Increased levels of SPARC (osteonectin) in human breast cancer tissues and its association with clinical outcomes

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Abstract

SPARC (secreted protein acidic and rich in cystein), also known as osteonectin and BM40, is a 32 kDa secreted glycoprotein that interacts with extracellular matrix (ECM) proteins to promote adhesion of cells from the matrix, thereby inducing a biological state conducive to cell migration. SPARC is also thought to play an important role in tissue remodelling, angiogenesis, embryonic development and tumourigenesis. The current study set out to examine both the transcript levels of SPARC and the presence of the molecule in breast cancer tissue and to demonstrate if a link existed between the levels of SPARC and the clinical outcome.

Breast tumour tissues ($n = 120$) and non-neoplastic mammary tissues ($n = 32$) were studied. Protein levels of SPARC were assessed using immunohistochemistry. Transcript levels of SPARC were analysed using RT-PCR. The levels were correlated with nodal status, grade, prognosis and long-term survival (10 years).

Transcript levels of SPARC were found to be significantly higher in tumour tissue when compared to normal background breast tissue. This fact was mirrored when comparing levels of SPARC in ductal tumours with levels in lobular and other types of tumour. A high level of SPARC was also found in Grades 3 and TNM2 and TNM4 tumours. Node-positive tumours also exhibited higher levels of SPARC than node-negative tumours. It was also noted that SPARC was present in high levels in NPI 2 and NPI 3 tumours. Over a 6 year follow-up, high levels of SPARC was seen to be significantly associated with the overall survival of the patients ($P = 0.0198$). However, there was no significant correlation with disease-free survival.

It is concluded that SPARC plays a crucial role in tumour development in breast cancer and as such has a significant bearing on patient prognosis and long-term survival.

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1. Introduction

SPARC (secreted Protein Acidic and Rich in Cystein), also known as osteonectin and BM40, is a secreted glycoprotein that has the ability to bind to a number of proteins of the extracellular matrix (ECM) and by doing so modulates growth factor efficacy, tissue remodelling, angiogenesis and embryonic development. SPARC has been seen to regulate morphogenesis and cellular differentiation by means of modulating cell matrix interventions [1,2]. The molecule has been found to be present in high levels in fibrocytes and endothelial cells which are involved in tissue repair.

The potential impact of SPARC on cancer cells has also inspired a number of clinical studies on clinical cancer. It has been found in many types of malignant tumour cells, especially those of the gastrointestinal tract, breast, lung, kidney, adrenal cortex and brain.

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SPARC has also been implicated in the development of cataracts and in the regulation of angiogenesis during the wound healing process [3]. SPARC/osteonectin is also found in the bone matrix and in blood platelets that form megakaryocytes [4]. Sato et al. [5] have demonstrated that SPARC is found in high levels in pancreatic cancer cells, whilst Yamashita [6] has also demonstrated this phenomenon in relation to oesophageal cancer cells. Golembieski [7] and Kempel [8] confirmed that SPARC is present in high levels in neurogliomas and melanomas, respectively. Yamanaka et al. [9] demonstrated that SPARC is implicated in Grade 2 and 3 bladder cancers. Sakai [10] has implicated the raised levels of SPARC found in Renal Cell Carcinomas with subsequent tumour development and neovascularisation.

Whilst Sakai [10] has implicated the raised levels of SPARC in neurogliomas and melanomas, respectively. Kempel [8] confirmed that SPARC was evident in high levels in pancreatic cancer cells, whilst Yamashita [6] have demonstrated that SPARC is found in high levels in neurogliomas and melanomas, respectively. Despite the progress in the past decade, there is little information on the role of SPARC in human breast cancer. In particular, there has been little information on the relationship between SPARC and a long-term clinical outcome.

This study set out to determine the level of SPARC in human breast cancer tissue and to ascertain the implications this might have on patient prognosis. We report here that high levels of SPARC in human breast cancer are associated with the aggressive nature of breast cancer and, most interestingly, high levels of the molecule are associated with a poor clinical outcome and shorter long-term survival.

2. Materials and methods

2.1. Materials

Breast cancer tissues (n = 120) and normal background tissues (n = 32) were collected immediately after surgery and stored in deep freezer until use. Patients were routinely followed after surgery. The median follow-up period was 72 months. The presence of tumour cells in the collected tissues was verified by examination of frozen sections using H & E staining, by a consultant pathologist (ADJ), with details previously given [13].

RNA extraction kit and RT kit were obtained from AbGene Ltd., Surrey, England, UK. PCR primers were designed using Beacon Designer (California, USA) and synthesised by Invitrogen Ltd. (Pasley, Scotland, UK). Molecular biology grade agarose and DNA ladder were from Invitrogen. Mastermix for routine PCR and quantitative PCR was from AbGene.

Mouse monoclonal anti-human SPARC and anti-actin antibodies were purchased from Haematologic Technologies Inc. (Essex, VT, USA) and Santa-Cruz Biotechnologies Ltd (Santa Cruz, California, USA), respectively.

2.2. Tissue processing, RNA extraction and cDNA synthesis

Frozen sections of tissues were cut at a thickness of 5–10 μm and were kept for immunohistochemistry and routine histology [13,14]. A further 15–20 sections were homogenised using a hand-held homogeniser in ice-cold RNA extraction solution. The concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was carried using a RT kit with an anchored oligo-dt primer supplied by AbGene, using 1 μg total RNA in 96-well plate. The quality of cDNA was verified using β-actin primers.

2.3. Quantitative analysis of CCN family members

The transcript level of the CCN family members from the above-prepared cDNA was determined using a real-time quantitative PCR, based on the Amplifluor™ technology [15], modified from a method previously reported [16,17]. Briefly, a pair of PCR primers were designed using the Beacon Designer software (version 2, California, USA). To one of the primer (routinely to the antisense primer in our laboratory), an additional sequence, known as the Z sequence (5′-actgaacctgaccgtaca-3′), which is complementary to the universal Z probe [15] (Intergen Inc., England, UK), was added. A Taqman detection kit for β-actin was purchased from Perkin-Elmer. The primers used were SPARCF 5′-aggcgtggtacctt3′, SPARC 5′-aagacctgcgtatcctt-3′ and SPARCZR 5′-actgaacctgcgtacagatcactgtagctgaa-3′. β-actin forward and reverse primers were 5′-atgatatcgccgegtgcatc-3′ and 5′-gtcggtggtgacact-3′. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence, 10 pmol of FAM-tagged probe (Intergen Inc.), and cDNA from approximate 50 ng RNA (calculated from the starting RNA in the RT reaction) [18]. The reaction was carried out using IcyclerIQ™ (Bio-Rad) which is equipped with an optic unit that allows real-time detection of 96 reactions, using the following condition: 94 °C for 12 min, 50 cycles of 94 °C for 15 s, 55 °C for 40 s and 72 °C for 20 s [16,17]. The levels of the transcripts were generated from an internal standard [13] that was simultaneously amplified with the samples, and are shown here in two ways: levels of transcripts based on equal amounts of RNA, or as a target/CK19 ratio.
2.4. Immunohistochemistry

Frozen sections of breast tissue were cut at 6 μm thickness on a cryostat. The sections were subsequently air dried and fixed in a mixture of 50% alcohol and 50% acetone for 15 min. They were then allowed to air dry before storing at −20°C. When required, the sections were rehydrated for 5 min in Optimax wash buffer (Biogenex). Following rehydration, sections were treated for 20 min with horse serum diluted in wash buffer prior to the application of the primary anti-SPARC antibody. After a 1-h incubation, the primary antibody was drained off and the sections washed thoroughly in wash buffer before application of the biotinylated secondary antibody (Vector Labs) and incubation for a further 30 min. The sections were then once again thoroughly washed before Avidin Biotin Complex was administered for a 30 min incubation period. The ABC complex was then drained off and the sections washed extensively in buffer before the application of the chromogen DAB for 5 min. The sections were then washed for 10 min in running tap water before a nuclear counterstain (Gill’s Haematoxylin) was applied for 1 min. This was followed by a further 10 min wash in tap water before the sections were dehydrated in ascending grades of alcohol, cleared in xylene and mounted under a cover slip using clear mounting medium.

3. Results

3.1. SPARC protein is widely stained in mammary tissues

We first examined the distribution of SPARC protein in mammary tissues. As shown in Fig. 1 (left panel), mammary epithelial cells and stromal cells stained positive for SPARC. There was a very low level of staining of SPARC in tissue stroma. In breast cancer tissues, however, the staining of SPARC was substantially increased (Fig. 1 right panel). The increased staining was primarily seen in cancer cells and in matrix.

3.2. Expression of SPARC mRNA in breast tumours

We went on to analyse the level of expression of SPARC mRNA in mammary tissues, using quantitative PCR. As shown in Fig. 2, breast tumours had a significantly increased level of SPARC transcript \( (P = 0.04) \).

When the breast tumours were divided into node-positive and node-negative tumours, it was shown that node-positive tumours had higher levels of SPARC than the node-negative tumours (Fig. 3A); however, this difference is not statistically significant \( (P = 0.08) \).

The levels of SPARC were also high in Grade 3 tumours and in ductal carcinoma (compared with lobular carcinoma) (Table 1).

Fig. 1. Immunohistochemical staining of SPARC in mammary tissues. Left: normal tissue; middle: DCIS; and right: invasive breast cancer.
3.3. Levels of SPARC is associated with prognosis

In the current study, we used two parameters to assess prognosis at the time of surgery: the Nottingham prognostic index (NPI) and the clinical outcome. As shown in Fig. 3B, patients had stepwise increase in the levels of SPARC from good, moderate to poor prognosis. This trend of increase was proved to be significant ($P < 0.01$, Kruskal–Wallis test).

Over the 6 year follow-up, patients were divided into those who remained disease free, who developed metastasis, with local recurrence, or who died of breast cancer. As shown in Fig. 4, patients who died with breast cancer as the cause of death had significantly higher level of SPARC ($P = 0.04$).

3.4. SPARC transcript is associated with overall survival

The relationship between SPARC transcript and survival was analysed using Kaplan–Meier survival analysis and the Cox Proportion analysis. As shown in Fig. 5, High levels of SPARC were significantly correlated with a poor overall survival ($P = 0.0198$). Although high levels were also linked to a poor disease-free survival, this is yet to reach statistical significance ($P = 0.12$).

4. Discussion

Our study reports that SPARC RNA was found in significantly higher levels in tumour cells when compared with normal background breast tissue. Furthermore, the level of SPARC RNA was found to be significantly higher in ductal tumours when compared with other tumour types. This fact was mirrored when we compared SPARC levels in Grade 3 tumours and TNM2 and TNM4 tumours. This study has also demonstrated that SPARC levels are higher in node-positive than node-negative tumours and this is also true when comparing NPI2 and NPI3 tumours.

Yamashita [6] has also demonstrated that high levels of SPARC mRNA is associated with lymph node metastasis and poor prognosis. Research into bladder cancer [9] has also determined that SPARC is found in high levels in Grade 2 and Grade 3 bladder cancers when compared with Grade 1 cancers. These high levels of SPARC were also associated with highly invasive tumours and subsequent poor prognosis. Similarly, Thomas [20], demonstrated that SPARC is found in high levels in prostate cancer metastases with a conversely low level in normal prostate tissue. Other
workers [19] have also postulated that high levels of SPARC play a significant and important role in stimulating mobility and invasive behaviour in breast cancer cells. In a similar vein it has been shown [21] that SPARC, and specifically its NH2 terminal, is a contributory factor in the protein pathway involved with tumour invasion. The current study has therefore provided further evidence that SPARC is indeed linked to the prognosis and aggressiveness of human breast cancer.

However, despite these observations on the pivotal role of SPARC in human cancer, there are controversial reports to show that SPARC may have a different role to play in breast cancer cells. For example, SPARC has been indicated to be potentially inhibitory to breast cancer cells [3]. In ovarian cancer cells, over expression

<table>
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<th>Grade 3</th>
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<tr>
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<td>$P &gt; 0.05$ vs. ductal</td>
<td>$P &gt; 0.05$ vs. ductal</td>
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of SPARC results in cells with increased apoptosis [22,23]. These controversies may rise as a result of different cell lines, different expression system used (i.e. retroviral vs. bacterial system), and different ways the tested were conducted. However, the majority of the studies, in vitro and clinical studies including the current study, have strongly indicated SPARC as a molecule that contributes to the aggressive nature of breast cancer cells and clinical breast tumours.

The other striking observation from the current study is that SPARC is an independent prognostic factor and is linked to long-term overall survival. As already shown in Fig. 5, patients with high levels of SPARC had a significantly shorter overall survival, and marginal disease-free survival. This significance is independently achieved with any other factors. This has strongly pointed to SPARC as a promising prognostic marker in human breast cancer. Furthermore, the study has indicated SPARC as being a potential therapeutic target in cancer therapy. In an accompanying paper, we have reported that an anti-cancer polyunsaturated fatty acid, namely gamma linolenic acid (GLA), is able to inhibit the secretion and expression of SPARC in breast cancer cells.

References


