Serum metastasin mRNA is an important survival predictor in breast cancer

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ABSTRACT

This study investigates the possible prognostic role of serum metastasin messenger RNA (mRNA) in breast carcinoma as a non-invasive screening tool, and determines metastasin mRNA in the serum of breast cancer patients with high sensitivity (85%) and specificity (100%). A significant difference ($P=0.05$) was observed between serum metastasin mRNA and the number of involved lymph nodes. Patients with higher expression of serum metastasin showed poor survival (six times worse) than those with lower levels. Patients negative for serum metastasin mRNA suffered recurrences, while those positive for serum metastasin mRNA suffered distant metastases. The results of this study suggest that serum metastasin mRNA represents an important survival marker in breast carcinoma.

KEY WORDS: Calcium-binding proteins.
Carcinoma, intraductal.
Nucleic acids.
Prognosis.
S100A4 protein, human.

Introduction

A simple blood test to detect cancer has been the quest of many researchers. Therefore, the finding that cell-free circulating nucleic acids in the plasma and serum of cancer patients have the characteristics of tumours has produced much interest.[1–4] Many tumour-related RNAs have been detected in the plasma and serum of cancer patients, including in those with breast cancer.[5,6] Although ribonuclease activity has been reported to be increased in the plasma of cancer patients,[7] circulating RNA molecules have shown integrity due to their association with lipid, protein, lipoprotein or phospholipid moieties.[8,9] These secretory complexes appear to represent communication channels between the tumour and the organism.[6]

Metastasin belongs to the family of EF-hand calcium-binding proteins, which includes 24 members, the expression of which is elevated in a number of pathological conditions.[10] Although it is well documented that metastasin is expressed in cancer cells and contributes to tumour cell motility and metastatic progression, the exact underlying mechanisms remain elusive.[11] Several studies suggest that formation of homo- and hetero-dimers, binding of Ca^{2+} and interaction with effector molecules play an essential role in the development and progression of many cancers, including those affecting the breast.[10–12]

To the authors' knowledge, no study has examined the presence of serum metastasin messenger RNA (mRNA) in breast cancer patients, or its prognostic and/or diagnostic role.

Materials and methods

This study comprised 50 females (15 with benign breast lesions, 20 with breast carcinoma and 15 healthy controls). Patients were recruited according to the ethical rules of the Belmont report (http://ohsr.od.nih.gov/guidelines/belmont.html). Pathological investigations, angiogenesis and clinical follow up were performed as described by El-Abd *et al*.[13] Prognostic index was calculated using the Nottingham index.[14]

Total RNA was extracted from serum samples or tissue specimens using Trizol reagent (Invitrogen). A reverse transcriptase–polymerase chain reaction (RT–PCR) method was performed using ready-to-go RT–PCR beads (Amersham), according to the manufacturer's instructions. Specific primers for human metastasin (M80563; from 172 to 380) and β-actin (X00351; from 781 to 935) were used to generate 209-bp and 155-bp fragments, respectively.

The PCR protocol was 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 50°C (for metastasin) or 60°C (for β-actin) for 1.5 min, and 72°C for 1 min. After a final extension at 72°C for 7 min, the reaction was stopped by adding the gel loading dye. The amplified products were then separated on 2% agarose gel containing ethidium bromide and visualised under ultraviolet (UV) light.[15] The relative expression level of metastasin (metastasin to β-actin) was quantified using the Scion image program for Windows.

The results were analysed using SPSS (Statistical Package for Social Sciences, IBM PC/XT) version 12. The survival curve was constructed using Kaplan-Meier plots and Wilcoxon-Gehan statistics.[16]

Results

Study population

Age range was 34–75, 16–51 and 33–57 years (mean±SD: 50.2±10.7, 29.9±9.1 and 44.1±7.7) in the breast cancer

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patients, benign breast group and healthy controls, respectively. In total, 50% of the breast cancer patients, 86.7% of the benign group and 53.3% of the healthy controls were premenopausal. The benign breast lesions included six cases of fibroadenosis, four cases of fibroadenomatous hyperplasia, three cases of fibrolipomatous tissue, one case of atypical ductal hyperplasia and one case of lactating adenoma (as diagnosed by fine-needle aspiration cytology). The histopathological characteristics of the breast cancer patients are summarised in Table 1.

Angiogenesis

Analysis of CD31 immunostaining on all paraffin blocks prepared from the malignant breast masses for the determination of vascular proliferative index showed that angiogenesis in cancerous tissue was significantly higher (173.1±63.1, $P<0.001$) than that detected in matching adjacent normal tissue (60.0±2.1), and represented a 2.9-fold increase.

Angiogenesis varied in the tumour specimens, as shown in Figures 1a and 1b. Counts in the range 76–93 microvessels/mm^2 were associated with negative lymph node metastasis (LNM) status, whereas >190 microvessels/mm^2 was associated with positive LNM status. Counts of 94–190 microvessels/mm^2 were predominantly associated with positive LNM status, but some cases of negative LNM status were found in this group.

Table 1. Histopathological characteristics of the breast cancer patients.

	No	(%)
Histopathological type		
Intraductal carcinoma	17	(85)
Lobular and Paget's disease	3	(15)
Oestrogen receptors		
Negative	3	(15)
Positive	17	(85)
Progesterone receptors		
Negative	3	(15)
Positive	17	(85)
Lymph node metastasis		
Negative	5	(25)
Positive	15	(75)
1–3	3	(20)
4–9	9	(60)
≥10	3	(20)
Tumour size (cm)		
<2	3	(15)
≥2	17	(85)
Histological grade		
II	17	(85)
III	3	(15)
Outcome		
Non-metastatic	14	(70)
Metastatic	6	(30)
Died	8	(40)
Alive	12	(60)

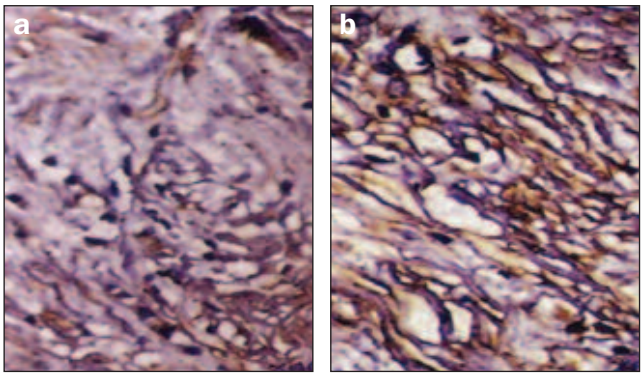

Fig. 1. Immunohistochemical staining of intraductal carcinoma by specific CD31 antibody to score angiogenesis: a) low-grade angiogenesis, b) high-grade angiogenesis. See the images in colour at www.bjbs-online.org

No significant correlation was observed between angiogenesis in malignant tissues and various histopathological parameters (LNM, tumour size, tumour grade or hormone receptors), or with prognosis. However, among the 10 patients who developed metastasis and/or died, seven (70%) had a vascular density of ≥100 microvessels/mm^2.

Serum metastasin mRNA

RNA per serum aliquot corresponding to 30–50% of the RNA extracted from 500 µL of serum from the breast cancer patients demonstrated the ability of the assay to detect metastasin mRNA in serum (Fig. 2). To verify the presence, integrity and equal loading, all sera were assayed also for human β-actin mRNA, which was detected in all of the samples examined.

In all amplification assays, the metastasin-positive malignant breast tissue (positive control) and complementary DNA (cDNA)-absent sample (negative control) were tested appropriately. Metastasin mRNA was detected in the sera of 17 (85%) patients in the malignant group. However, expression of metastasin was observed in two (13.3%) of the benign cases characterised by hyperplasia. In contrast, none of the 15 normal control sera tested positive for metastasin mRNA. Negative results showed no detectable band, while the positive results were subdivided into two groups (<1 and ≥1) according to the relative expression level.

Serum metastasin mRNA exhibited a statistically significant difference ($P<0.001$) between the three groups studied (Table 2), and serum metastasin showed high sensitivity and specificity (85% and 100%, respectively). Nine malignant specimens were selected randomly to compare the expression of tissue and serum metastasin. All the tissue sample results reflected the serum results (Fig. 2).

Lymph node metastasis

A significant difference ($P=0.05$) was observed between serum metastasin mRNA and the number of involved lymph nodes. Expression of serum metastasin mRNA was detected in 100% (12/12) and 33.3% (1/3) of cases with positive LNM, ranging from 1–9 and ≥10, respectively.

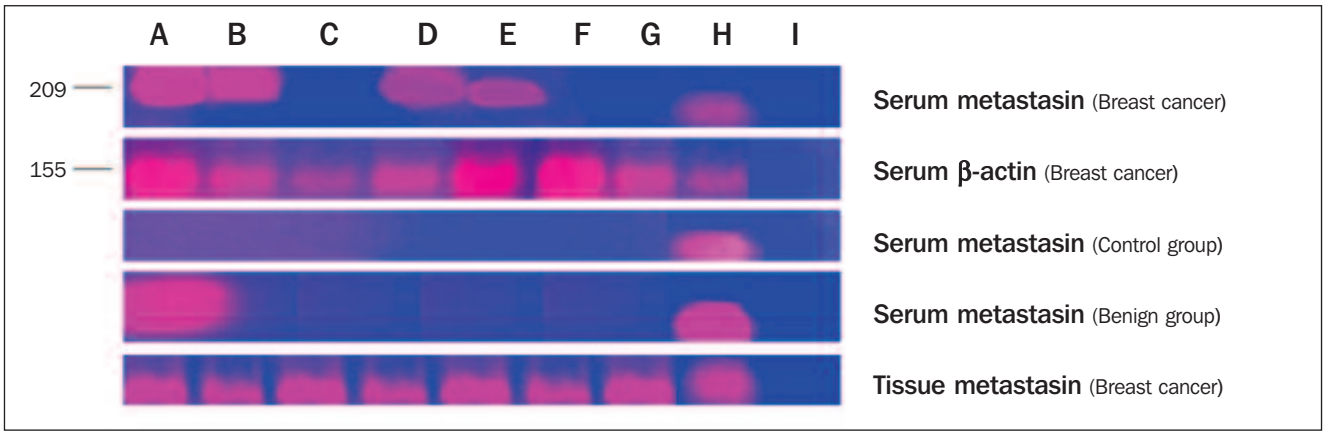

Fig. 2. Gel electrophoresis of serum metastasin RT-PCR products. Lanes A–G represent a random selection of typical results for the selected group. Lane H: positive control; lane I: negative control.

Tumour size and vascular density

Although high expression of serum metastasin was associated with tumour size ≥2 cm and microvessels ≥100/mm^2, this correlation was not statistically significant.

Prognosis

Survival and metastasis rates were 60% (12/20) and 30% (6/20), respectively. Based on the prognostic index, which includes tumour size, grade and LNM, 65% (13/20) and 35% (7/20) of the patients were expected to have a five-year survival rate >60% (group with better survival) and <60% (group with poor survival), respectively.

The actual survival rate for the first group (PI<4.5) was 84.6% (11/13), while that for the second group (PI>4.5) was 14.3% (1/7) over the 30-month follow-up period. The rate of metastasis was 15.4% (2/13) and 57% (4/7) in the two groups, respectively.

The discrepancy in survival and metastatic rates between the two groups was partially explained by the 1.8- and 2.2-fold increase in relative expression of metastasin (≥1, Fig. 3) and angiogenesis (≥100 microvesseles/mm^2), respectively.

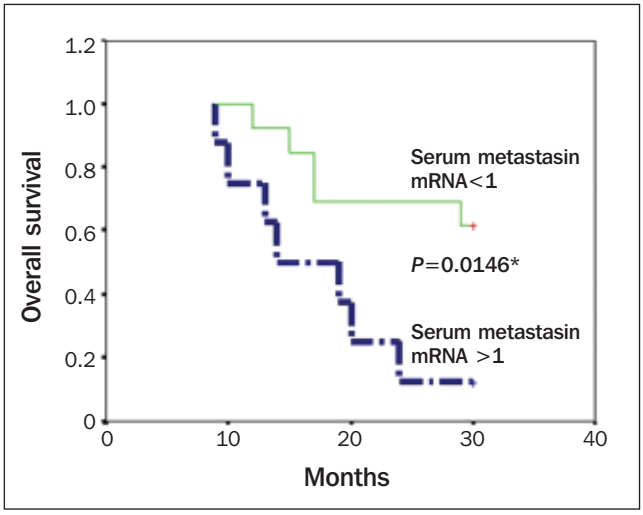

Fig. 3. Association of metastasin mRNA level with overall survival of patients.

Discussion

Metastasin protein is a well-established marker of tumour progression, invasion and metastasis formation, as well as an indicator of poor prognosis.[10–12] Strong overexpression of metastasin protein has been found in different types of tumours, including breast,[17] oesophageal squamous[18] and colon carcinomas,[19] invasive pancreatic carcinomas,[20] non-small cell lung cancers,[21] primary gastric cancers[22] and bladder cancer.[23]

To the authors' knowledge, no previous investigations have been performed to examine the presence of metastasin mRNA in the serum of breast cancer patients. The present study aimed to provide opportunities to establish a non-invasive test for the early detection of breast cancer using serum metastasin mRNA.

In the present study, a 7.2-fold increase (P<0.001) was observed between serum metastasin levels in benign cases

Table 2. Qualitative expression of serum metastasin mRNA.

	Group I (n=15) Control No (%)	Group II (n=15) Benign lesions No (%)	Group III (n=20) Breast carcinoma No (%)	P value
Positive	0 (0)	2 (13.3)	17 (85)	0.0001*
Negative	15 (100)	13 (86.7)	3 (15)	
Range	0	0–1.004	0–1.65	<0.001*
Mean±SD	0	0.131±0.346	0.930±0.454	

*Statistically significant.

and malignant cases. As the positive benign cases were associated with hyperplasia, metastasin might be useful as a marker to monitor cancer development and/or progression.

These results were consistent with work conducted on cell lines and human specimens.[24] The mRNA of metastasin (S100A4) was expressed at three- to 25-fold higher levels in cultured infiltrating ductal carcinoma (IDC) cell lines than in normal or benign human breast cell lines. A statistically significant higher mean level of S100A4 mRNA was detected in invasive carcinoma (124 cases: 113 IDC and 11 carcinoma *in situ*) than in the benign lesions (20 cases: five fibrocystic disease and 15 fibroadenomas).

Although other investigators[6] were able to detect several genes in healthy subjects, no metastasin mRNA was detected in our controls. Nikitenko *et al*.[25] demonstrated the presence of S100A4 mRNA in the stromal regions surrounding the epithelial ducts in normal breast tissue specimens. Analysis of 68 tumour biopsies showed that S100A4 protein is expressed preferentially by macrophages, fibroblasts and activated lymphocytes present in the tumour microenvironment, rather than by the tumour cells.[26] Moreover, it has been shown that it is externalised by the stromal cells to the fluid of the tumour microenvironment. A significantly higher concentration of S100A4 protein was detected in the tumour interstitial fluid (TIF) compared to that in the normal counterparts. The absence of metastasin mRNA in control subjects reflects its special differential role in health and disease.

Serum β-actin mRNA was detected in all tested subjects, confirming the presence and integrity of serum RNA, and of equal loading. The same results were achieved using GAPDH RNA as a reference gene.[27] Serum GAPDH mRNA was detected in all cancer patients, those with benign disease, and in the controls.

Nine breast tumour specimens from the group of patients tested for serum metastasin mRNA were selected randomly to investigate the expression of tissue metastasin mRNA. Serum metastasin was representative of tissue metastasin in all nine tested samples. Therefore, serum metastasin mRNA may offer a new non-invasive approach for cancer screening and/or monitoring of hyperplastic activity.

The cases in the present study were mainly positive for LNM (15/20), but serum metastasin mRNA was expressed in both LNM-negative and LNM-positive cases. However, expression showed significant variation ($P=0.05$) between the number of involved lymph nodes, which ranged from 1–9 and ≥10. This might further confirm involvement of metastasin in early metastasis. While other investigators[28, 29] have shown an association between metastasin protein and tumour spread to the regional lymph nodes, specifically in IDC, others have not detected a significant correlation between S100A4 mRNA and the number of nodes affected.[24] As the number of affected lymph nodes is an important prognostic indicator in breast cancer, these conflicting results require further study.

Vascular density was significantly higher in tumour tissue than in normal tissue ($P<0.001$). In the tumour specimens, vascular density varied considerably and showed no significant relationship to any histopathological parameter or to serum metastasin mRNA level. In contrast, extracellular metastasin protein has been shown to have an angiogenic and motility stimulating effect.[30] The blood vessel network of S100-positive tumours is more pronounced than in S100-negative tumours. This discrepancy could be due simply to functional difference between serum metastasin mRNA and the extracellular metastasin oligomeric form of the protein, which modulates angiogenesis via inhibition of the thrombospodin gene and interaction with the annexin II receptor.[31,32]

Non-significant correlation was observed between hormone receptors and serum metastasin in the present study, while a non-significant or only weakly significant correlation has been reported by other workers;[33,34] however, an inverse relationship was detected between S100A4 mRNA and oestrogen receptor level.[35]

Similar non-significant correlation was observed between tumour grade and serum metastasin. In contrast, a significant correlation between S100A4 protein and histological grade was detected in a study of 62 breast carcinomas.[36] This discrepancy between the results of the present study and those of other studies might be explained by differences in sample size.

Extracellular S100A4 protein level has been shown to increase in aged mice;[37] however, no comments on age were reported in patients with colorectal cancer.[38] No significant correlation between age and serum metastasin mRNA level was detected in the present study, perhaps because only four patients were aged over 60 years. The remainder of the patients ($n=16$) were in the 35–58 age range.

A significant relationship with prognosis was detected in the present study. Therefore, high serum metastasin mRNA levels may prove to be an indicator of poor prognosis in breast cancer patients. The same poor prognosis was demonstrated for survival of S100A4 patients in other studies.[17,39]

Patients who were serum metastasin mRNA-positive developed distant metastases in the lung, bone and liver, while patients who were serum metastasin mRNA-negative developed local recurrence. Similar results have been reported previously,[24] where the level of S100A4 mRNA proved to be predictive of the time of distant metastasis but not local metastasis. Biochemical and cell-based studies have demonstrated the involvement of metastasin in remodelling of the extracellular matrix[40] and cellular motility,[41,42] which are two steps in the metastatic cascade.

In conclusion, the present study shows that analysis of metastasin in serum serves as an important prognostic marker in breast carcinoma and may identify patients who are at high risk of metastasis and/or death. □

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