PERGAMON

European Journal of Cancer 36 (2000) 1631–1639

European Journal of Cancer

www.ejconline.com

Individual characterisation of the metastatic capacity of human breast carcinoma

R. Heimann *, S. Hellman

Department of Radiation and Cellular Oncology, The Pritzker School of Medicine, The University of Chicago, MC 9006, 5758 South Maryland Avenue, Chicago, IL 60637, USA

Received 5 April 2000; accepted 22 May 2000

Abstract

The clinical implications of understanding the invasive and metastatic proclivities of an individual patient's tumour are substantial because the choice of systemic therapy needs to be guided by the likelihood of occult metastasis as well as by knowing when the metastases will become overt. Malignant potential is dynamic, progressing throughout the natural history of a tumour. Required of tumours is the development of critical phenotypic attributes: growth, angiogenesis, invasion and metastagenicity. Characterisation of the extent of tumour progression with regard to these major tumour phenotypes should allow the fashioning of individual therapy for each patient. To examine the clinical parameters and molecularly characterise the metastatic proclivity we have been studying a series of regionally treated breast cancer patients who received no systemic therapy and have long follow-up. Clinically we describe two parameters: metastagenicity — the metastatic proclivity of a tumour, and virulence — the rate at which these metastases appear. Both attributes increase with tumour size and nodal involvement. However, within each clinical group there is a cured population, even in those with extensive nodal involvement, underscoring the heterogeneity of breast cancers within each group and the need for further molecular characterisation. Using biomarkers that characterise the malignant phenotype we have determined that there is progression in the phenotypic changes. Angiogenesis and loss of nm23 are earlier events than the loss of E-cadherin, or abnormalities in *TP53*. The strongest biomarkers of poor prognosis are p53 and E-cadherin, but even when both are abnormal 42% of node-negative patients are cured indicating that other determinative steps need to occur before successful metastases are established. Identification of these critical later events will further increase the efficacy of determining the malignant capacities of individual tumours.

Keywords: Metastasis; Prognostic markers; Breast cancer; Angiogenesis; nm23; E-cadherin; *TP53*

1. Introduction

Cancer, while the most curable of the major life-threatening chronic diseases, is the most dreaded because of the terrifying mystique of relentless growth, invasion and metastasis, even after apparent long periods without evidence of disease. It is the second leading cause of death in the USA where in the year 2000 approximately 1 220 100 new invasive cancers will be diagnosed (excluding basal cell and squamous cell carcinoma of the skin) and an estimated 552 200 people will die of the disease [1]. Invasion and metastases to distant sites via lymphatic or blood vessels are the hallmark of malignancy. They result in significant morbidity and ultimately lead to the patient's death.

The clinical implications of understanding the invasive and metastatic proclivities of an individual patient's tumour are substantial. Upon diagnosis, the critical questions facing the patients and the physicians are; what is the most effective treatment, and what are its side-effects? The use of systemic therapy in patients with seemingly localised disease needs to be guided by the likelihood of occult metastasis, as well as by knowing when the occult metastasis will become overt. To design a treatment plan, we must ascertain these characteristics for the individual patient. A thorough understanding of the clinical and molecular characteristics of tumour progression will aid in this determination and allow the fashioning of a specific tailored therapy.

* Corresponding author. Tel.: +1-773-702-5194; fax: +1-773-834-7340.

E-mail address: heimann@rover.uchicago.edu (R. Heimann).

0959-8049/00/$ - see front matter
PII: S0959-8049(00)00151-9

Cancer is heterogeneous. Clinical staging systems have been developed to group patients in categories with similar prognosis. Most staging systems group patients into four stages based primarily on anatomical considerations, with some subdivisions within the various stages [2,3]. However, even within subgroups the prognosis varies significantly since these groups comprise a spectrum of patients some of whom can be cured by local therapy while others will have occult metastases. Improvements in outcome and cure rates are likely to come from the selection of therapies based on the molecular characterisation of the tumour by providing prognostic markers as well as predicting which treatments will be most effective by identifying in the tumour the presence of the intended molecular target.

To address the heterogeneity of the current tumour stages and to predict therapeutic efficacy we have been examining the natural history of breast cancer, its clinical manifestations and the molecular characteristics of the metastatic phenotype. We have been using a database collected by Donald Ferguson at The University of Chicago which includes more than 2000 women who underwent local and regional therapy between 1927 and 1987, and have a long follow-up [4–8]. We excluded patients who received adjuvant systemic therapy in order to be able to examine the unconfounded natural history of locally treated breast cancer correlated with clinical characteristics and molecular biomarkers. Thus, 1590 patients who received local therapy only are the subject of these studies.

2. Progression during the natural history of clinical breast cancer

Several hypotheses have been proposed to explain the natural history of breast cancer. At the turn of the 20th century, Halsted put forward the hypothesis that breast cancer is an orderly disease spreading in a contiguous fashion from the primary site by direct extension, through the lymphatics to the lymph nodes and subsequently to distant sites [9,10]. This suggested that wide extirpation of the tumour, draining lymphatics and regional nodes would result in cure, providing the rationale for radical and extended mastectomy. Since, this hypothesis does not explain the development of metastatic disease in node-negative breast cancer, an alternative hypothesis was promulgated proposing that breast cancer is a systemic disease in which nodal involvement does not merely represent contiguous disease but rather serves as a marker of occult distant disease [11]. This latter hypothesis minimises the importance of local therapy and presumes that if metastases are to appear they must have spread before clinical detectability. However, this hypothesis is also incomplete since it does not satisfactorily explain the clinical natural history of all breast cancers. Many node-positive breast cancer patients are cured by local regional therapy only. To capture the virtues of both theories, as well as to deal with their shortcomings, ''the spectrum'' hypothesis has been put forward [12]. It assumes that breast cancer comprises a spectrum of diseases extending from tumours which are destined to remain localised to those that are systemic when first detected. The spectrum exists because tumours become progressively more malignant during their clinical evolution. This process of mutation, selection and amplification is stochastic, and the metastatic capacity is acquired at different rates, by redundant mechanisms in different tumours. Small tumours in general are less likely to have spread not only because of the number of tumour cells, or the length of their presence, but also because the tumour cells themselves are less malignant. While generally this is true, tumours of any given size are heterogeneous with some small tumours having extensive metastatic capability, as well as some larger tumours having lessened malignant capacity. The ''spectrum'' hypothesis also postulates that lymph node involvement serves as both a prognostic marker and a potential source of distant metastases, and untreated or suboptimally treated local or regional disease may progress to be a source for further metastasis. This hypothesis then provides the rationale for a treatment plan designed to optimise local tumour control as well as for adjuvant systemic therapy for potential occult metastases.

Clinically, cancer patients can be grouped into three groups: those with overt clinical evidence of metastatic disease at diagnosis; those with covert metastatic disease; and those with no metastases. Early diagnosis in general increases the proportion of patients in the third group, while delay in diagnosis or untreated disease will increase the proportion of patients with micrometastases and overt metastases. With no treatment, there will most likely be a gradual progression from no metastases to covert and then overt metastases [13–15].

The concept of malignant progression is well accepted in the studies of cancer development from normal to premalignant and then to malignant cells, but receives much less attention after the development of an invasive neoplasm. There is the suggestion by those proposing the ''systemic'' hypothesis that by the time of clinical detectability tumours have acquired all of their malignant capacity, but there is ample clinical evidence that progression occurs during the clinical natural history of breast cancer [15]. Screening mammography trials have demonstrated a 30% reduction in breast cancer deaths in the screened population [16–20]. In general, mammographically detected tumours are smaller than clinically detected tumours. For example in the two-county Swedish screening mammography trial the diameter of

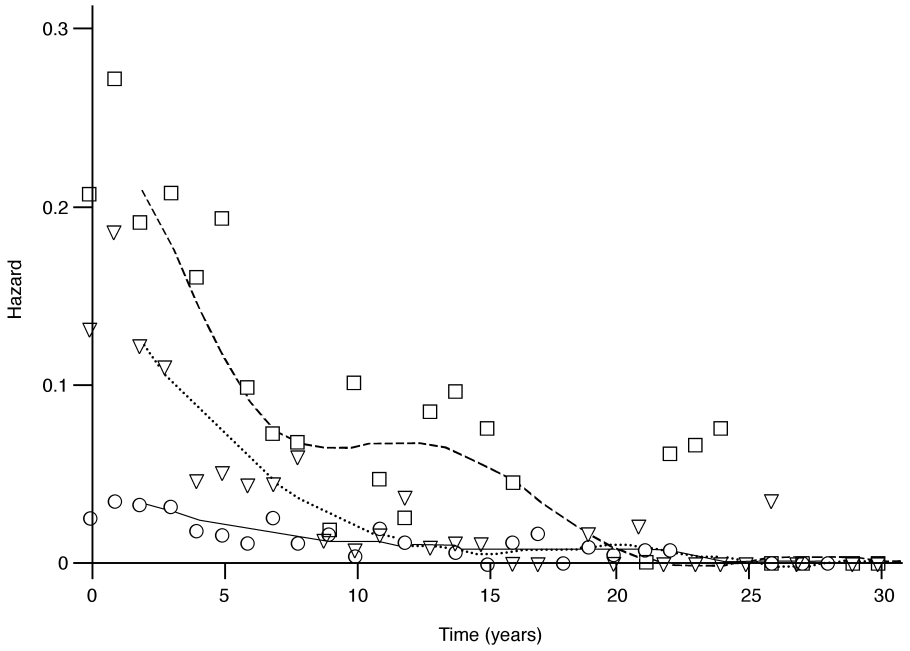

Fig. 1. Annual hazard rate of distant disease in: axillary node negative (–○–); 1–3 node positive (···▽···); and ⩾4 node positive (- -□- -) breast cancer. None of the patients received systemic treatment.

the tumours mammographically detected was 1.4 cm compared with 2.2 cm for those detected clinically [21]. At the University of Chicago the median tumour size of mammographically detected breast cancers was 1.0 cm compared with 1.8 cm for the clinically detected breast cancers [22]. The improved survival of the small mammographically detected tumours compared with the larger clinically detected ones can be explained by the higher number of clonogens, longer time for metastases to develop, and tumour progression as suggested by the 'spectrum' hypothesis.

The concept of progression during the clinical phase of cancer has important implications because it suggests that smaller tumours are more curable not only because they had less opportunity but also because they are less proficient in their ability to invade and metastasise and can acquire this ability during their clinical course if they remain undetected or untreated. This is reflected in the breast cancers we are seeing today, that, as a result of screening mammography and more patient awareness, are smaller, less likely to have axillary node involvement, and are of lower grade, thus are less malignant [23]. Progression can also happen in persistent local or nodal disease and serve as a source for subsequent metastasis. Tumour progression may even occur in patients with node-positive breast cancer. We have previously shown that for patients with a limited number of involved lymph nodes the likelihood of disease metastasis increases with the size of the tumour, suggesting that a greater proportion of the smaller tumours are able to metastasise only to lymph nodes since they have not yet acquired the capacity to metastasise to distant sites [6,24]. This is not so if the number of involved lymph nodes exceeds four.

3. Parameters of malignancy

In order to quantify the extent, rapidity and likelihood of distant metastases we have separated two clinical characteristics of the metastatic proclivity: metastagenicity — the ultimate likelihood of metastases which we defined as (100−plateau)/100 of the actuarial distant disease-free survival (DFS) curve and, virulence — the rate at which the metastases become clinically apparent, obtained from the initial slope of log-linear distant DFS plot [15,24]. Both measures are important in answering the two questions facing the physician and patient upon diagnosis: will metastases develop and if so when will the first of these lesions present themselves clinically? Not only is this information prognostically important but it impacts upon the choice of an individualised treatment plan.

Both virulence and metastagenicity increase with the size of the tumour and number of lymph nodes involved [24]. Virulence can be assessed as well by the annual hazard rate for metastases. As shown in Fig. 1, the hazard of distant disease recurrence is very low in node-negative patients but it completely abates only after more than 20 years. The metastagenicity in this group (shown in Table 1) is 0.29 indicating that 71% of node-negative patients treated only with local regional therapy are cured of the disease. Both the virulence and metastagenicity increase with increasing nodal involve-

ment. While the metastagenicity is 0.62 in those with 1–3 positive nodes and 0.86 in patients with $\geqslant 4$ nodes (Table 1), there is still, even in the most advanced group, a population cured by local therapy only (14%). This illustrates the significant heterogeneity within the clinical groups. It is true also when considering T1 ($\leqslant 2$ cm) tumours since even within groups of patients having these small cancers we are unable to separate those patients with occult metastases. Currently, treatment recommendations based primarily on tumour size and nodal involvement do not reflect this heterogeneity.

A large number of clinical trials demonstrate a benefit from adjuvant chemotherapy in most breast cancer patients [25]. The relative survival benefit is approximately 30%, i.e. 30% of those patients with occult metastases are cured, but the absolute survival benefit depends on the proportion of patients with occult micrometastases. In node-negative breast cancer patients, the long-term survival with local therapy only, without systemic therapy is 70–80%. Thus if all node-negative patients are treated systemically the large majority will receive this therapy unnecessarily. If these patients could be identified they could be spared systemic therapy and a more intense programme could be employed for those patients at the greatest risk, perhaps increasing their likelihood of survival. Chemotherapy is usually recommended for patients in whom the disease has spread to the lymph nodes. But many patients with limited lymph node involvement, particularly if the tumour size is small will be cured of their disease without adjuvant chemotherapy. Among those patients in our series who survived 20 years 31% had involved axillary nodes and of these almost half, had more than one positive node [15]. Involved axillary lymph nodes do not always indicate distant metastases; thus not all node-positive breast cancer patients need chemotherapy. A specific molecular characterisation of each tumour may allow a more complete evaluation of the tumour's metastatic proclivities allowing a better tailored therapy. Currently the inability to characterise an individual tumour's capacity for invasion and metastasis has resulted in treatment plans that are too much in some patients and insufficient in others.

4. Molecular biomarkers of progression in breast cancer

Both invasion and metastasis are well regulated multistep processes in which multiple genes and gene products are involved, but not all changes are equally important. Growth controlling genes are important in the development of malignant cells, but a different set of genes participate in enabling proliferating cells to invade and metastasise. The resulting metastatic phenotype is the biological expression of multiple genetic changes following the principles of convergent evolution [26].

Table 1
Metastagenicity as a function of positive axillary lymph nodes

No. lymph nodes positive	Metastagenicity
0	0.29
1–3	0.62
$\geqslant 4$	0.86

Tumour cells progressively acquire the characteristics that allow them to invade and spread. Multiple redundant pathways appear to be involved, ultimately leading to invasion and metastasis. This results in significant heterogeneity of tumours within any clinical stage and group.

The steps needed for successful metastasis include but, are not limited to: (1) tumour growth; (2) angiogenesis and access to vascular or lymphatic channels; (3) decrease in cell surface molecules that control cell adhesion; (4) increased locomotion; (5) activation of enzymes to allow invasion of basement membrane, extracellular matrix and vascular wall; and (6) activation of cellular receptors that allow interaction of tumour cells with extracellular matrix proteins and are permissive of cell motility [27–29]. Mechanisms that protect the tumour cells from the host defences also need to be activated. These functions are required at both the primary and metastatic sites. Many of these characteristics of the metastatic tumours are also found in normal tissues, particularly embryonic, and regenerating tissues, but in the malignant cells the control of these activities is tilted in favour of growth, invasion and metastases.

Based on the clinical data and the notion of clinical tumour progression we proposed a new strategy for the development of prognostic biomarkers, in which the markers are selected based on their function in tumour growth, angiogenesis, invasion and metastatic progression. Such a strategy of using as biomarkers gene products that characterise the malignant phenotype may not only serve a prognostic purpose, but in the process it may allow us to learn about the progression of human breast cancer during its clinical expression. Table 2 shows some examples of potential biomarkers for the malignant phenotype. This is not meant as an exhaustive list but rather as a work in progress.

Table 2
Malignant tumour phenotypes and potential biomarkers

Growth	PCNA
	Ki67
Neovascularisation	Microvessel count (MVC)
Homophilic adhesion	E-cadherin
Invasion, locomotion, metastasis	nm23
	Vimentin
	Integrins
	Proteolytic enzymes (uPA, MMP)

Proliferating cell nuclear antigen (PCNA) is a cell cycle kinetic marker that can be detected in paraffin-embedded tissue [30–33]. In node-negative patients we have shown that PCNA detected immunohistochemically is a marker of virulence only [34]. Irrespective of the level of PCNA, the long-term DFS was similar but 80% of those patients having metastases developed them by 2.5 years if PCNA was high, while in patients with low levels it took 10 years for 80% of the metastases to become evident. While the ultimate metastagenicity is the same, this difference in virulence may lead to a very different therapeutic plan depending on patients' age and other coexisting morbidities.

On archival tissue we determined microvessel count (MVC) using immunohistochemical staining, for nm23-H1, E-cadherin, p53 and vimentin protein expression [35–38]. MVC is a measure of angiogenesis that accompanies tumour progression and metastasis [35,36,39,40]. Nm23-H1 (referred to hereafter as nm23), a metastasis suppressor gene product has a possible function in signal transduction, tumour cell shape, motility and basement membrane protein deposition [41–43]. E-cadherin

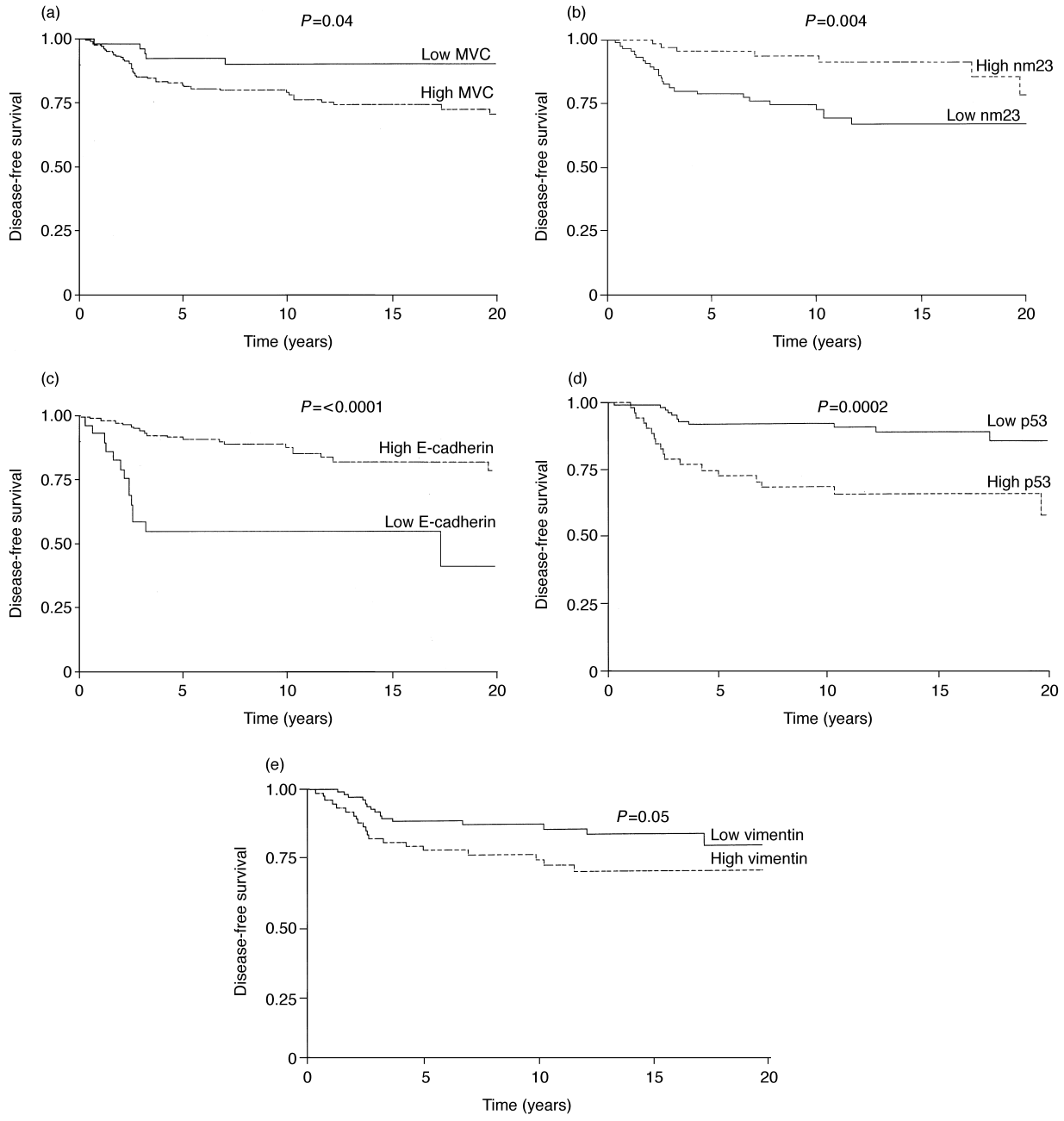

Fig. 2. Disease-free survival (DFS) in patients with node-negative breast cancer as a function of: (a) microvessel count (MVC); (b) nm23; (c) E-cadherin; (d) p53; and (e) vimentin.

Table 3
Multivariate analysis of prognostic factors for DFS

Variable	HR (95% CI)	*P*
Size	0.99 (0.95–1.03)	0.7
Grade (1–3)	0.5 (0.2–1.1)	0.1
MVC (high, low)	1.6 (0.4–6.6)	0.4
nm23 (high, low)	0.4 (0.1–1.1)	0.09
Vimentin (high, low)	1.3 (0.5–3.4)	0.5
E-cadherin	0.2 (0.1–0.6)	0.004
p53	4.1 (1.5–11.6)	0.006

DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; MVC, microvessel count.

Table 4
Biomarkers and tumour size in node-negative breast cancer

		T1 %	T2 %	*P*
MVC	Low	30	18	0.04
	High	70	82	
nm23	High	48	36	0.1
	Low	52	64	
p53	Low	72	62	0.1
	High	28	38	
E-cadherin	High	86	74	0.07
	Low	14	25	

is a calcium-regulated homophilic adhesion molecule. Decreased E-cadherin-mediated adhesion is one of the changes characterising the invasive phenotype [44–47]. p53 a tumour suppressor gene product, regulates directly or indirectly multiple cellular functions including cell cycle checkpoints, apoptosis, angiogenesis as well as the expression of some metastasis-related genes [48,49]. In several studies, p53 overexpression has been shown to be associated with poor prognosis in breast cancer [50–53]. Vimentin is the mesenchymal cell's intermediate filament protein. Breast carcinomas usually would not be expected to express this intermediate filament protein, but a subset that does appear to have a more invasive phenotype, possibly with a higher capacity for cell invasion and motility [54–57]. Each of these biomarkers regulate important steps in the malignant cascade. In Figs. 2(a–e) we show the long-term DFS in node-negative breast cancer patients as a function of MVC, nm23, E-cadherin, p53 and vimentin. The 14-year DFS in patients with either low MVC or high nm23 is more than 90%. These two markers are excellent in identifying good prognosis patients, but not as good in identifying poor prognosis patients since approximately two-thirds of patients with high MVC and loss of nm23 are still cured with local therapy only. E-cadherin appears to be better in identifying "bad" prognosis patients while not as good in identifying good prognosis patients. p53 which has multiple regulatory functions and is at the crossroads of multiple cell functions appears to be a strong prognostic factor separating good and bad prognosis patients. Vimentin, however, does not appear to add much prognostic information. In addition, we examined the prognostic value of oestrogen receptor (ER), and found no significant ($P = 0.6$) difference in DFS between ER-positive and ER-negative patients. The multivariate analysis shown in Table 3 demonstrates that E-cadherin and p53 are the most significant biomarkers for predicting metastasis.

Fig. 3 describes a putative malignant progression scheme as determined from the proportion of $\leqslant 2$ cm node-negative breast cancers having each abnormality: high angiogenesis (MVC) (70%), loss of nm23 (52%), abnormal p53 (28%) and loss of E-cadherin (14%). This suggests that the capacity for significant angiogenesis followed by the loss of nm23 precedes the other changes — if either is normal then metastases are rare. Abnormalities in p53 and E-cadherin occur latter and correlate with poor prognosis, thus they may be some of the necessary changes permissive for successful metastases. This can be seen in Fig. 4 where the outcome as a function of a combination of the markers is analysed. Increase in angiogenesis (as measured by MVC) is a frequent early event of little significance if the latter changes do not happen. This is followed by a change in nm23. Mutations in p53 and loss of E-cadherin occur later. In node-negative patients, as shown in Fig. 5, if p53 is low and E-cadherin is fully expressed the DFS is 92%, while if p53 is high and E-cadherin is low the DFS

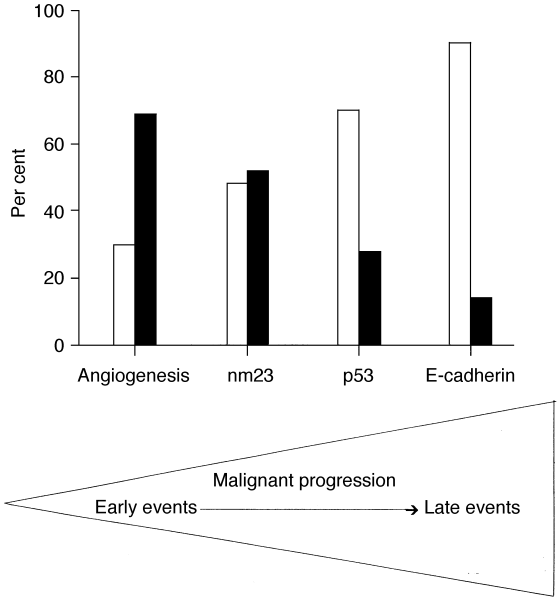

Fig. 3. Progression in node-negative breast cancer from early to late events as determined by the proportion of tumours with cancer with increased angiogenesis (high MVC), low nm23, high p53 and low E-cadherin — ■ respectively; compared with low angiogenesis, high nm23, low p53 and high E-cadherin — □. ■ Abnormal phenotype.

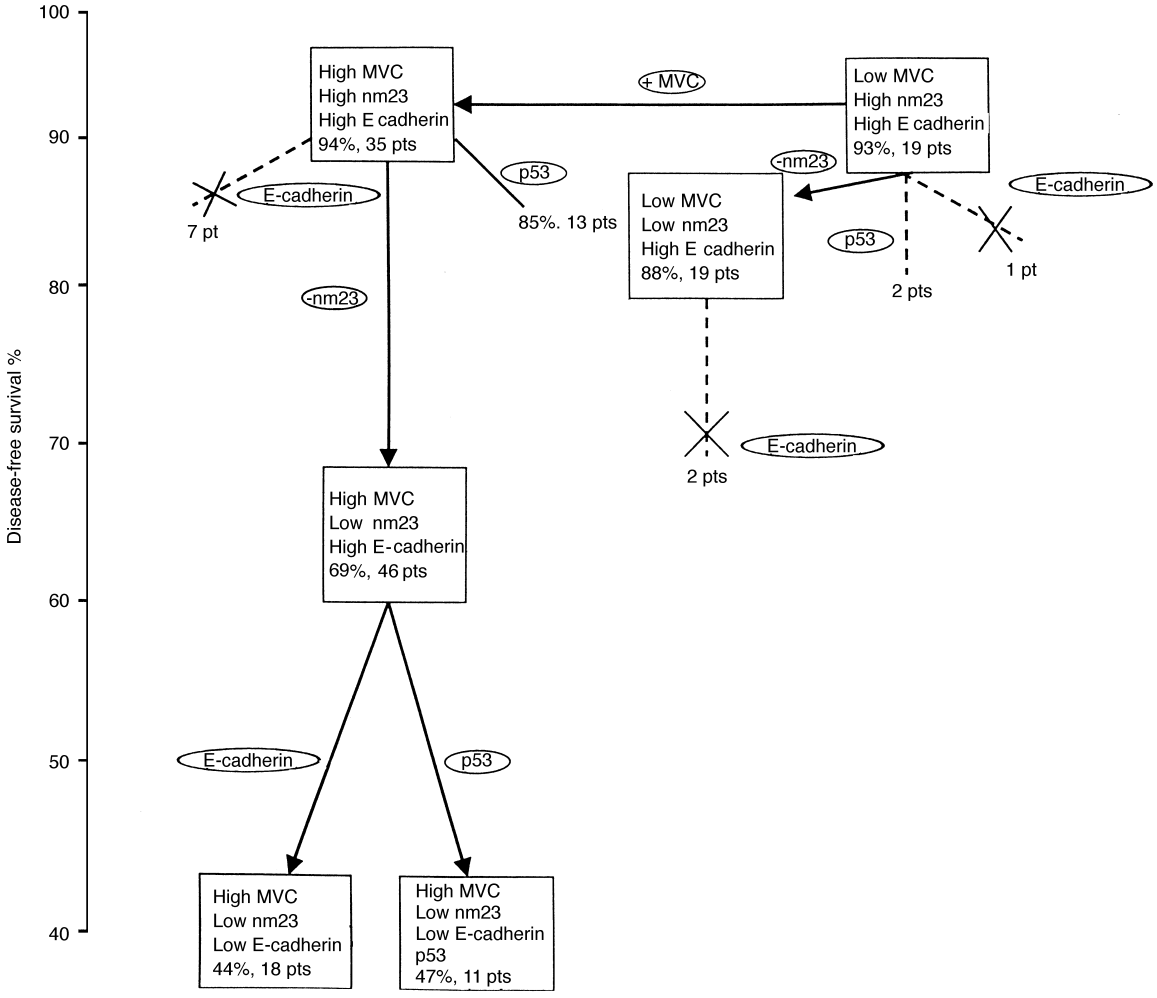

Fig. 4. Number of patients with node-negative breast cancer and their long-term disease-free survival (DFS) as a function of combinations of microvessel count (MVC), nm23, E-cadherin and p53 as found at diagnosis. The biomarker expression, patient numbers and long-term DFS (%) are detailed in the boxes. In the ovals, the change or loss of expression that had to occur (before diagnosis) is shown. The broken lines reflect changes with low likelihood, since there were much fewer patients in these groups. The vertical position of the boxes is indicative of the DFS as shown on the left-side scale. Note that because the tumour characteristics of the patients at diagnosis are presented, the number of patients in consecutive bins cannot add up and there can be either more, less or an equal number of patients in a lower bin compared with a higher one. pts, patients.

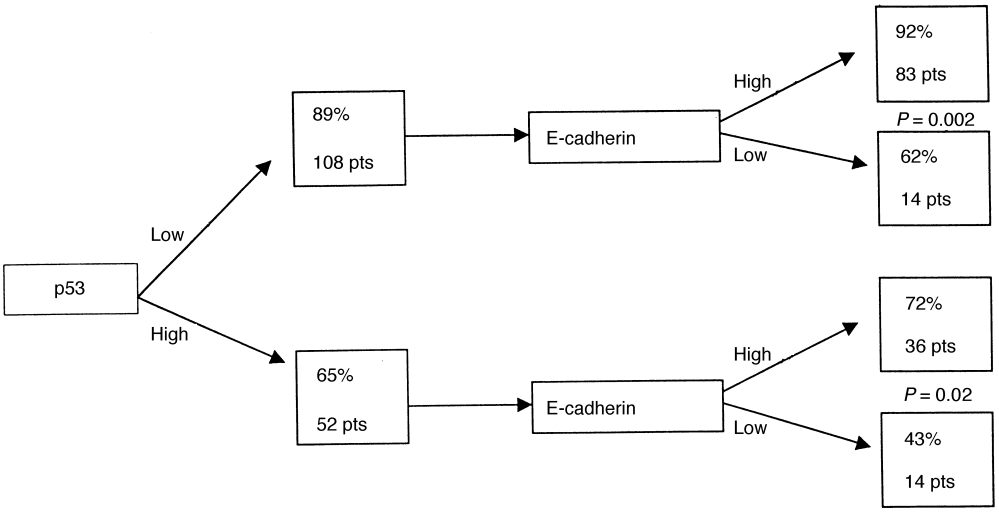

Fig. 5. Long-term disease-free survival (DFS) in node-negative breast cancer patients as a function of p53 and E-cadherin. pts, patients.

is 43%. If either one is normal but the other marker is abnormal the DFS is an intermediate figure of 63–72%. The molecular biomarkers appear to be more important than tumour size both from multivariate analysis (Table 3) and the data shown in Table 4, where the proportion of T1 and T2 tumours with the respective abnormality are presented. There is a trend for more abnormalities in the larger tumours.

This analysis of biomarkers of metastatic progression offers an opportunity to improve prognostication as well as increasing our understanding of the malignant cascade. Because even in our worst outcome group 43% of the patients are long-term survivors there must be additional necessary steps required for metastasis. These could include interaction with the basement membrane via integrins or specific receptors (laminin), and matrix degradation [58–61]. Integrins are cell surface receptors for extracellular and basement membrane components like fibronectin and collagen. They consist of alpha and beta subunits and connect the extracellular matrix to the cytoskeleton. Integrins may help cell survival in the circulation by contributing to the interaction between tumour cells, leucocytes and platelets and thus the formation of multi-cell emboli. Furthermore, interaction with integrins result in the destruction of extracellular matrix by activating matrix metalloproteinases. Matrix metalloproteinases may degrade proteins directly and additionally activate other proteases like urokinase plasminogen activator. This may clear the way for tumours en route to a distant site. We are currently studying these processes for further prognostic information.

5. Conclusions

Increasing our knowledge of invasion and metastasis has obvious implications for the diagnosis and therapy of cancer. Many current therapies particularly aimed at tumour growth are toxic and are not always successful. In order to limit aggressive therapy to patients with poor prognosis we must determine whether a tumour has the capacity to metastasise. Molecular biomarkers for invasion and metastatic phenotypes have the potential to identify the patients who need therapy and spare those without occult metastatic disease. Ideally anticancer therapy should be specifically tailored to the characteristics of the individual tumour. Advances in cure can be achieved by both better selecting existing therapies, as well as by using new therapies aimed at particular tumour characteristics. This requires the understanding of the pathways of malignant progression. Although the required phenotypes — proliferation, angiogenesis, invasion and metastases can be similar — the specific molecular pathways leading to them may be quite different in each tumour.

References

1. Greenlee RT, Murray T, Bolden S, Wingo PAW. Cancer Statistics, 2000. *CA Cancer J Clin* 2000, **50**, 7–33.
2. American Joint Committee on Cancer. *Cancer Staging Manual*. Philadelphia, J.B. Lippincott Co, 1997.
3. Yarbro JW, Page DL, Fielding LP, Partridge EE, Murphy GP. American Joint Committee on Cancer prognostic factors consensus conference. *Cancer* 1999, **86**, 2436–2446.
4. Ferguson DJ, Meier P, Karrison T, Dawson PJ, Straus FH, Lowenstein FE. Staging of breast cancer and survival rates. An assessment based on 50 years of experience with radical mastectomy. *J Am Med Assoc* 1982, **248**, 1337–1341.
5. Quiet CA, Ferguson DJ, Weichselbaum RR, Hellman S. Natural history of node-negative breast cancer: a study of 826 patients with long-term follow up. *J Clin Oncol* 1995, **13**, 1144–1151.
6. Quiet CA, Ferguson DJ, Weichselbaum RR, Hellman S. Natural history of node-positive breast cancer: the curability of small cancers with limited number of positive nodes. *J Clin Oncol* 1996, **14**, 3105–3111.
7. Heimann R, Ferguson D, Powers C, Suri D, Weichselbaum RR, Hellman S. Race and clinical outcome in breast cancer in a series with long follow-up evaluation. *J Clin Oncol* 1997, **15**, 2329–2337.
8. Karrison TG, Ferguson DJ, Meier P. Dormancy of mammary carcinoma after mastectomy. *J Natl Cancer Inst* 1999, **91**, 80–85.
9. Halsted WS. The results of operation for the cure of cancer of the breast performed at the Johns Hopkins Hospital from June 1889 to January 1894. *Johns Hopkins Bull* 1894–95, **4**, 297–319.
10. Halsted WS. The results of radical operations for the cure of carcinoma of the breast. *Ann Surg* 1907, **46**, 1–6.
11. Fisher B. Laboratory and clinical research in breast cancer — a personal adventure: The David Karnovsky Memorial Lecture. *Cancer Res* 1980, **40**, 3863–3874.
12. Hellman S. Karnofsky Memorial Lecture. Natural history of small breast cancers. *J Clin Oncol* 1994, **12**, 2229–2234.
13. Bloom HJG, Richardson WW, Harries EJ. Natural history of untreated breast cancer (1805–1933). *Br Med J* 1962, **ii**, 213–221.
14. Koscielny S, Tubiana M, Le MG, *et al*. Breast cancer: Relationship between the size of the primary tumour and the probability of metastatic spread. *Br J Cancer* 1984, **49**, 709–715.
15. Heimann R, Hellman S. Aging, progression, and phenotype in breast cancer. *J Clin Oncol* 1998, **16**, 2686–2692.
16. Chu KC, Smart CR, Tarone RE. Analysis of breast cancer mortality and stage distribution by age for the Health Insurance Plan clinical trial. *J Natl Cancer Inst* 1988, **80**, 1125–1132.
17. Nystrom L, Rutqvist LE, Wall S, *et al*. Breast cancer screening with mammography: overview of Swedish randomized trials. *Lancet* 1993, **341**, 973–978.
18. Roberts MM, Alexander FE, Anderson TJ, *et al*. Edinburg trial of screening for breast cancer: mortality at seven years. *Lancet* 1990, **335**, 241–246.
19. Tabar L, Fagenberg CJ, Gad A, *et al*. Reduction in mortality from breast cancer after mass screening with mammography: randomised trial from the breast cancer screening working group of the Swedish national board of health and welfare. *Lancet* 1985, **1**, 829–832.
20. Miller A, Baines CJ, To T, Wall C. Canadian national breast screening study: 1. Breast cancer detection and death rates among women aged 50 to 59 years. *Can Med Assoc J* 1992, **147**, 1477–1488.
21. Tabar L, Fagenberg G, Duffy SW, Day NE, Gad A, Grontoft O. Update of the Swedish two-county program of mammographic screening for breast cancer. *Radiol Clin North Am* 1992, **30**, 37–59.
22. Heimann R, Bradley J, Hellman S. The benefits of mammography are not limited to women of ages older than 50 years. *Cancer* 1998, **82**, 2221–2226.

23. Duffy SW, Tabar L, Fagerberg G, *et al*. Breast screening, prognostic factors and survival — results from the Swedish two county study. *Br J Cancer* 1991, **64**, 1133–1138.
24. Heimann R, Hellman S. Clinical progression of breast cancer malignant behavior: what to expect and when to expect it? *J Clin Oncol* 2000, **18**, 591–599.
25. Polychemotherapy for early breast cancer: an overview of the randomised trials. *Lancet* 1998, **352**, 930–942.
26. Hellman S. Darwin's clinical relevance. *Cancer* 1997, **79**, 2275–2281.
27. Killion JJ, Fidler IJ. The biology of tumor metastasis. *Semin Oncol* 1989, **16**, 105–115.
28. Kohn EC, Liotta LA. Invasion and metastasis: new approaches to an old problem. *Oncology* 1993, **7**, 47–62.
29. Stracke ML, Liotta LA. Molecular mechanism of tumor cell metastasis. In Mendelsohn J, Howley P, Israel A, Liotta LA, eds. *The Molecular Basis of Cancer*. Philadelphia, W.B. Saunders Company, 1995, 233–247.
30. Celis JE, Celid A. Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. *Proc Natl Acad Sci USA* 1985, **82**, 3262–3266.
31. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. Cyclin-PCNA is the auxiliary protein of DNA polymerase delta. *Nature* 1987, **326**, 515–517.
32. Garcia RL, Coltera MD, Gown AM. Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed, embedded tissues. *Am J Pathol* 1989, **134**, 733–739.
33. Bianchi S, Paglierani M, Zampi G, *et al*. Prognostic value of proliferating cell nuclear antigen in lymph node-negative breast cancer patients. *Cancer* 1993, **72**, 120–125.
34. Heimann R, Ferguson D, Recant WM, Hellman S. Breast cancer metastatic phenotype as predicted by histologic tumor markers. *Cancer J Sci Am* 1997, **4**, 224–229.
35. Heimann R, Ferguson D, Powers C, Recant WM, Weichselbaum RR, Hellman S. Angiogenesis as a predictor of long-term survival for patients with node-negative breast cancer. *J Natl Cancer Inst* 1996, **88**, 1764–1769.
36. Heimann R, Ferguson D, Gray S, Hellman S. Assessment of intratumoral vascularization (angiogenesis) in breast cancer prognosis. *Breast Cancer Res Treat* 1998, **52**, 147–158.
37. Heimann R, Ferguson D, Hellman S. The relationship of nm23 and angiogenesis to the metastatic propensity of node-negative breast cancer. *Cancer Res* 1998, **58**, 2766–2771.
38. Heimann R, Lan F, McBride R, Hellman S. Separating favorable from unfavorable prognostic markers in breast cancer: the role of E-cadherin. *Cancer Res* 2000, **60**, 298–304.
39. Weidner N, Folkman J, Pozza F, *et al*. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992, **84**, 1875–1887.
40. Gasparini G, Weidner N, Bevilacqua P, *et al*. Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. *J Clin Oncol* 1994, **12**, 454–466.
41. Leone A, Flatow U, VanHoutte K, Steeg P. Transfection of human nm23H1 into the human MDA-MB-435 breast carcinoma cell line: effects on tumor metastatic potential, colonization and enzymatic activity. *Oncogene* 1993, **8**, 2325–2333.
42. Steeg PS, De La Rosa A, Flatow U, MacDonald NJ, Benedict M, Leone A. Nm23 and breast cancer metastasis. *Breast Cancer Res Treat* 1993, **25**, 175–187.
43. Kantor JD, McCormick B, Steeg PS, Zetter BR. Inhibition of cell motility after nm23 transfection of human and murine tumor cells. *Cancer Res* 1993, **53**, 1971–1973.
44. Bracke ME, Van Roy FM, Mareel MM. The E-cadherin/catenin complex in invasion and metastasis. *Curr Top Microbiol Immunol* 1996, **213**, 123–161.
45. Frixen UH, Behrens J, Sachs M, *et al*. E-cadherin-mediated cell–cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 1991, **113**, 173–185.
46. Oka H, Shiozaki H, Kobayashi K, *et al*. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res* 1993, **53**, 1696–1701.
47. Siitonen SM, Kononen JT, Helin HJ, Rantala IS, Holli KA, Isola JJ. Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. *Am J Clin Pathol* 1996, **105**, 394–402.
48. Kirsch DG, Kastan MB. Tumor-suppressor p53: implications for tumor development and prognosis. *J Clin Oncol* 1998, **16**, 3158–3168.
49. Sun Y, Wicha M, Leopold WR. Regulation of metastasis-related gene expression by p53: a potential clinical implication. *Mol Carcinogenesis* 1999, **24**, 25–28.
50. Isola J, Visakorpi T, Holli K, Kallioniemi OP. Association of overexpression of tumor suppressor protein p53 with rapid cell proliferation and poor prognosis in node-negative breast cancer patients. *J Natl Cancer Inst* 1992, **84**, 1109–1114.
51. Thor AD, Moore DH, Edgerton II SM, *et al*. Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst* 1992, **84**, 845–855.
52. Silvestrini R, Benini E, Daidone MG, *et al*. p53 as an independent prognostic marker in lymph node-negative breast cancer patients. *J Natl Cancer Inst* 1993, **85**, 965–970.
53. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L. Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. *Nat Med* 1995, **1**, 1029–1034.
54. Domagala W, Lasota J, Dukowicz A, *et al*. Vimentin expression appears to be associated with poor prognosis in node-negative ductal NOS breast carcinomas. *Am J Pathol* 1990, **137**, 1299–1304.
55. Domagala W, Striker G, Szadowska A, Dukowicz A, Harezga B, Osborn M. p53 protein and vimentin in invasive ductal NOS breast carcinoma — relationship with survival and sites of metastases. *Eur J Cancer* 1994, **30A**, 1527–1534.
56. Raymond WA, Leong AS. Vimentin — a new prognostic parameter in breast carcinoma? *J Pathol* 1989, **158**, 107–114.
57. Thompson EW, Paik S, Brunner N, *et al*. Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. *J Cell Physiol* 1992, **150**, 534–544.
58. Monteagudo C, Merino MJ, San-Juan J, Liotta LA, Stetler-Stevenson WG. Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. *Am J Pathol* 1990, **136**, 585–592.
59. Duffy MJ, Reilly D, McDermott E, O'Higgins N, Fennelly JJ, Andreasen PA. Urokinase plasminogen activator as a prognostic marker in different subgroups of patients with breast cancer. *Cancer* 1994, **74**, 2276–2280.
60. Price JT, Bonovich MT, Kohn EC. The biochemistry of cancer dissemination. [Review] [643 refs]. *Crit Rev Biochem Mol Biol* 1997, **32**, 175–253.
61. Talvensari-Mattila A, Paakko P, Hoyhtya M, Blanco-Sequeiros G, Turpeenniemi-Hujanen T. Matrix metalloproteinase-2 immunoreactive protein. A marker of aggressiveness in breast carcinoma. *Cancer* 1998, **83**, 1153–1162.