Pergamon

European Journal of Cancer Vol. 32A, No. 10, pp. 1686–1692, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

PII: S0959-8049(96)00154-2

Original Paper

Comparison of Prognostic Markers Detected by Immunohistochemistry in Male and Female Breast Carcinomas

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Male infiltrating breast carcinomas are rare and seem to have different characteristics, prognosis and sensitivity to hormonal treatment than those of female breast carcinomas. Our aim was to determine whether markers which have an established rôle in women are also important in men. 66 male infiltrating-ductal breast carcinomas were compared with 190 female breast carcinomas of the same type. Various markers were studied using immunohistochemistry. Tumour size at diagnosis, grade, number of axillary metastases and prognosis were comparable in male and female breast carcinomas. However, male breast carcinomas were characterised by a higher percentage of oestrogen receptor (OR) reactivity, and weekly associated with markers that, in women, are under oestrogen control. Male breast carcinomas were positive for markers under androgen control. Male breast carcinomas also differed from female carcinomas by the low percentage of p53$^+$ and the high percentage of bcl-2$^+$ tumours. The phenotype of male breast carcinomas has characteristics that could have repercussions on prognosis and on the choice of hormonal treatment. Only a few male breast cancers are p53$^+$. OR, which are frequently present in male tumours, are probably not functional. In contrast, androgen receptors seem efficient, as several markers under androgen control are expressed. Therefore, the selection of hormonal therapy should not be based on OR status only. Copyright © 1996 Published by Elsevier Science Ltd

Key words: immunohistochemistry, male breast carcinomas, p53, bcl-2, hormonal receptors, oestrogens, androgens, markers, pS2

Eur J Cancer, Vol. 32A, No. 10, pp. 1686–1692, 1996

INTRODUCTION

THE INCIDENCE of male breast carcinoma is low compared to that of female breast cancer [1, 2]. However, they have many similarities. The number of positive axillary lymph nodes, the size of the tumour and histological grade have prognostic value in both sexes, although the prognosis of male breast carcinoma seems generally poorer. Several markers, which have been well-studied in female breast carcinomas, have also been found immunohistochemically in male carcinomas. Cathepsin D [3], Ras [4], B72.3 [4, 5], pregnancy specific β1 glycoprotein [5], and EGF-receptor [6] have been shown with a frequency almost equal to that of female breast carcinoma. However, in male breast carcinomas, there is a high incidence of oestrogen receptor (OR) positivity [3, 6], but it is not known whether these receptors are functional under the low oestrogen levels of the male environment. Moreover, the importance of OR in male breast cancer treatment is not as well established as it is in female breast carcinomas. Hormonal treatment has not always been based on OR status [7], and the response to such treatment has been variable [8–10]. We therefore studied 66 male breast carcinomas.

Our purpose was to see whether the markers that have an established rôle in women, are also important in men. We chose to study hormonal receptors and antigens under oestrogen control (protein pS2, cathepsin D, heat shock protein 27) and also antigens isolated from breast cyst fluid which mark apocrine differentiation and are under androgen control

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Received 1 March 1995; revised and accepted 22 Apr. 1996.

(apolipoprotein D, alpha-2-Zn-glycoprotein, and GCDFP-15). We also studied prognostic markers such as p53, bcl-2 and c-erbB-2.

We wanted to compare the frequency of these markers in male with that in female breast carcinomas and attempted to determine whether hormonal receptors in men are functional.

MATERIALS AND METHODS

The samples collected for the study came from cases recorded at the tumour register of Geneva and Lausanne between 1968 and 1991. They were analysed at the Institute of Pathology of Geneva and Lausanne. All the samples were reviewed by one immunohistochemist and one pathologist. Of the 89 patients, 17 were excluded due to an insufficient amount of tissue or inadequate clinical information. Out of 72 cases, there was 1 medullary carcinoma, 2 infiltrating papillary carcinomas, 3 intraductal papillary tumours, and 66 infiltrating-ductal carcinomas. The characteristics of these ductal carcinomas are summarised in Table 1. Histological grade was established according to Bloom and Richardson [11]. These 66 cases were the focus of our study. The staging classification was based on the tumour size described on the pathological reports of the patients. Because clinical follow-up of these cases was limited and incomplete, a meaningful correlation between different markers and prognosis was not possible.

The group of 66 male invasive-ductal carcinomas was compared to a group of 190 female invasive-ductal breast carcinomas. The latter group has been the object of several studies [12–15] and its principal characteristics are summarised in Table 1. All the breast cancer samples (male and female) were obtained from archival paraffin-embedded material that had been formalin fixed. Five micrometre sections were cut from an appropriate block containing carcinoma and stained with haematoxylin–eosin.

Immunohistology

The various antigens were immunostained according to the PAP method of Sternberger and associates [16] using diaminobenzidine as substrate. The sections were first incubated for 10 min with normal swine serum diluted 1/30. The incubation time in the first-step antisera is indicated in Table 2. The incubation time in the second-step antisera and in PAP was 30 min, each separated by washing in tris-buffered saline (TBS). Mouse and rabbit peroxidase–anti-peroxidase complexes (diluted 1/800 and 1/200) were obtained from Dakopatts A/S, Copenhagen, Denmark and from Sternberger Monoclonals Inc., Baltimore, Maryland, U.S.A. Finally, all sections were washed in TBS and stained for 10 min in the diamino/hydrogen peroxide substrate (40 mg 3-3′ diaminobenzidine in 100 ml TBS containing 35 µl H_2O_2). Nuclei were weekly stained with haematoxylin.

Table 1. Comparison of female and male ductal invasive breast carcinomas

		Female		Male	
		n	(%)	*n*	(%)
Tumour size (cm)	Unknown	3	(1.6)	5	(7.6)
	<2.0	43	(22.6)	22	(33.3)
	2–2.9	70	(36.8)	19	(28.8)
	>2.9	74	(38.9)	20	(30.3)
Grade	I	60	(31.6)	32	(48.5)
	II	78	(41.1)	21	(31.8)
	III	52	(27.4)	10	(15.2)
	Unknown			3	(4.5)
Axillary metastases	0	85	44.7	31	(47.0)
	1–3	53	27.9	10	(15.2)
	>3	46	24.2	9	(13.6)
	Unknown	6	3.2	16	(24.2)

In control slides, the first antiserum was replaced by normal rabbit serum or a supernatant from a hybridoma without specificity for the tissues examined. With some antisera, enzymatic digestion was carried out prior to incubation with the antiserum: protease VII (Sigma 5255 1 mg/1.3 ml phosphate-buffered saline) was employed for 10 min at 37°C. In some cases, antibody incubation was preceded by microwave treatment in citrate buffer according to a method (Gerdes and colleagues [17]) modified from that of Shi and colleagues [18]. p53 was detected with the avidin–biotin complex technique (ABC) described by Hsu and colleagues [19]. To minimise background staining, sections were preincubated with normal horse serum (1/30).

Antisera

All of the antisera employed in the first step are listed in Table 2. Non-immune serum from swine and swine anti-rabbit IgG serum (diluted 1/30) were obtained from Dakopatts A/S. Goat anti-mouse immunoglobulins (diluted 1/100), mouse and rabbit peroxidase–anti-peroxidase complexes (diluted 1/800 and 1/200) were obtained from Sternberger Monoclonals Inc. Monoclonal Clonab LN-C, used as control, was obtained from Biotest Diagnostics, Dreieich, Germany. Non-immune serum from rabbit, used as control, was obtained from Pel-Freez Biologicals, Rogers, Arizona, U.S.A. Normal horse serum was obtained from a local slaughterhouse. Biotinylated horse antibodies specific for mouse Ig (diluted 1/200) and ABC (Vectastain Elite) were obtained from Vector Laboratories Inc., Burlingame, California, U.S.A. Two antisera specific for p53 were used, since this combination allows for better detection of the antigen on fixed tissues [12, 14]. Monoclonal antibody 1801 described by Banks and associates [20], reveals epitopes between amino acids 32 and 79. Rabbit antiserum CM1, described by Midgley and colleagues [21], is specific for both wild-type and mutant forms of the p53 protein. Rabbit antisera specific for GCDFP-15 and for cathepsin D, produced in our laboratories, have been described in previous papers [13, 22]. Rabbit antiserum specific for apolipoprotein D was produced in our laboratory. Apolipoprotein D was isolated from breast cyst fluid by chromatography on Sephadex G 200, preparative isoelectric electrophoresis and preparative polyacrylamide electrophoresis (data not shown). This antiserum reveals only one band on Western blot of breast cyst fluid. For PR (progesterone receptor), OR and p53, tumours were defined as positive even if only 10% of the cells were labelled. For all the other markers, tumours were considered positive if 30% of their cells were labelled.

Statistical method

Chi-square and Fisher's exact test were used for univariate analysis.

RESULTS

The results are summarised in Tables 3 and 4.

Table 2. Antibodies used in this study

Antibody	Specificity	Dilution	Incubation time	Pretreatment	Source
Monoclonal	Bcl-2	1/200	30 min	Microwave	Dakopatts A/S, Copenhagen, Denmark
Polyclonal	Cathepsin D	1/400	30 min	Proteolytic digestion	Our laboratory
Monoclonal	Protein pS2 (pS2)	1/5	30 min	Proteolytic digestion	CIS, Saint-Quentin, Yvelines, France
Monoclonal	Oestrogen receptor	1/50	Overnight	Microwaves	Dakopatts A/S
Monoclonal	Progesterone receptor	1/40	Overnight	Microwaves	Dianova, Hamburg, Germany
Monoclonal 1801	p53	1/4000	Overnight	–	Cambridge Research Biochemicals, Northwick, U.K.
Polyclonal CM1	p53	1/2000	Overnight	–	Novocastra, Newcastle upon Tyne, U.K.
Monoclonal	Heat shock protein 27	Prediluted	30 min	–	Biogenex Laboratories, San Ramon, U.S.A.
Polyclonal rabbit	Alpha-2-Zn-glycoprotein	1/500	30 min	–	Nordic Immunology, Tilburg, The Netherlands
Polyclonal rabbit	Apolipoprotein D	1/300	30 min	–	Our laboratory
Monoconal	c-erbB-2	1/500	30 min	Microwave	Cambridge Research Biochemicals
Polyclonal rabbit	GCDFP-15	1/250	30 min	Proteolytic digestion	Our laboratory

Table 3. Positivity for various markers: comparison between mammary-infiltrating ductal carcinomas from men and women

	Women*				Men				
Markers	Positive cases	(%)	Negative cases	(%)	Positive cases	(%)	Negative cases	(%)	*P* value
Alpha-2-Zn-glycoprotein	91	(50)	92	(50)	45	(68)	21	(32)	0.007
Apolipoprotein D	78	(42)	106	(58)	61	(92)	5	(8)	<0.001
GCDFP-15	95	(50)	95	(50)	32	(48)	34	(52)	0.47
OR	142	(75)	47	(25)	54	(82)	12	(18)	0.17
PR	116	(62)	72	(38)	51	(77)	15	(23)	0.010
Cathepsin D	131	(69)	58	(31)	25	(38)	41	(62)	<0.001
pS2	83	(44)	106	(56)	18	(27)	48	(73)	0.011
Heat shock protein 27	73	(38)	116	(62)	16	(24)	50	(76)	0.026
c-erbB-2	101	(53)	88	(47)	34	(52)	32	(48)	0.44
Bcl-2	91	(48)	99	(52)	44	(67)	22	(33)	0.006
p53	42	(22)	148	(78)	9	(14)	57	(86)	0.09

*In some cases, data were not complete. OR, oestrogen receptor; PR, progesterone receptor.

Table 4. Relationship between OR and markers under oestrogen control in ductal carcinomas in men and women

	Women			Men		
	OR^+	OR^-	*P*	OR^+	OR^-	*P*
Number of cases	142	47		54	12	
pS2	66	17	0.04	15	3	0.04
Heat shock protein 27	66	7	<0.001	16	0	0.03
Cathepsin D	107	24	0.001	19	6	0.33
PR	113	3	<0.001	45	6	0.01

OR, oestrogen receptor; PR, progesterone receptor.

Oestrogen receptors and proteins under oestrogen control

Eighty-two per cent of male breast carcinomas were positive for OR, as compared to 75% in female carcinomas. This difference was not significant ($P = 0.17$) (Table 3). There was a relationship between age and OR, only when the group of patients under 60 years of age (17 cases) was compared with that over 60 years (49 cases). Eleven tumours were OR^+ in the first group and 43 in the second ($P = 0.03$). Positivity for OR was always nuclear (Figure 1).

Positivity for progesterone receptors (PR) was also more frequent (77%) in male than in female carcinomas (62%). The difference was significant ($P = 0.01$). Positivity for PR was nuclear (Figure 1).

The proteins under oestrogen control were expressed differently in the two series. Cathepsin D, protein pS2 and heat shock protein 27 were respectively present in 69, 44 and 38% of the female breast carcinomas but only in 38, 27 and 24% of the male breast carcinomas (Table 3). These markers were cytoplasmic. Cathepsin D was also present in macrophages infiltrating the tumour, but only the positivity of tumoral cells was taken into account (Figure 2). This difference was still apparent if the proteins under oestrogen control were related to OR^+ and OR^- tumours (Table 4). In the female breast carcinomas, expression of these various markers, with the exception of pS2, was high in OR^+ and low in OR^- carcinomas and the difference was significant. In the male carcinomas, however, the difference was not significant for cathepsin D, and less marked for heat shock protein 27 and PR.

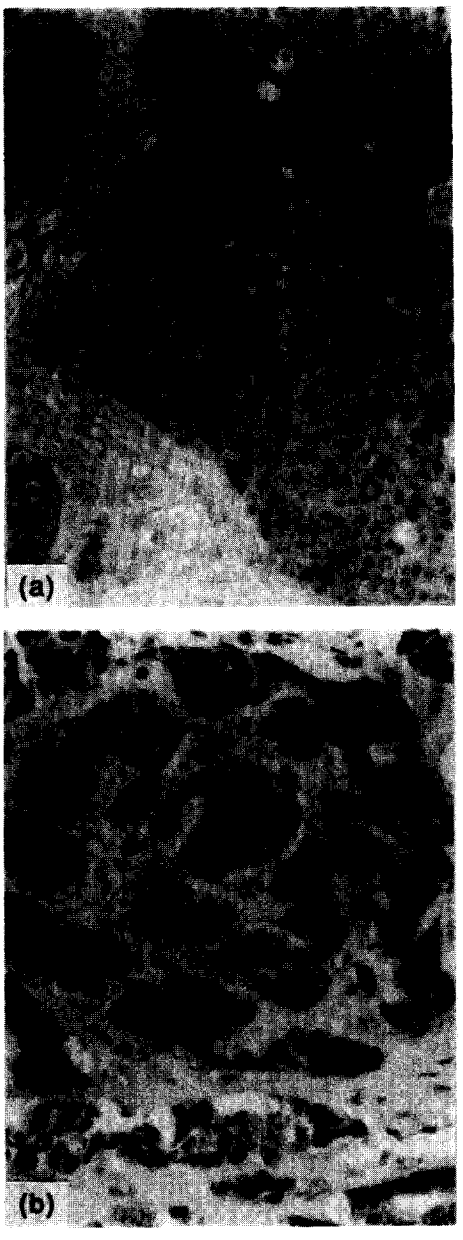

Figure 1. Immunohistochemical detection of hormone receptors: (a) oestrogen receptors; (b) progesterone receptors. Nuclei are positive.

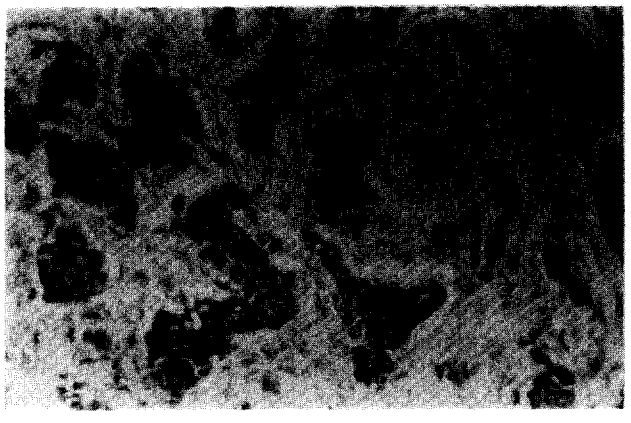

Figure 2. Immunohistochemical detection of cathepsin D.

Markers under androgen control

Alpha-2-Zn-glycoprotein and apolipoprotein D were present in the majority of male breast carcinomas. In female breast cancers these two markers were less frequent and the difference between the two series was significant ($P = 0.017$ and $P < 0.001$). The positivity in male and female carcinomas was cytoplasmic, with staining intensity and the number of positive cells being variable (Figure 3). Several cases showed a mosaic staining pattern with an alternation of negative and positive cells. Almost half of the cases were positive for GCDFP-15 in the female and male series.

Oncogenes and tumour suppressor genes

Almost half of the carcinomas in the two series was positive for c-erbB-2. For p53, the percentage of positive tumours was higher in female carcinomas than in male carcinomas, but the

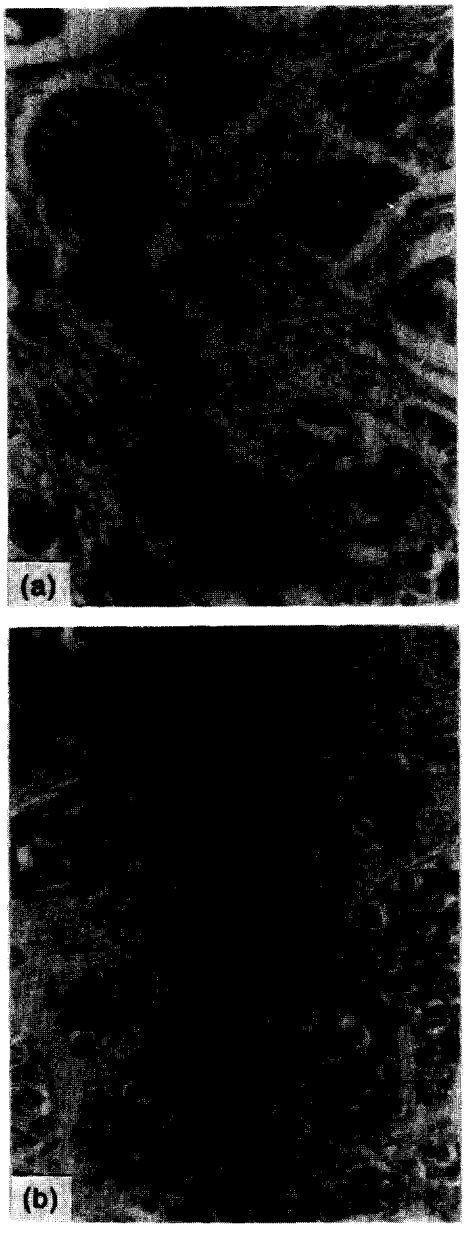

Figure 3. Immunohistochemical detection of proteins under androgen control: (a) apolipoprotein D; (b) alpha-2-Zn-glycoprotein.

difference was not significant (Table 3). The p53$^+$ tumours were generally OR$^-$ and PR$^-$. Of the 57 p53$^-$ male carcinomas, 50 and 48 were, respectively, positive for OR and PR. Of the 9 p53$^+$ male tumours, only 4 and 3 were, respectively, positive for OR and PR ($P = 0.002$ for OR and <0.001 for PR).

For bcl-2, 48% of female breast carcinomas were positive as compared with 67% of male carcinomas ($P = 0.006$). The bcl-2$^+$ tumours were generally p53$^-$ and OR$^+$. Of the 44 bcl-2$^+$ male tumours, 3 were positive for p53 but 39 were positive for OR. Of the 22 bcl-2$^-$ male tumours, 6 were positive for p53 and 15 for OR ($P = 0.02$ for $p53$ and $P = 0.04$ for OR).

DISCUSSION

Analysis of male breast carcinomas has shown similarities as well as differences with female breast carcinomas. The size and grade of the male breast carcinomas were similar to those of female cancers as were the number of metastatic axillary lymph nodes. This observation has also been made by Morimoto and colleagues [8]. Two of the markers, GCDFP-15 and c-erbB-2, labelled the same proportion of female and male tumours. GCDFP-15 is a breast cystic fluid protein which is synthesised by breast cells—*in vitro* and *in vivo*—under androgen influence [23–26]. This protein, present in 50–86% of female breast carcinomas [22, 27, 28] is associated with androgen receptors and apocrine characteristics [24]. GCDFP-15 synthesis is inhibited by oestradiol [29], an effect which is reversed with anti-oestrogens. However, this protein is probably under intricate hormonal control. This could explain some observations [30] where GCDFP-15 synthesis was not stimulated by oestrogens nor by androgens. This could also explain why we did not find more male carcinomas positive for this protein, as we did for two other proteins under androgen control—apolipoprotein D and alpha-2-Zn-glycoprotein. The marker c-erbB-2 was also present in almost half of the male and female carcinomas. Only one report concerning c-erbB-2 [5] has shown divergent results, with 17% positivity in males and 33% in females. This could perhaps be explained by the use of a different antiserum, and another cut-off for positivity.

However, in this study, male breast carcinomas have shown significant differences with female carcinomas. Male patients were older than female patients, in accordance with most studies in the literature [8, 10, 31–33]. The number of OR$^+$ tumours was high in the male series. This high percentage has previously been reported [3, 5–8, 33, 34] with figures between 65 and 89%. This positivity seems related to age since, in the group under 60 years of age, 64% of the tumours were OR$^+$ as compared to 87% in the group over 60 years of age. This relationship has also been observed by Everson and colleagues [7]. This high percentage of OR$^+$ tumours is probably not due to the low levels of oestrogens, since immunohistochemical detection is not dependent upon oestrogens. However, it is possible that low levels of circulating oestrogens or abnormal oestrogen metabolites could influence the synthesis of receptors [5].

Proteins under oestrogen control such as pS2 [30, 35–37], heat shock protein 27 [30, 38, 39], cathepsin D [30, 40, 41] and progesterone receptors [30], were more frequent in female breast carcinomas than in male carcinomas. Only Kardas and associates [42] have found a high percentage of pS2 in male breast carcinomas. This is probably due to the fact that tumours with 10% positive cells were considered positive. Contrary to female breast cancer, cathepsin D was not associated with OR in male carcinomas. This is in agreement with the results of Kardas and associates [42] concerning pS2 and Rogers and associates [3] for cathepsin D. These results contrast strongly with most results published on female breast carcinomas [13, 36, 37, 39]. All these facts could suggest that ORs in male breast carcinomas do not have the same function as ORs in female carcinomas. This could explain the variable success reported for antihormonal treatment [7, 33]; we agree with Everson and colleagues [7] that therapeutic intervention must not be based on OR status. Proteins under androgen control were frequent in male breast carcinomas. Alpha-2-Zn-glycoprotein is synthesised *in vitro* by mammary cells with increased production under androgens and progestin [25]. This protein has been shown to be present in female mammary carcinomas [22, 43], which are generally well-differentiated [43], and have a rather favourable prognosis [44]. Apolipoprotein D, previously described as GCDFP-25 [45], has also been shown to be under androgen influence [46, 47]. These two proteins were, respectively, seen in 68 and 92% of male breast carcinomas, as compared to 50 and 42% of female breast carcinomas ($P = 0.007$ and $P < 0.001$). Although these proteins do not seem to have prognostic value, they could be important in the choice of hormonal treatment. In male breast carcinomas, the incidence of bcl-2$^+$ cancers was higher than in the female series ($P = 0.006$), while the incidence of p53$^+$ carcinomas was low in male carcinomas and high in female carcinomas ($P = 0.09$). Both in female and male carcinomas [15], we found that bcl-2$^+$ carcinomas were less frequently positive for p53. The correlation between p53 and bcl-2 proteins is surprising. As in women, the presence of p53 protein in male breast carcinomas would be associated with absence of apoptosis. Bcl-2 protein protects cells against apoptosis. Therefore, one would not expect an inverse relationship between these two proteins. As for female breast carcinomas [15], we do not have an explanation in male carcinomas for this intriguing fact.

Contrary to most reports [6, 8–10, 32, 33, 48], we did not find worse prognosis for male than for female breast carcinomas. However, several authors [10, 31, 49, 50] have published results analogous to ours.

The prognostic value of usual parameters seems different for men than for women. We did not find prognostic value for tumour size and grade nor for axillary metastases. This is unusual, and most reports have shown the importance of these parameters [48–51]. These divergences can be explained by the small number of patients in our series and particularly by the fact that 24% of the men did not have axillary node dissection.

Contrary to the female series, bcl-2 does not seem to have prognostic value, and, inversely, p53 has independent prognostic value for overall survival at 5 years.

In conclusion, male and female breast carcinomas seem to differ in several aspects. The most striking being that in men, there is a high frequency of OR$^+$ tumours, a predominance of tumours positive for proteins under androgen control, and a low frequency of p53$^+$ tumours [52]. The small number of proteins under oestrogen control, together with a large amount of proteins under androgen control could explain the difficulties encountered with hormonal treatment in male breast carcinomas. This particular phenotype of male breast carcinomas could be useful for establishing an adequate hormonal treatment. Our study shows the wide range of results obtained in immunohistochemical studies. This might be

related to the lack of a standardised protocol, including the use of different primary antibody, variations in tissues fixation (and thus antigen preservation/availability for antibody interaction) and immunostaining methodology, particularly the interpretive criteria for considering a case "positive" or "negative". Indeed, unless the above parameters are standardised, comparison between immunohistochemical study will remain difficult. The short term of follow-up and the low number of male patients do not allow conclusions about the prognostic value of immunocytochemical markers in breast carcinoma to be drawn.

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Acknowledgements—This work was supported in part by La Ligue vaudoise contre le cancer (JH). The authors thank M.M. Bertholet, S. Laurini, M. Correvon, S. Burki and J. Maillardet for their technical assistance; D. Vala, S. Gebhard and G. Perriard for their help in the preparation of the manuscript, as well as Dr S. Widgren, Institut universitaire de Pathologie, Geneva, for allowing us to use 19 cases from this Institute. The authors thank Prof. J.M. Kurtz for his help in reviewing the manuscript.