J.F.R. Robertson, I.O. Ellis and R.W. Blamey

The percentage of oestrogen receptor (ER) positive cells in a series of 118 breast cancers has been examined by immunohistochemistry in relation to patients' response to endocrine therapy. Positive and negative predictive values have been used to calculate appropriate cut-off points. The rate of response to treatment was significantly higher in women with receptor positive tumours, especially where the tumours contained more than 70% positive cells. Tumours that were apparently negative for ER expression rarely responded to endocrine therapy. The hormone sensitivity of ER positive breast cancer was also influenced by the rate of tumour cell proliferation, with tumours expressing high levels of Ki67 immunostaining rarely responding to therapy.
Eur J Cancer, Vol. 27, No. 7, pp. 908–913, 1991

INTRODUCTION

THE PREDICTION of hormone sensitivity in breast cancer has occupied clinical and biochemical laboratories for many years. This preoccupation is based on the relatively complication free and lengthy tumour remissions that may be achieved in advanced breast cancer patients with endocrine therapy and the need to preselect patients with primary breast cancer for adjuvant therapies. Some success has already been achieved in this area using the hormone receptor status of either the primary or secondary disease [1, 2], especially when quantitative aspects of the assays are taken into account [3] or when more than one hormone receptor is measured [4]. Unfortunately, the steroid binding assays [1–4], and more recently the enzyme immunoassays [5] that have been employed in these studies have required the solubilisation of the receptor prior to its measurement and have necessitated access to appreciable quantities of tissue. Thus, information on the heterogeneity of hormone receptor expression is lost and tumour tissue is often not available for other assays. These technical difficulties have, however, now been overcome through the use of monoclonal antibodies to

oestrogen and progesterone receptors in immunohistochemical procedures [6–8].

The object of this study was, therefore, to firstly investigate the relationship between the heterogeneity of oestrogen receptor (ER) expression in the tumour cell population and the responsiveness of breast cancer to endocrine therapy and then to examine the modifying influence that cell proliferation, as measured by Ki67 immunostaining, has on these events. We have previously shown that each of these parameters has an influence on the rate of recurrence of breast cancer after mastectomy in the absence of adjuvant endocrine treatment [9, 10].

PATIENTS AND METHODS

118 samples of primary tumour tissues obtained from patients with histologically proven breast cancer presenting to R.W.B. at the City Hospital, Nottingham, during the period July 1984 to August 1987 were included in the study. Histology was confirmed according to previously described procedures [11]. No patient had previously received any form of adjuvant endocrine or cytotoxic therapy.

All patients received systemic endocrine therapy either for locally advanced primary tumours (diameter >5 cm, $n=45$) or for local ($n=11$) or distant ($n=62$) recurrences. Premenopausal or perimenopausal patients received the LH-RH agonist goserelin (3.6 mg depot/28 days) alone ($n=23$) or in combination with antioestrogen tamoxifen (20 mg twice daily, $n=3$), while the majority of postmenopausal women received tamoxifen (20 mg twice daily, $n=88$). 4 postmenopausal women received Megase (160 mg twice daily).

Patients were assessed for response, static disease (no change) or progression at 2–3 monthly intervals by UICC criteria [12]. As recommended by the British Breast Group, response and static disease were only reported following a minimum duration of remission of 6 months [13]. The overall numbers of patients with responsive (complete and partial), static and progressive disease following endocrine measures were 30, 29 and 59, respectively. No statistical differences in response rate were observed within the groups of women receiving tamoxifen (22 responders, 23 statics and 43 progressors) or goserelin with or without tamoxifen (7 responders, 5 statics and 14 progressors). Of women treated with megase 1 woman responded, 1 had static disease and 2 women progressed despite therapy.

Preparation of tissue and immunohistochemical procedures

Samples of primary tumour tissue were rapidly frozen upon excision, stored at −70°C and transported on dry ice to the Tenovus Institute, Cardiff, for analysis. A representative portion of the tissue was blocked for cryostat sectioning and immunohistochemistry.

ER-immunohistochemical assay (ICA)

All immunological reagents, including H-222, the primary rat anti-human ER antibody, were supplied in the monoclonal ER-ICA kit (Abbott). Details of the staining procedures and validity of results have been discussed elsewhere [9] and are relatively unmodified here. Briefly, fixation of 5 μm cryostat sections was in 3.7% formaldehyde in 0.01 mol/l phosphate-buffered saline (PBS) for 15 minutes followed by immersion in cold (−20°C) baths of methanol (5 minutes) and acetone (2–3 minutes). Fixed sections can be stored at this point at −20°C for up to 1 week prior to staining, according to the method of Crawford [14]. Non-specific attachment of antibody was prevented by preincubation of slides for 15 minutes with a blocking reagent (2% normal goat serum), followed by primary or control rat antibody incubations (30 minutes). Receptor localisation was revealed by the indirect peroxidase anti-peroxidase (PAP) procedure [15].

Ki67 immunostaining

The details of this assay have been previously described [10]. Briefly, fixation of 5 μm cryostat sections was in cold (−10 to −25°C) acetone (10 minutes) followed by air drying. Sections were incubated at room temperature with 10% normal goat serum for 15 minutes. Excess serum was then removed and the slides incubated for a further 45 minutes with mouse monoclonal Ki67 antibody (1.4 μg/ml, Dakopatts, Denmark). The slides were then rinsed in PBS and primary antibody binding was revealed by the indirect PAP procedure [15] using goat anti-mouse bridging antibody containing normal human serum followed, after washing in PBS, with a mouse peroxidase antiperoxidase complex.

Specimen evaluation

All specimen evaluation was performed on an Olympus microscope (BH-2) first using an ocular magnification of × 10 in order to enable the localisation and subsequent avoiding of normal and benign areas within the section. This initial examination also allows the heterogeneity of immunostaining within the tumour components to be assessed and thus ensures that adequate sampling of all areas is performed. All subsequent evaluations were carried out using an ocular magnification of × 40. ER and Ki67 immunostaining patterns were evaluated by two personnel (ER-ICA, R.M. and R.I.N.; Ki67, N.B. and R.I.N.) using a dual-viewing attachment to the microscope. Control slides were checked for non-specific binding before assessing the percentage of tumour cells stained by the primary antibody (minimum of 2000 tumour cells evaluated). The intensity of ER-ICA staining in target cells were assessed semiquantitatively using a score of 0 to 3 corresponding to negative, weak, intermediate and strong staining intensities. The percentage of tumour cells in each of these categories was used to calculate an index (I) for each tumour based on: I = [(% cells showing an intensity value of 1 × 1) + (% cells showing an intensity value of 2 × 2) + (% cells showing an intensity value of 3 × 3)]/100. Unlike ER-ICA, the nuclear Ki67 immunostaining patterns were not uniform within individual nuclei and showed substantial granulation in some tumours. Since this does not allow an accurate semiquantitative analysis to be performed, an index was not calculated for Ki67 immunostaining.

Negative and positive predictive values for the ER and Ki67 assays were calculated using varying cut-offs for the assays according to the formulae:

$$\text{ER-ICA negative predictive value (Fig. 1a)} = \frac{\text{ER}- \text{ progressors}}{\text{ER}- \text{ responders} + \text{ER}- \text{ progressors}}$$

$$\text{ER-ICA positive predictive value (Fig. 1c)} = \frac{\text{ER}+ \text{ responders}}{\text{ER}+ \text{ responders} + \text{ER}+ \text{ progressors}}$$

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Revised 25 Mar. 1991; accepted 9 Apr. 1991.

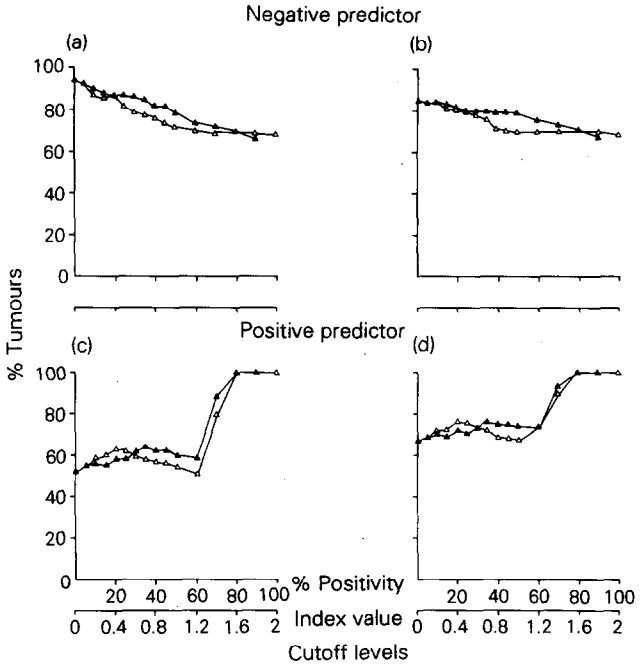

Fig. 1. Negative and positive predictive values for the ER-ICA. Data derived from percentage ER-ICA positive cells (▲) and index values (△) are presented. (a) and (c) = response vs. progression, (b) and (d) = response static vs. progression.

Ki67 negative predictive value (Fig. 2a)

$$= \frac{\text{Ki67+ progressors}}{\text{Ki67+ responders + Ki67+ progressors}}$$

Ki67 positive predictive value (Fig. 2c)

$$= \frac{\text{Ki67− responders}}{\text{Ki67− responders + Ki67− progressors}}.$$

Static disease was included as a response in Figs 1b, d and 2b, d. Ki67 immunostaining was carried out on a subset (n=52) of the tumours stained with the ER-ICA (n=118). The response rate of these tumours to the endocrine measures (29% responders, 26% static and 44% progressors) was similar to the total group (25%, 25% and 50% respectively) and they were not disproportionately biased towards any of the staging groups (locally advanced disease, n=20, local, n=4, and distant, n=28, recurrences). They were similarly well matched for patient age (total group, 26–78 years, median 59; Ki67 group, 28–78, median 61) and hence type of endocrine therapy administered. The overall ER positivity (42/52) in the Ki67 stained group was slightly, but not significantly, higher than in the total tumour group (86/118).

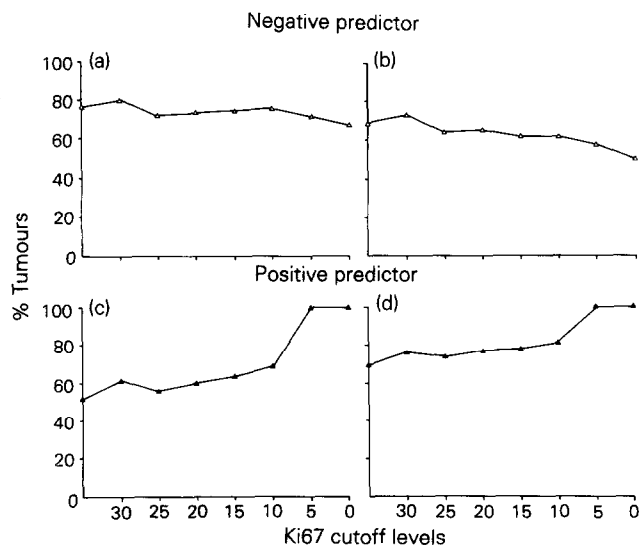

Fig. 2. Negative and positive predictive values for the Ki67 assay. (a) and (c) = response vs. progression. (b) and (d) = response static vs. progression.

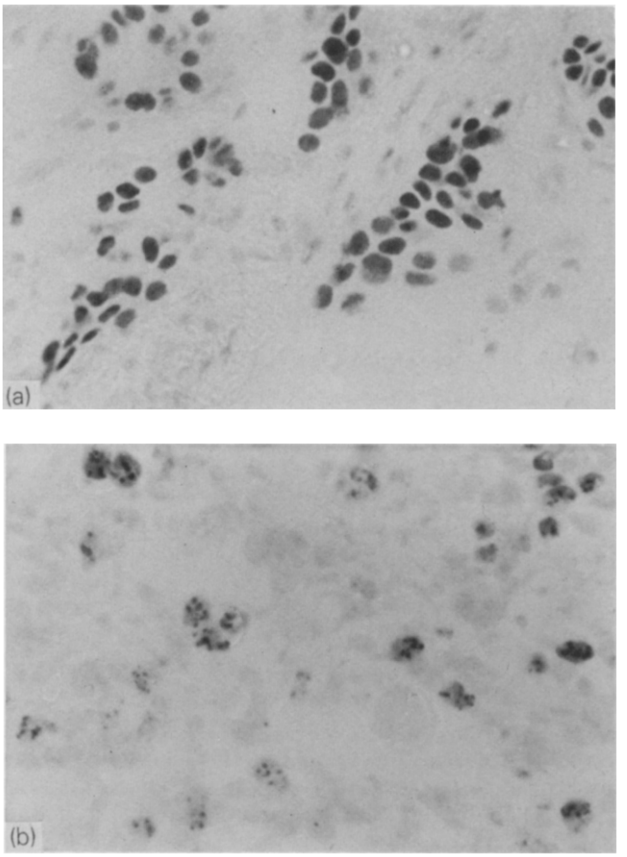

Fig. 3. Immunohistochemical staining of tumours with monoclonal antibodies H222 (a) and Ki67 (b).

Statistical analysis

Data were analysed using a χ^2 statistic for contingency table analysis and χ^2 for trend according to Armitage [16].

RESULTS

ER immunostaining

Figure 3a shows a typical immunohistochemical staining pattern using the antihuman ER rat monoclonal antibody H222 and demonstrates its nuclear staining characteristics. Of the 118 tumours examined 86 (72%) were classified as ER-ICA positive (any tumour cells ER-ICA positive). Within these tumours ER staining was heterogeneous with up to 95% of tumour cells expressing detectable quantities of ER. Figure 4 shows the relationship of this heterogeneity of ER expression to response to endocrine therapy. Although no continuous trend is obvious between tumour receptor levels and the responsiveness of breast cancer to endocrine measures, at the extremes of receptor positivity and negativity there appears to be an enrichment for hormone responsive and hormone unresponsive tumours respectively. This was apparent when either the percentage of

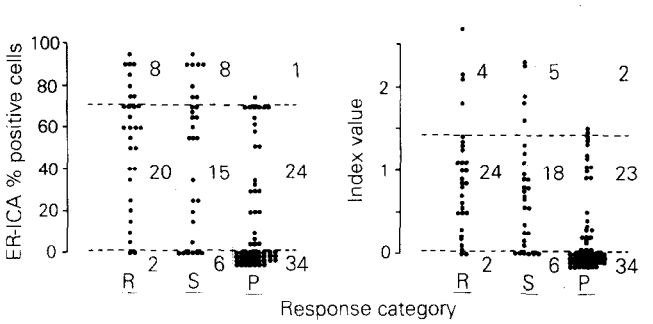

Fig. 4. Relationship between ER and response to therapy. The number of responsive (R), static (S) and progressive (P) tumours in each of the three defined categories of receptor positivity are presented.

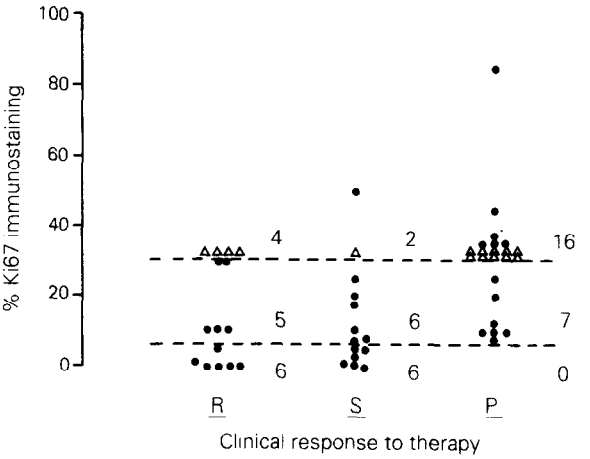

Fig. 5. Relationship between Ki67 immunostaining and response to therapy. Tumours which show high focal Ki67 positivity are shown by (△).

ER-ICA positive cells was considered (Fig. 4a) or ER-ICA indices (Fig. 4b). Negative and positive predictive values were calculated for ER-ICA cut-off values ranging from 0 to 100% positive cells (or index values 0 to 2). The 89 patients who showed responsive or non-responsive disease were used to calculate the data shown in Figs 1a, c. Patients with static disease ($n=29$) were included in the results shown in Figs 1b, d. The negative predictive value of the ER-ICA declined from 94% at a cut-off of 0% positive cells (or an index value of 0) in Fig. 1a and 85% in Fig. 1b, to approximately 70% in each instance. Examination of the positive predictive value for the ER-ICA assay showed that using cut-off points between 20 and 60% ER-ICA positive tumour cells or index values between 0.4 and 1.2, the calculated value was approximately 60–70% (Fig. 1d). Above these cut-off limits, however, the calculated predictive value rose to 100%.

Based on these observations tumours have been defined as ER-ICA positive if they contain any ER-ICA positive cells and highly positive if they contain more than 70% positive cells or have index values over 1.4. As may be observed in Fig. 4 (dotted lines) this classification scheme shows a highly significant correlation between increasing ER expression and increased likelihood of tumour response to endocrine therapy (Fig. 4a: ER-ICA positive cells, χ^2 for trend, 28.9, $P<0.001$; Fig. 4b: index value, χ^2 for trend, 22.3, $P<0.001$).

Although in this study postmenopausal women were more likely to be ER positive (69/92, 75%) than their premenopausal counterparts (17/26, 65%), the differences were not statistically significant and subdivision of the above data according to patient menopausal status was not undertaken.

Ki67 immunostaining

Figure 3b illustrates the Ki67 immunostaining pattern in a highly positive tumour. Again a nuclear staining pattern is evident. Of the 52 tumours examined 44 (85%) showed Ki67 positive cells (Fig. 5). Interestingly, in 29 of the 44 positive tumours intratumour Ki67 immunostaining was relatively uniform and a mean value for the tumour was estimated. The estimated values ranged from 2 to 85% of cells positive. In the remaining positive tumours, however, Ki67 staining was focal and showed areas of high positivity (more than 30% cells involved) against a background of lower positivity or negativity. These tumours are identified in Fig. 5, but have been classified elsewhere as high Ki67 immunostaining tumours.

Subdivision of the Ki67 immunostained tumours according to the clinical response of the patients to endocrine therapy showed patients with progressive disease more often contained highly Ki67 positive tumours than those women with endocrine responsive tumours (Fig. 5). Calculation of a negative predictive value for the Ki67 assay using cut-off values ranging from 35 to 0% positive cells demonstrates a lower efficiency of prediction of failure to respond to endocrine therapy than when the ER-ICA was used (Figs 2a, b). The maximum value was observed at 30% Ki67 positivity and included all of the high focal Ki67 positive tumours. The greatest positive predictive value for the assay was observed at a cut-off value of 5% Ki67 cellular positivity, where 12/12 patients responded to endocrine measures (Figs 2c, d).

Based on these observation tumours have been defined as Ki67 positive if they contain >5% positive cells and highly positive if they contain >30% positive cells. As may be observed from Fig. 5 (using dotted lines) this classification scheme shows an association between increasing Ki67 immunostaining and loss of hormone sensitivity in breast cancer (χ^2 for trend, 12.0, $P<0.001$).

Combination of ER-ICA and Ki67 results

Figure 6 shows the relationship between the ER and Ki67 immunohistochemical assays. Although an inverse relationship between these parameters may exist, with ER-ICA negative tumours tending to show higher levels of Ki67 immunostaining than their highly ER-ICA positive counterparts, nevertheless,

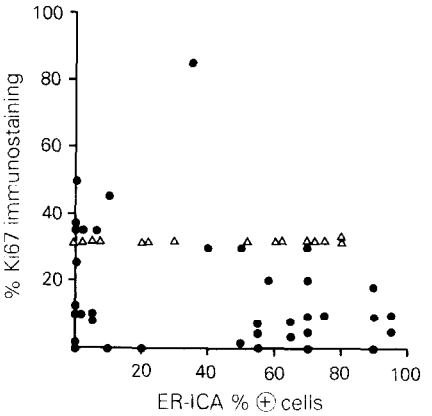

Fig. 6. Relationship between the ER-ICA and Ki67 assays. Tumours which show high focal Ki67 positivity are shown by (△).

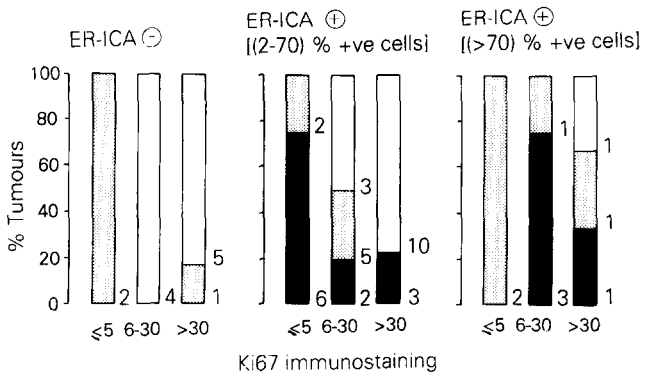

Fig. 7. Relationship between the ER-ICA and Ki67 assays and response to therapy. The number of patients with responsive ■, static ▨ and progressive □ disease are presented.

considerable numbers of ER-ICA positive tumours with high Ki67 staining patterns have been identified. Subdivision of the ER-ICA response data according to the Ki67 assay showed a tendency for the acquisition of hormone insensitivity in weakly ER positive tumours (2–70% positive cells) with increasing Ki67 immunostaining (Fig. 7). Indeed, while all 8 patients with low or absent (<5% positive cells) Ki67 staining derived some benefit from endocrine therapy, 10/13 patients with highly Ki67 positive tumours failed to respond. Insufficient numbers of tumours in the ER-ICA negative and highly positive groups prevented further analysis of the data.

DISCUSSION

A number of publications have shown a relationship between the ER-ICA and the therapeutic response of patients to endocrine therapy [17–19]. Response is almost solely restricted to ER positive disease, where approximately half of the patients undergo a clinically defined remission. Significantly, however, these studies have been on relatively small groups of patients and, with the exception of one recent publication [19], have not examined quantitative aspects of the assay. In the current study, using calculations of the negative and positive predictive values for various cut-offs for the ER-ICA, we have determined that three subgroups of patients may be defined (Fig. 4). Firstly, patients with tumours in which ER-ICA staining was not observed in any tumour cell and which show very few tumour remissions on endocrine therapy. Secondly, a group of tumours which show modest ER expression and contain approximately equal proportions of hormone sensitive and insensitive tumours. Thirdly, a group of highly ER-ICA positive tumours, where hormone insensitivity is a rare event. These data, which are paralleled by ER-ICA indices, support the notion that the proportion of tumour cells which are hormone receptor positive contributes to the sensitivity of the disease to endocrine measures, and directly confirms the quantitative association between ER level and hormone sensitivity shown by our group and others using ligand binding assays [1–3]. However, cellular ER positivity/negativity is not the only influence on the hormone responsiveness of the disease and in the current study we have extended our observations to show that the proportion of breast cancer cells engaged in cell proliferation, as measured by Ki67 immunostaining, also has a modifying or determining role in patients with ER-ICA positive tumours. Indeed, this antibody, which detects a poorly characterised nuclear protein, the presence of which directly relates to the rate of recurrence of breast cancer after mastectomy [10], when expressed in a high proportion of tumour cells is frequently associated with a failure of ER positive tumours to respond to endocrine therapy, especially those which display intermediary ER levels. Taken together these results provide immunohistochemical data to support the clinical observations that rapidly growing tumours are unlikely to be responsive to endocrine therapy [20]. The present information, however, may be gained in advance of therapy and if confirmed in a prospective study may be useful in the planning of treatment regimes in both primary and advanced breast cancer.

In addition to the obvious clinical relevance of our observations, they are also of biological interest since they more clearly define scenarios in which hormone receptor positive tumours fail to respond to endocrine measures and hence gain an autonomous state. Detailed investigation of these immunohistochemically defined tumours should now be undertaken to establish the nature of the mitogenic signals which appear to play such an important role in the subversion of hormone sensitivity in ER-ICA positive tumours. Immunohistochemical studies to investigate the involvement of growth factor pathways and oncogenic events in the clinical loss of hormone sensitivity are currently underway in our laboratory. Significantly, the current study employed four treatment groups, with the majority of women receiving either goserelin or tamoxifen. Since these therapies are believed to have a common mechanism of action by reducing the availability of oestrogens to the tumour cells [21], the study has not differentiated between these treatments. In the future, however, recruitment of a larger group of patients will allow individual therapies to be studied separately.

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What Healthy Women Think, Feel and Do about Cancer, Prevention and Breast Cancer Screening in Italy

Deborah R. Gordon, Antonella Venturini, Marco Rosselli Del Turco, Domenico Palli and Eugenio Paci

In preparation for a major campaign to encourage participation in a breast screening programme for all women between 50–70 years old in Florence, Italy, an in-depth study of 200 women's attitudes and practices regarding cancer and its prevention was undertaken. 72% participated in semistructured interviews, and inclination to participate was analysed in terms of Yes (39%), Uncertain (36%), and No (20%) (5% were unspecified). Age ($P = 0.03$), area of birth ($P = 0.01$), education level of husband ($P = 0.04$) and prior Pap smear ($P = 0.00$) or mammography ($P = 0.00$) were among the variables statistically associated with inclination to participate. While the women shared more or less common images of cancer, concern for health, and belief in the importance of God and destiny, those less inclined to participate felt less personally vulnerable, had less faith in medicine's ability to cure or prevent cancer, were more distant from the medical system and culture in general and regarded information about health or illness more as unnecessary or dangerous than beneficial. Understanding and consideration of both "popular" and "medical" cultures need to be encouraged.
Eur J Cancer, Vol. 27, No. 7, pp. 913–917, 1991

INTRODUCTION

IN PREPARATION for a major campaign to encourage participation in a free breast cancer screening programme beginning in 1990 for about 60 000 women between 50–70 years old in Florence, Italy, an in-depth study of women's attitudes and behaviour related to cancer—specifically breast cancer—was undertaken.

The first aim of the study was to describe how Italian women experience, understand and approach cancer and its prevention in their daily lives. It was assumed that women's perceptions of and reactions to a proposed screening test—including not participating—would be culturally grounded in popular, lay logic(s) that would likely differ from medical logic.

A second aim was to identify attitudes, beliefs and behaviours of potential participants and refusers of the programme to help plan campaign messages to reach women with different inclination to participate.

We describe how cancer is perceived among this healthy population in general as well as the differential characteristics of groups of women with varying inclination to participate in the screening programme.

METHODS

The study was based on a random sample of 200 women 50–70 years old selected from the demographic registry of two representative districts of the municipality of Florence. Women were first contacted by letter and then by telephone.

A semistructured interview questionnaire elaborated from a pilot study of 36 women was used. The interviews were carried out by 3 skilled interviewers at the site chosen by the woman: either at home or at the Center for the Study and Prevention of Cancer (CSPO), or when absolutely necessary, by telephone. Direct interviews were tape-recorded. All the interview questions, barring four, were open-ended yet predominantly precoded and a number of answers were recorded with up to three lines of quotation from the women. Inter-rater reliability was

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