Translational Medicine
ISSN: 2161-1025

Perrier et al. Translational Medic 2012, S:1
DOI: 10.4172/2161-1025.S1-003

Research Article Open Access

Migration Stimulating Factor (MSF): A Novel Biomarker of Breast Cancer Progression

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Abstract

Migration Stimulating Factor (MSF) is a novel oncofetal biomarker previously identified in a number of human tumours. The aim of this study was to evaluate the possible association between MSF expression and disease progression in breast cancer. Archival breast tissues examined included malignant tumours (T; n=23), benign tumours or pathologies (B; n=8) and histologically normal breast from either reduction mammoplasties (NB; n=19) or from tumour patients (NB-T; n=18). Sections were stained with MSF-specific antibodies and assessed by consensus of 3-4 independent observers in terms of various MSF indices, which represented overall, epithelial and stromal MSF expression. MSF was heterogeneously expressed by epithelial and stromal cells, with significant inter-group quantitative differences in the sequence NB<NB-T=B<T. The percentages of specimens in the various groups showing moderate or strong MSF expression were 0% (NB), 45% (NB-T), 50% (B), and 78% (T). MSF was also observed in ductal carcinoma in situ and in carcinoma tissue cores in microarrays. In both histologically normal tissues (NB, NB-T), MSF expression was inversely associated with patient age, and epithelial and stromal MSF indices were significantly and directly correlated; in contrast, such associations were not observed in tumours (B, T). MSF expression in T was not associated with its expression in the paired NB-T. These data indicate that MSF is a biomarker of early breast cancer and disease progression. Epithelial and stromal MSF may be differently regulated and may carry different diagnostic or prognostic value. MSF expression in histologically normal and benign tissues suggest the presence of "field cancerisation", defined as the presence of predisposing genetic or epigenetic lesions which increase the risk of developing subsequent malignant lesions.

Keywords: MSF; Breast cancer; Cancer progression; Biomarker; Field cancerisation

Abbreviations: MSF: Migration Stimulating Factor; NB: Histologically normal breast tissues from reduction mammoplasties; NB-T: Histologically normal breast from patients with breast carcinoma; B: Benign breast tumours/pathologies; T: Breast carcinomas; DCIS: Ductal carcinoma in situ; TMA: Breast carcinomas in tissue microarrays

Introduction

There remains an urgent need to improve cancer diagnosis, prognosis and our capacity to design individualized therapeutic strategies [1-5]. This is likely to involve the use of complementary biomarkers identifying perturbations in homeostatic regulatory pathways operative in the normal breast. Migration Stimulating Factor (MSF) is a candidate novel biomarker for consideration.

MSF is an oncofetal regulatory protein constitutively expressed by a variety of cell types during fetal development, not expressed by the majority of cells in the healthy adult, but persistently re-expressed by both carcinoma and associated stromal cells in patients with breast and other human cancers [6-12]. MSF is a 70 kDa genetically truncated isoform of fibronectin [7]. Its message is generated from the fibronectin gene transcript by a variation of standard alternative splicing involving the retention of intron12, followed by a two step series of intra-intronic cleavage events to produce a 2.1 kb mature mRNA [7, 13]. These events are consistent with other reported variations in alternative splicing associated with cancer pathogenesis [14]. Approximately 20 "full-length" fibronectin isoforms have been identified, all with molecular masses in the region of 250-280 kDa; MSF protein terminates in a unique intron12-derived decamer not present in any of the full-length fibronectin isomers [7]. A panel of MSF identification antibodies have been raised against a synthetic peptide containing this unique decamer sequence; these are highly specific for MSF and may be used to demonstrate the presence of MSF protein in archival tissue sections [7, 12].

MSF displays a number of highly potent bioactivities, including the stimulation of normal and neoplastic cell migration, matrix remodeling and angiogenesis [7, 9-12]. Taken together with its oncofetal pattern of expression, this spectrum of bioactivities suggests that MSF may contribute to tumour progression.

The aim of the present study has been to obtain initial information regarding the association between MSF expression and disease progression in breast cancer. Towards this end, MSF expression was assessed in archival specimens of benign and malignant breast tumours, as well as in histologically normal breast, both from reduction mammoplasty and adjacent to malignant tumours.

Materials and Methods

Specimens

Archival specimens of breast tissues were obtained through the

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Received November 13, 2011; **Accepted** February 20, 2012; **Published** February 22, 2012

Citation: Perrier S, Woolston A, Purdie CA, Kazmi S, Preece PE, et al. (2012) Migration Stimulating Factor (MSF): A Novel Biomarker of Breast Cancer Progression. Translational Medic S1:003. doi:10.4172/2161-1025.S1-003

Tayside Tissue Bank, Ninewells Hospital, Dundee, and from the Department of Pathology Christie Hospital, Manchester. Tissue microarrays were purchased from the Cooperative Human Tissue Network and the Tissue Array Research Program (T-BO-1 Breast/Ovary TARP5. TARP, NIH, Bethesda, MD, USA). The study was performed according to the Declaration of Helsinki and approved by the appropriate local Ethics Committees. The groups of tissues examined were classified as follows:

NB: Histologically normal breast tissues obtained from reduction mammoplasties (n= 5 frozen, 19 paraffin-embedded);

NB-T: Histologically normal breast from patients with breast carcinoma (n= 3 frozen, 18 paraffin-embedded);

B: Benign breast tumours and other benign pathologies (n= 8 paraffin-embedded), from patients with no evidence of malignant tumours;

DCIS: Ductal carcinoma in situ (n=4, paraffin-embedded), from patients with concurrent invasive ductal carcinoma;

T: Breast carcinomas in tissue blocks (n= 5 frozen, 23 paraffin-embedded);

TMA: Breast carcinomas in tissue microarrays (n= 28, paraffin-embedded). Some tissue cores were lost or un-assessable. Only those specimens (n=28) that had at least two good quality tissue cores were assessed.

Among the NB-T specimens, 4/18 were present in the same section as the tumour, whereas 14/18 were in separate blocks, taken from the tumour margin. These 14 paired T and NB-T blocks were analyzed separately in some instances. Further details are shown in Table 1.

Detection of MSF by immunohistochemistry (IHC)

Mouse monoclonal (mab7.1, mab2.1) and rabbit polyclonal (Rp2) MSF-identification antibodies were raised against the unique C-terminal sequence of MSF (VSIPPRNLGY), which is not present in any full-length fibronectin isoform. Antibodies were prepared as previously described [7]. In an initial study, frozen and paraffin embedded blocks from the same specimen were examined in a small number of cases, including NB (n=5), NB-T (n=3) and T (n=5). These tissues were stained with MSF-identification antibodies mab7.1 and Rp2. Paraffin-embedded breast tumour cores in tissue microarrays (TMA, n=28) were stained with antibodies mab7.1 and mab2.1. Subsequent studies were performed with formalin-fixed paraffin embedded tissues (described in Table1), stained with antibody mab7.1 as previously published [7,12]. Briefly, de-paraffinised sections were incubated with 3% (v/v) hydrogen peroxide in phosphate buffered saline (PBS) for 20 minutes to inhibit endogenous peroxidase activity. This was followed by an avidin/ biotin blocking step (Avidin/Biotin Blocking Kit, Vector Labs, Peterborough, UK) and incubation with 20% (v/v) normal goat serum in PBS (NGS-PBS) for 30 minutes. Sections were then incubated overnight at 4°C with the MSF-specific primary antibody (mab7.1, 20 µg/ml) in NGS-PBS. Detection was achieved by treatment with 6 µg/ml biotinylated goat anti-mouse IgG (Vector Labs, UK) in NGS-PBS, followed by avidin-biotin complex for 30 minutes at room temperature. All the above stages were separated by PBS washes. Immunostaining was visualized by developing the slides in diaminobenzidin (DAB) for 10 minutes and counter-staining with Mayer's Haematoxylin.

At least two non-consecutive sections were stained for each antibody and specimen analysed. Negative controls were provided by incubating sections with non-immune normal (mouse/rabbit) IgG, as appropriate, (Dako, Cambridgeshire, UK) instead of primary antibody. MSF antibody previously adsorbed to MSF was used as an additional negative control. Early in the study, four calibration slides were selected, by consensus of two observers, on the basis of the intensity of the staining (from 0 to 3). As several staining runs were required to complete the study, at least one previously stained positive slide and/or a calibration slide were included in each run as positive controls.

Assessment of MSF expression in breast tissue sections

Each type of assessment described below was carried out by 3-4 independent observers and the final results (as presented) were obtained by consensus. MSF staining was first evaluated at x100 magnification, scanning the whole section. The overall distribution of staining was recorded and the following semi-quantitative parameters were then evaluated by comparison to pre-selected calibration slides:

(i) Overall MSF expression (MSF grades 0-3). Specimens were initially graded as negative (0), weak (1), moderate (2), or strong (3) positive. At least 5% of the whole area stained was chosen as the cut-off point between grade 0 and grade 1. Although such overall MSF evaluation includes epithelial and stromal compartments, it mainly reflects the former, as higher magnification is required to assess the stroma. TMAs and DCIS were assessed by this method only. In all other tissues, the epithelial and stromal compartments were also evaluated separately.

(ii) Epithelial MSF expression was defined by four indices [12,15]: The percentage of area stained (1-100%) was estimated, and the intensity of the staining was graded from 0 (negative) to 3 (strong) by comparison to calibration slides. The highest intensity or "hot spot" intensity (present in at least 10% of the epithelium) was also recorded. Final score (0-300) was derived by multiplying the % area stained by the intensity of the staining. Heterogeneous staining was common regarding the intensity and the percentage area stained; this is reflected in the final score. For example, 30% area stained with intensity 1 and 20% area with intensity 2 gives a final score of 70.

(iii) Stromal MSF expression was classified using higher magnification as either positive or negative for two constituent stromal cell types: fibroblasts and micro vascular. These stromal cell types were identified on the basis of standard

Tissue group	n	Histology / nodal status (*)	Age (**)
NB	19	Normal breast from non-tumour patients	**30.7 ± 7.7 29 (24-35)**
NB-T	18	Normal breast from breast tumour patients	**62.7 ± 11.5 64 (55-72)**
B	8	Benign tumours/pathologies: IDP (2); DE (1); FA (2); Cys (2); CysH (1)	**51.4 ± 15 49.5 (39-58)**
T	23	Malignant tumours: IDC (19); ILC (3); IPC (1); N0 (15); N1/2 (8).	**59.4 ± 12.6 60 (53-70)**
paired			
NB-T	14	Normal breast from breast tumour patients	**64.6 ± 10.7**
T	14	Malignant tumours: IDC (10); ILC (3); IPC (1); N0 (9); N1/2 (5).	**66 (57-74)**

(*) Histology/nodal status. Benign tumours/pathologies: IDP = intraductal papilloma; DE = duct ectasia; FA = fibroadenoma; Cys =Fibrocystic change; CysH= Fibrocystic change with hyperplasia of usual type, Malignant tumours: IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; IPC = invasive papillary carcinoma; N0 = no nodal involvement; N1/2 = nodal involvement. Number of specimens within brackets. All specimens as paraffin embedded archival blocks.
(**) age in years.First line: mean ± SD; second line: median (interquartile range)

Table 1: Breast tissues examined and age of the patients.

cytological characteristics, as generally accepted and used in routine pathology.

Statistical analysis

Statistical analyses were carried out with the Prism 5 software package (Graphpad, Inc., La Jolla, Ca, USA). Differences among groups of tissues were determined either by Chi square and Fisher's Exact Tests or Kruskal-Wallis and two-tailed Mann-Whitney tests, as appropriate. Significance differences were defined at 95% level of confidence ($p<0.05$).

Results

Immunolocalisation of MSF in breast tissues

In an initial study, we compared MSF staining in duplicate frozen and paraffin embedded blocks from a small number of tissue specimens, including NB (n=5), NB-T (n=3) and T (n=5). These specimens were stained with three different MSF-identification antibodies (mouse monoclonal antibodies mab7.1 and mab2.1 and rabbit polyclonal Rp2). The same results were obtained using frozen and paraffin embedded breast tissues and the various MSF-identification antibodies (not shown). Optimization of the staining [16] indicated that pre-treatment of paraffin-embedded sections for antigen retrieval was not required for any of the antibodies or tissues tested. The optimal antibody concentration was identical for tissues that had been processed by standard routine pathology methods in different Centers and at different times. On the basis of these results, MSF expression in all archival paraffin-embedded blocks was assessed following staining with monoclonal antibody mab7.1 [7,12]. The specimens included NB (n=19), NB-T (n=18), B; (n=8) and T (n=23). Among the NB-T specimens, 4/18 were present in the same section as the tumour, and 14/18 were in separate blocks, taken from the tumour margin (Table1). We also examined breast carcinoma cores in tissue microarrays (TMA; n=28) and ductal carcinoma in situ (DCIS; n=4).

In malignant tumours (T, TMA), MSF was heterogeneously expressed by both the carcinoma cells and the associated stromal fibroblasts and blood vessels. Cells of the inflammatory infiltrate were usually negative, but positive cells were also present in some specimens. MSF staining was negative or weak positive in most NB specimens, whereas a positive staining was frequently observed in NB-T. B specimens included benign breast tumours and other benign pathologies (Table 1); positive staining was observed in both types of tissues. As discussed below, quantitative differences were observed among the various breast tissues examined, while heterogeneous expression in epithelial and stromal cells was the usual finding whenever MSF was present. The extracellular matrix commonly appeared negative, although high background staining was occasionally observed. Such background is consistent with MSF binding to certain matrix constituents, as it contains both the collagen- and heparin/fibrin-binding domains of fibronectin [7]. In this regard, high background staining was commonly present in the dense matrix of the normal breast interlobular stroma, but not in the looser intralobular stroma. No staining was apparent in the negative controls (not shown). Representative examples of the MSF staining patterns observed in the various breast tissues are presented in Figure 1.

Differential expression of MSF in normal, benign and malignant breast tissues

MSF expression was assessed according to various semi-quantitative indices (Materials and Methods). The tissues were first classified according to their MSF grade (overall expression) from negative (0) to strong positive (3). MSF-positive and negative specimens were observed in all tissue groups, although to significantly different extents. To facilitate the comparison of the different tissues, the number of NB, NB-T, B, T and TMA specimens showing MSF grades 0 to 3 are presented in Figure 2A as a percentage of the total numbers examined. Four samples of DCIS were classified as MSF grade 2 or 3. For statistical analyses, both the actual number of specimens and the percentage of specimens (as in Figure 2A) showing the various MSF grades were analyzed by Chi square and Fisher's exact tests. MSF grades were combined (0/1 v 2/3) when required, due to the presence of excessive zero values or small numbers (for NB and B), and analyzed by Fisher's exact test.

The results indicate that overall MSF expression increased in a step-wise manner from NB to T (Figure 2A). The percentage of MSF-positive specimens (MSF grades 1-3) in NB, NB-T, B, T and TMA tissues was 26%, 67%, 88%, 91% and 82%, respectively. The equivalent percentage of specimens showing moderate or strong MSF expression (MSF grades 2-3) was 0%, 45%, 50%, 78% and 57%, respectively.

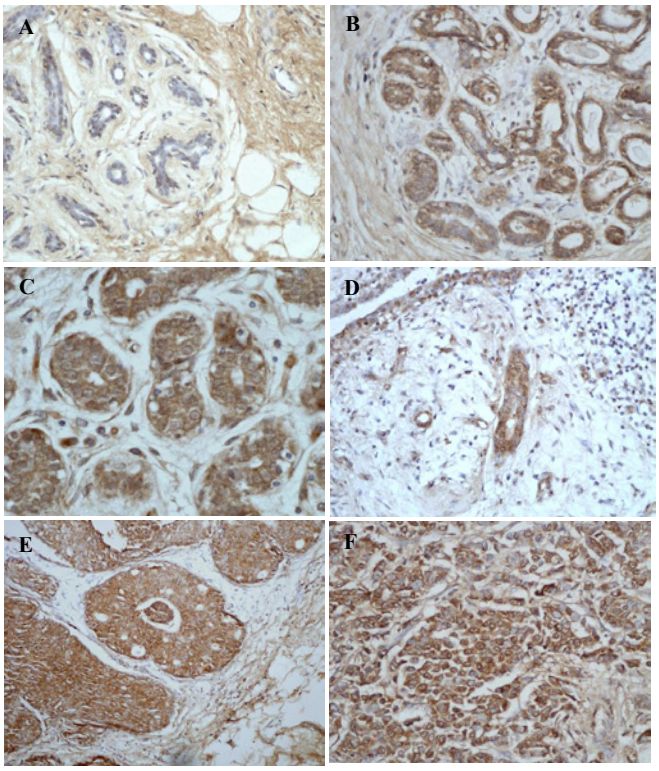

Figure 1: MSF expression in breast tissues. Paraffin-embedded tissues were stained with MSF-specific antibody and counter-stained with haematoxylin. Brown colour denotes positive staining.
A. Histologically normal breast tissue from a non-tumour patient (NB). No MSF expression in the normal breast lobules or associated stroma. High background in the extracellular matrix of the interlobular stroma, but not in the intralobular stroma. **B**. Histologically normal breast tissue from the excision margin of a breast carcinoma (NB-T). MSF-positive lobular epithelium, negative stromal cells. **C**. Normal/benign breast lobule from a patient with fibrocystic change (B). Positive staining in the lobular pithelium, associated fibroblasts and vessels. **D**. Benign breast tumour, fibroadenoma (B). Positive staining in the tumour epithelium, fibroblasts and vessels. Mostly negative inflammatory cells **E**. Ductal carcinoma in situ (DCIS). Homogeneous epithelial staining. **F**. Malignant breast tumour, infiltrating lobular carcinoma of pleomorphic type (T). Heterogeneous epithelial staining.
Original magnification x200 (A, B, D, E, F) and x400 (C).

Expression was low or negligible in NB, this being significantly lower than in NB-T (p= 0.001). The difference between NB-T and B was not statistically significant, whereas the difference between NB and B was so (p= 0.004). Malignant breast tumours were examined as two tissue groups: either standard block from routine pathology (T, n=23) or TMA cores (n=28). The difference between T and TMA was not significant according to the actual number of specimens showing the various MSF grades, but significant (p=0.0024) when comparing the percentages of specimens. However, a comparison of malignant tumours with the other tissue groups indicated that similar results were obtained when using either T or TMA: That is, MSF grade was significantly higher in T than in NB (p<0.0001) or in NB-T (p=0.02). The difference between T and B was significant (p<0.0001) when comparing the percentages of specimens showing the various grades,

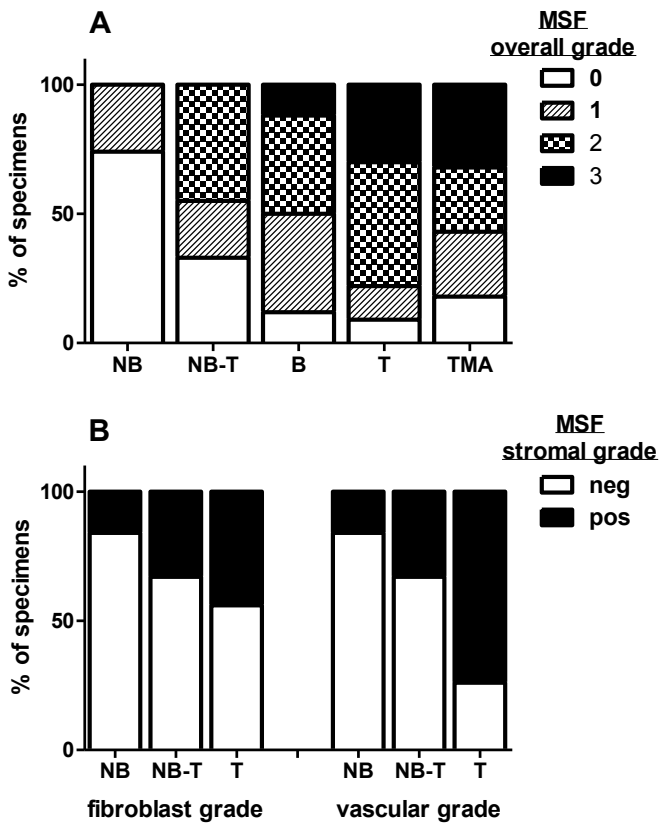

Figure 2: Overall MSF expression (MSF grade) and stromal grade in breast tissues.
Paraffin-embedded tissues stained with MSF-specific antibody included:
NB (n=19): Histologically normal breast tissues from reduction mammoplasties;
NB-T (n=18): Histologically normal breast from patients with breast carcinoma;
B (n=8): Benign breast tumours/pathologies;
T (n=23): Breast carcinomas, from routine pathology blocks; and
TMA (n=28): Breast carcinomas cores in tissue microarrays.
For statistical analyses, both the number of specimens (n) and the percentage of specimens (as in graph) showing the various MSF grades were analysed by Chi square and Fisher's exact tests
A. Overall MSF grade, classified as negative (0), weak positive (1), moderate (2), or strong positive (3). No significant differences were found between NB-T and B. Significant differences were found between all other tissues (p=0.02-0.0001).
B. Stromal grade, classified as either negative or positive for fibroblasts and the microvasculature. Differences between NB and NB-T were significant when comparing the percentages of specimens examined (p=0.008 for both fibroblast and vascular grades), but not for the actual number of specimens. Differences between NB and T were significant for both stromal grades (p=0.002-0.0001), whereas differences between NB-T and T were significant only for the vascular grade (p=0.01-0.0001).

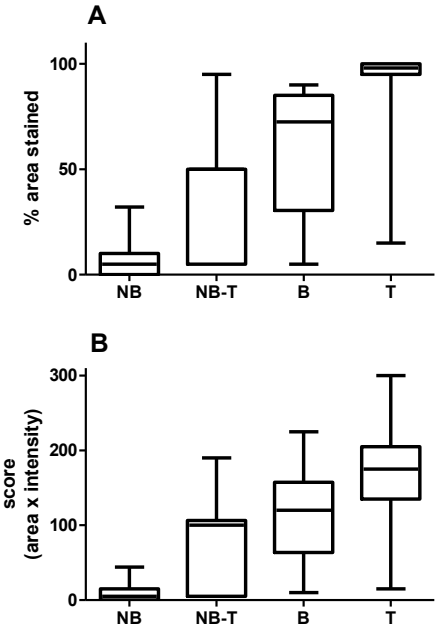

Figure 3: Epithelial MSF expression in breast tissues.
The tissues examined included NB (n=19); NB-T (n=18); B (n=8) and T (n=23), as described in Table 1 and Fig 2. Following staining with MSF-specific antibody, MSF expression in the epithelium was evaluated according to: **(A) the percentage of area stained**, and **(B) the final score** (product of area and intensity). Differences among tissues were analysed by Kruskall-Wallis and unpaired two tailed Mann Whitney tests. No significant differences were found between NB-T and B. The difference between B and T was significant for the area stained (p=0.001) and near significant for the epithelial score (p=0.07). Significant differences were found between all other tissues (p=0.001-0.0001).

but not by the actual number of specimens. Although an evaluation of overall MSF expression/grade includes epithelial and stromal compartments, it mainly reflects the former, as higher magnification is required to assess the stroma. MSF staining in stromal fibroblasts and microvessels was classified in NB, NB-T and T tissues as either negative or positive. The results indicate that stromal MSF expression also changed in the sequence NB < NB-T < T. Differences among tissues were more apparent for the vascular grade, as differences in fibroblast grade were only significant when comparing the percentages of specimens examined (Figure 2B).

Epithelial MSF expression was further assessed in the same NB, NB-T, B and T specimens, according to the percentage of area stained, intensity of the staining, final score (product of area and intensity) and highest intensity (Materials and Methods). Percentage of area stained and score are shown in Figure 3. Both also appeared to increase in a stepwise manner from NB to NB-T, B and T. However, the difference between NB-T and B specimens was not significant. Significant differences were found between all the other tissues examined, including NB and NB-T. Comparison between B and T indicated that the percentage area stained was more informative than the final score. Comparison of the tissues according to the MSF highest intensity (not shown) produced similar results as the overall MSF grade (Figure 2A).

Association between MSF expression and clinical parameters

The age of the NB group (Table 1) was significantly lower than that of the other groups, including NB-T (p=0.0001). Therefore we examined the possibility that lower MSF expression in NB than NB-T might

reflect patient age. The results obtained argue against this possibility, as MSF expression was found to be higher in younger patients, reaching significant or nearly significant differences for NB and NB-T groups, respectively. In contrast, there was no association between age and MSF expression in tumour patients (Table 2). Within the small number of malignant tumours examined (T; n=23), no associations were found between any of the MSF indices and other clinical parameters such as tumour grade, size or nodal status (data not shown). Among the B specimens (n=8) there was no apparent association between type of pathology and MSF expression. For example, 2 specimens diagnosed as fibrocystic change and fibrocystic change with hyperplasia had MSF grades of 3 and 0 respectively. Similarly, 2 fibroadenomas showed MSF grades 1 and 2.

Correlations among MSF indices

As expected, overall MSF grade was directly and significantly associated with both *epithelial* and stromal MSF indices in all tissues; although the correlation with the latter was stronger in normal tissues than in tumours. A difference between normal tissues (NB and NB-T) and tumours (B and T) was most apparent when comparing *stromal MSF* indices (fibroblast v vascular) or epithelial MSF v stromal MSF indices. In these cases, MSF indices were significantly correlated in the normal tissues but not in the tumours. These results are summarized in Table 3.

Comparison of MSF expression in tumours and adjacent normal tissues

Paired T and NB-T specimens from the same patients (n=14, Table 1) were used to examine the possible association between tumours and histologically normal breast tissue from the tumour margin regarding MSF expression. The results indicated a lack of association for the various MSF indices (Spearman r=-0.41-0.05); p=0.14-0.96) (results not shown). These two groups of paired specimens did not differ from the larger groups of T (n=23) and NB-T (n=18) regarding patient characteristics (Table 1) or differences between each other and other tissue groups regarding MSF expression (not shown).

Discussion

Several biomarkers are currently employed in the clinical management of patients with breast cancer, including hormone and growth factor receptor status, oncogene expression and cell adhesion receptors [1-5,17-20]. Previous reports have suggested that MSF may be a novel tumour biomarker by virtue of its pattern of expression and potent bioactivities. Using a small number of specimens, MSF has been previously identified in various human cancers, including breast, oral, lung, oesophagus, colon, skin and prostate tumours [7,9,11,12 and unpublished data]. Co-expression of MSF mRNA and protein has been confirmed in breast tumours [7]. MSF has also been reported to be expressed by skin fibroblasts explanted from patients with a variety of common human cancers [21-24]. These observations have been extended in the current communication by examining the possible association between MSF expression and breast disease progression. The results obtained indicate that MSF is a biomarker of early breast cancer and disease progression. We specifically report that:

(i) MSF was highly expressed in over 70% of the breast tumours (T) and in 4/4 DCIS examined. In contrast, MSF expression was low or negligible in normal breast from non-cancer patients (NB);

(ii) There was a significant association between disease progression and MSF expression in the sequence normal breast<benign pathology<malignant tumour;

(iii) Histologically normal breast tissue adjacent to a carcinoma (NB-T) exhibited significantly higher levels of MSF expression than histologically indistinguishable NB;

(iv) MSF was heterogeneously expressed by epithelial and stromal cells;

(v) The levels of tumour-epithelial and tumour-stromal MSF were not associated with each other or with the levels of MSF in the normal breast tissue (NB-T) from paired specimens.

(vi) Consistent results were obtained when using either frozen or paraffin-embedded tissues, different MSF-specific identification antibodies and tissue blocks from different Centres.

A significant difference in MSF expression between benign and malignant salivary gland tumours has also been observed [12]. The heterogeneity in MSF expression opens the possibility that its relative level may convey diagnostic and/or prognostic information, an eventuality suggested by recently obtained data [9 and unpublished data]. When assessing immunostaining, conflicting results may be obtained depending on the particular parameter/index examined (e.g. intensity, area stained, etc) as well as a result of inter- and intra-observer variations [15,16]. To avoid these potential problems, MSF expression was assessed by consensus of 3-4 independent observers according to various semi-quantitative *MSF indices* that measured overall (MSF grade), epithelial (% area stained, intensity) and *stromal* staining (fibroblasts, vessels). Stromal indices were simplified (positive or negative) in order to achieve observer consensus. Further assessment of stromal MSF can be best accomplished with computer-assisted image analysis (unpublished data). The percentage of positive tumours in tissue microarrays (TMA: 82% positive, 57% moderate/strong positive) was lower than in standard pathology blocks (T: 91% positive, 78%moderate/strong positive). Given the heterogeneity in MSF expression and the small size of the TMA tissue cores, this difference is likely to be due to sampling error, which may influence the accuracy of the results when evaluating prognostic biomarkers in TMAs.

The majority of biomarkers utilized for patient management involve the assessment of features of the carcinoma cell population [17-19]. It is now apparent, however, that cells of the tumour-associated stroma, including fibroblasts, microvasculature endothelial cells and adipocytes, may also display aberrant features relevant to tumour progression [25-29]. This realization coincided with a growing awareness of the importance of "cell system" interactions by which cancer inception and progression are profoundly influenced by dynamic and reciprocal interactions between epithelial and stromal cells [6,8,21,23,24,30-32]. The aberrant expression of MSF by both epithelial and stromal cells and its association with disease progression

Age divided by median	NB (n=19)	NB-T (n=18)	T (n=23)
above	5.5 ± 5.9 5 (0-9)	48 ± 48 37 (5-100)	178 ± 45 192 (137-216)
below	17 ± 17 8.5 (5-40)	101 ± 63 100 (52-155)	149 ± 97 160 (46-214)
Difference (p value)	0.0084	0.064	0.3557

MSF expression was determined in NB, NB-T and T tissues. Tissues and age of the patients are described in Table 1. Results indicate the MSF epithelial score, as mean ± SD (first line), median and interquartile range (second line). The difference between older and younger patients was determined by unpaired two-tailed Mann Whitney test

Table 2: MSF expression according to the age of the patients.

Correlation coefficient (Spearman r) and p value in the indicated tissues				
MSF indices compared	**NB (n=19)**	**NB-T (n=18)**	**B (n=8)**	**T (n=23)**
Overall MSF grade v. epithelial area	r=0.769 p=0.0001	r=0.900 p=0.0001	r=0.768 p=0.028	r= 0.558 p=0.005
Overall MSF grade v. epithelial score	r=0.745 p=0.0003	r=0.903 p=0.0001	r=0.951 p=0.001	r=0.794 p<0.0001
Overall MSF grade v. vascular grade	r=0.724 p=0.0005	r=0.723 p=0.0007	r= 0.699 p=0.06	r=0.433 p=0.039
Fibroblast grade v. vascular grade	r=1.000 p=0.0001	r=0.979 p<0.0001	**r=0.529 p=0.19**	**r=0.340 p=0.11**
Epithelial area v. fibroblast grade	r=0.464 p=0.045	r=0.677 p=0.002	**r=-0.085 p=0.84**	**r=0.353 p=0.10**
Epithelial area v. vascular grade	r=0.464 p=0.045	r=0.682 p=0.001	**r=0.387 p=0.32**	**r=0.309 p=0.15**
Epithelial score v. fibroblast grade	r=0.456 p=0.049	r=0.605 p=0.007	**r=0.261 p=0.53**	**r=0.532 p=0.09**
Epithelial score v. vascular grade	r=0.456 p=0.049	r=0.613 p=0.006	**r=0.548 p=0.17**	**r=0.319 p=0.13**

MSF expression was determined in NB, NB-T, B and T tissues according to various MSF indices (shown in Figures 2 & 3). Correlations were analysed for the indicated MSF indices and tissues. Values showing no significant correlation are highlighted

Table 3: Correlations among MSF indices.

is consistent with this paradigm. It should accordingly be noted that the expression of MSF and its precise effect on target cells is profoundly modulated by both the nature of the macromolecular matrix to which the cells are adherent and the presence of cell-produced soluble factors, including TGF-β and neutrophil-associated lipocalin [7,13,33,34]. The complexity of this hierarchy of autocrine and paracrine regulatory circuits underscores the importance of achieving a holistic appreciation of the factors *collectively* contributing to the dynamic process of tumour progression [35,36]. In this context, it is of interest that MSF expression in tumours did not correlate with MSF expression in the adjacent normal breast tissue. Furthermore, epithelial and stromal MSF indices were significantly correlated in the normal breast tissues but not in the tumours. Therefore, MSF expression by the carcinoma cells may have a different role and/or prognostic value than MSF expression by the tumour-associated stromal cells. Another difference between tumours and normal tissues was the association of MSF expression with age in the normal, but not in the tumours. The significance of these results is not clear at the moment, but may be related to the transition in MSF expression from fetal-like to adult-like phenotype, as previously reported to occur in tissue culture [37].

It is of interest to note that both B and NB-T tissues exhibited significantly elevated levels of MSF expression compared to normal breast from healthy controls (NB). We speculate that this difference may reflect the occurrence of "field cancerisation", here defined as the presence of functionally aberrant cells in the absence of overt histological abnormalities. The concept of field cancerisation was first proposed by Slaughter and colleagues [38] to indicate the induction of predisposing (initiating) genetic lesions in the target epithelial cells of a normal tissue due to exposure to genotoxic agents. This hypothesis was supported by later observation and extended to include the presence of predisposing genetic or epigenetic lesions in both epithelial and stromal cell population; such lesions would therefore significantly increase the risk of developing subsequent malignancies [39,40]. According to this view, the presence of functionally aberrant (MSF-expressing) cells may pre-date the generation of overt tumour cells and indicate tissue-wide enhanced susceptibility to the development of neoplastic disease. Alternatively, it is also possible that MSF-expressing cells may reflect the inductive influence of a pre-existing population of neoplastic cells. In this latter case, the presence of neoplastic cells would pre-date the expression of MSF by histologically normal cells. In either case, the detection of MSF in histologically normal tissue may indicate the presence of a pervasive functional aberration not apparent by its normal histology. This hypothesis is consistent with previous results reporting the isolation of MSF-expressing fibroblasts from histologically normal tissue adjacent to a breast carcinoma but not from reduction mammoplasty tissue [41].

The assessment of MSF expression in tumours may inform the development of novel MSF-targeted adjuvant therapies, these designed to inhibit its expression and/or manifestation of its bioactivity [7, 33, 34, 42]. Such a targeted "theranostic" approach has the potential of enhancing therapeutic efficacy.

Acknowledgements

This work was funded by the Breast Cancer Campaign, Cancer Research UK and Tayside Area Oncology Fund. We thank the assistance of Mrs J Cox, Ms MM Florence Dr IR Ellis and Dr SJ Jones with antibody production and Mrs J Cox and Mr J Carlile with staining and assessment. We thank Prof FA Carey, Tayside Tissue Bank, Ninewells Hospital, Dundee, and Prof N Bundred, Department of Pathology, Christie Hospital, Manchester for providing the specimens.

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This article was originally published in a special issue, **Discovering Novel Biomarkers** handled by Editor(s). Dr. K. StephenSuh, Hackensack University Medical Center, USA; Dr. Takemi Tanaka, Thomas Jefferson University, USA; Dr. Valli De Re, Centro di Riferimento Oncologico, Italy