Research article

G Protein Coupled Receptor-Associated Sorting Protein 1 (GASP-1) Granule as a Cancer Invasion and Progression Biomarker

Frank N Chang*, George P Tuszynski

PROPLEX Technologies, LLC, USA

*Corresponding author: Dr. Frank Chang, PROPLEX Technologies, LLC. 1412 Candlebrook Dr. Dresher, PA 19025, USA, Tel: +01-215-704-2243; E-mail: fnchang11@gmail.com

Abstract

We have previously identified that G-protein coupled receptor-associated sorting protein 1 (GASP-1) is required for breast cancer progression. We now report that GASP-1 granules are involved in cancer invasion and progression. Immunohistochemical analysis of biopsy samples from normal and various stages of breast cancer progression including hyperplastic lesions, benign tumors, malignant cancers, and metastatic cancers revealed that GASP-1 was minimally stained in the cytosol of normal tissues but staining intensity progressively increased as abnormal (tumor) cells appear and advance to metastatic cancers. The overexpressed GASP-1 in the cytosol are present in three different granular sizes: powdery, fine and coarse granules. GASP-1 appears to act as a proliferation factor for cancer progression. Overexpressed cytosolic GASP-1 in DCIS are predominantly present in powdery granular form; production of more GASP-1 and increased granular sizes lead to the invasion of tumors to surrounding tissues. In malignant cancer, the GASP-1 granules are predominantly in fine granular form and many are attached to cancer cell membranes. Continuous production of GASP-1 leads to further increase in granular size to coarse granules, and their attachment to cell membrane are characteristics of metastatic cancer. Therefore, overexpression of GASP-1 and production of GASP-1 granules are required for both cancer invasion and cancer metastasis. To semi-quantify the extent of GASP-1 overexpression during cancer progression, we have developed a new tumor staging system, the H-score system. We found a 10-fold increase in GASP-1 expression when normal cells reach the DCIS (benign tumor) stage and a 23-fold difference in GASP-1 expression between normal and metastatic cancers. GASP-1 ELISA using 60 breast cancer patients and 31 normal individuals revealed that GASP-1 levels were 13-fold higher in cancer patients as compared to normal individuals. Sera from patients with stage IV metastatic breast cancer contained 30-fold more GASP-1 as compared to normal and inflammatory breast cancer. These data provide a strong rationale that GASP-1 is a biomarker for breast cancer invasion and progression.

Keywords: GASP-1 granules, cancer invasion, cancer metastasis, breast cancer biomarker, serum breast cancer marker

Introduction

Cancer initiation starts after DNA in normal cell is mutated. The cell is then subjected to the effects of promoters which promote the proliferation of the cell. The growth of abnormal cells (or tumor cells) is a stepwise progression including hyperplasia in which the altered cells divide in an uncontrolled manner leading to an excess of cells in that region of the tissue [1]. Additional changes make the cells and tissues appear even more abnormal. When they reach the ductal carcinoma in situ (DCIS) stage, the cells are now spread over a larger area but they are still contained within the initial location with well-defined borders and have not yet crossed the basal lamina to invade other tissues [1]. In breast cancer staging system, DCIS is considered a benign tumor stage or Stage 0 [2].

Cells inside the tumor are usually tethered to a structur-al support system known as the extracellular matrix or ECM [3]. In order for tumor to become malignant, the tumor cells have to break away from the tissues. Proteins such as integrins located on cell surfaces form the anchors that hold the cells in place [4]. Once tumor cells acquire the ability to penetrate the surrounding tissues, the process of invasion is initiated as the motile tumor cells pass through the basement membrane and extracellular matrix, progressing to intravasation as they penetrate the lymphatic or vascular circulation [5]. The cells then journey through the circulatory system, invading the vascular basement membrane and extracellular matrix in the process of extravasation [5].Ultimately, to complete the metastatic process, these cells will attach at a new location and proliferate to produce the secondary tumor.

There has been considerable effort to identify novel

cancer biomarkers that might offer clinical utility. Despite all efforts, researchers have not delivered validated biochemical markers that can be used to optimally diagnose and manage breast cancer [6]. Only four of the Food and Drug Administration (FDA)-approved markers (CA 15-3, CA 27.29, HER−2/neu, and circulating tumor cells analysis of EpCAM, CD45, CK8, 18, and 19) can be measured and assessed longitudinally [6]. In the case of CA 15-3, the levels are higher than normal in most women with breast cancer that has spread to other parts of the body (metastatic breast cancer) [7]. However, not all types of breast cancer will cause CA 15-3 levels to rise, as some types of cancer cells do not overproduce the antigen [8]. CA15-3 is also not measured for early stage breast cancer because the levels of this protein are rarely higher than normal at this stage [8]. Currently, there are no FDA approved biomarkers for breast cancer diagnosis or screening.

Failure of protein markers in detecting early cancer coupled with recent advances in DNA sequencing led to the development of liquid biopsy for detecting cancer. Liquid biopsy analyzes circulating tumor DNA (ctDNA). The basis of this approach is that genetic mutations drive the growth of cancer cells, and dying cells shed some of both mutated and normal DNA into the blood. Enormous resources were invested in the hunt for single mutations, which has evolved into a hugely popular medical effort known as "precision oncology" [9]. The idea is to sequence tumors, identify the key mutations responsible for the uncontrolled growth of cells and block their action with a specific drug [10]. However, among the more common tumors tested, "actionable" mutations addressable by existing drugs were found only in 15% of cases [10]. A bigger disappointment is that even pairing a mutation to a drug did not guarantee results—only a third of the matched patients responded to the treatment, and half of those responses faded within six months [10,11]. The failure could be partially attributable to difficulty in finding so-called "driver mutations" -those that play an important role in the cancer's biology [12]. They may instead be "passenger" mutations, that is, changes that accompany the development of cancer but do not control its growth.

To improve the sensitivity of detection, a combination of analyzing ctDNA with 8 or more protein biomarkers including CA15-3 has also been introduced by Johns Hopkins as "CancerSeek" [13]. Although these procedures are effective in detecting late stage cancers, they have low sensitivity and can only detect early cancer (Stage 1) about 43% of the time. Due to inability of current proteomic and liquid biopsy approach mentioned above, it is of importance to search for a biomarker that can detect early cancer and also be used for monitoring cancer progression.

Previously, we have identified a protein cancer biomarker, G-protein coupled receptor-associated sorting protein 1 (GASP-1), which is required for breast cancer progression [14]. In knockdown experiments using triple negative breast cancer cells we found that cells expressing low levels of GASP-1 grew much more slowly than wildtype cells. In fact, when GASP-1 is completely downregulated, the cells die and cannot be propagated [15]. To further demonstrate that GASP-1 overexpression is required for cancer progression, we analyzed biopsy tissue samples from normal and four different stages of breast cancer ranging from hyperplasia, benign, malignant to highly metastatic

cancer via immunohistochemistry (IHC) by staining with an anti-GASP-1 polyclonal antibody.

We have now shown that GASP-1 overexpression starts when abnormal (tumor) cells first appeared and GASP-1 appears to guide the tumor cells to reach the hyperplasia stage. More GASP-1 is produced when hyperplasia stage is advanced to become DCIS. The overexpressed GASP-1 in the cytosol are present in three different granular sizes: powdery, fine and coarse granules. In DCIS, the GASP-1 are predominantly present in a powdery granular form. Continuing overexpression of GASP-1 leads to aggregation of GASP-1 powdery granules to become fine granules. Attachment of many fine GASP-1 granules to tumor cell membrane is apparently associated with DCIS becoming malignant cancer. In metastatic cancer, GASP-1 is even more highly overexpressed and is present mostly in aggregated (coarse) granular form. Because GASP-1 is required for cancer invasion and currently there is no method to assess the likelihood of DCIS becoming invasive cancer, we suggest GASP-1 granules as a valid cancer invasion biomarker. Unable to assess the likelihood of tumor becoming malignant is a cause for over-treatment of tumors when they are still in benign stage and not becoming malignant. To semi-quantify the extent of GASP-1 overexpression during cancer progression, we have developed a refined tumor grading system (the H-score system). Since cancer patient serum samples are readily available, an ELISA assay was developed to quantify serum GASP-1 levels in patient sera. In two studies totaling 60 breast cancer patients and 31 normal individuals, we can clearly differentiate normal individuals from cancer patients with 100% sensitivity and 100% specificity.

Materials and Methods
Antibody production

Anti-GASP-1 polyclonal antibodies against EEASPEAVAGVGFESK were produced by ABclonal Science, Inc (Woburn, MA).

Tissue staining

Anti-GASP-1 antibody was used in immunohistochemical (IHC) staining to detect GASP-1 in formalin-fixed, paraffin-embedded (FFPE) tumor tissues. In brief, tissue staining was performed by QualTek Molecular Laboratories (Newtown PA) according to standard procedures using optimized antibody concentration and antigen retrieval and the appropriate negative controls.

Using polyclonal antibody against a 16 amino acid residue, EEASPEAVAGVGFESK, we discovered that GASP-1 is very highly overexpressed in many cancers. The same results were obtained using four different GASP-1 antibodies against other regions of the GASP-1 protein. We found that the overexpressed GASP-1 in cytoplasm appears in three different granular sizes: powdery, fine, and coarse granules. A new IHC scoring system (called the "H-score") was developed by Dr. Richard Siderits at QualTek Molecular Laboratories (Newtown PA) to quantify the GASP-1 granules at various stages of cancers. QualTek also carried out the IHC analysis of cancer biopsy samples from both in-house and samples purchased from US Biomax (Rockville, MD). Currently, cancer progression is assessed by either cancer grading (Grade 1 to 3) or cancer staging (Stage I to

IV) system. These methods use morphological changes, size of tumor, and whether or not tumor has spread. As will be shown later, the expression of GASP-1 is correlated with cancer progression and the biochemically based H-score system is much more refined than the two systems mentioned above.

The GASP-1 granule quantification requires recording the percentage of cells (tumor or normal) with GASP-1 cytoplasmic staining at a corresponding differential intensity on a four-point scale semi-quantitatively (0, 1+, 2+, 3+). Cytoplasmic GASP-1 intensity is estimated by the pathologist using a scale from 0-3+ as follows:

0: No perceptible staining or no granules in cytoplasm (none)
1+: No granules observed at 20x, blush, or faintly-perceptible powdery granule at 40x (weak)
2+: Fine-size granular pattern and easily perceptible intensity (moderate)
3+: Dense and dark cytoplasmic coarse granules (strong)

The H-Score is calculated by summing the percentage of cells with intensity of expression (brown staining) multiplied by their corresponding differential intensity on scale from 0-3+. Scores range from 0 to 300.

H-Score = [(% at <1) x 0] + [(% at 1+) x 1] + [(% at 2+) x 2] + [(% at 3+) x 3]

An example of GASP-1 powdery, fine and coarse granules is shown in Figure 1.

Cytoplasmic Staining at 1+   Cytoplasmic Staining at 2+   Cytoplasmic Staining at 3+
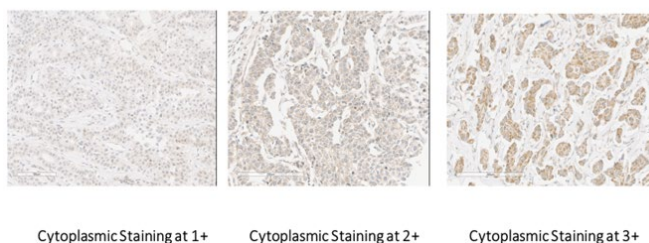
Figure 1. Examples of GASP-1 granules with cytoplasmic staining of 1+, 2+ and 3+.

GASP-1 ELISA

The ELISA procedure involves a conjugate of BSA (bovine serum albumin) and a GASP-1 peptide (BSA-GASP-1 conjugate) to coat a single plate to retain the GASP-1 peptide before detecting GASP-1 in a serum sample in a competitive assay. The sequence of the GASP-1 peptide is EEASPEAVAGVG-FESK. The serum samples were purchased from Discovery Life Sciences, Los Osos, CA.

Specifically, an ELISA plate having multiple wells was coated with 100 µl of a solution containing the BSA-GASP-1conjugate either overnight (e.g., 8-12 h) with shaking or for 2 h at room temperature. This was followed by the addition of 300 µl of 1% BSA solution and incubation for 20 min to block sites on the plate not covered by the BSA-GASP-1 conjugate. The solution was aspirated and washed once with Tris (tris-hydroxymethyl aminomethane)-buffered saline solution containing 0.05% Tween 20 (TBST). 50 µl of a diluted serum sample (or a standard GASP-1 peptide solution for standard curve construction) was

added to wells of the plate. This was followed by the addition of 50 µl of 1:8,000 biotinylated anti-GASP-1 antibody to the wells and incubation for 2 h at room temperature with shaking. The final dilution in the well was 1:16,000 for the antibody and 1:8 for the sample in a volume of 100 µl. The plate was then aspirated and washed 3 times with 300 µl of TBS-Tween buffer (TBST). The TBST solution was then aspirated before 100 µl of diluted Streptavidin-HRP was added and incubated for 30 min to one hour at room temperature. The mixture was then aspirated and the plate was washed 3 times with 300 µl of TBST buffer. The TBST buffer was then aspirated before 100 µl of TMB (tetramethylbenzidine) solution was added and incubated with shaking for 10 to 30 min (stop the reaction if the control well was highly blue) for color development. 50 µl of 1 N sulfuric acid was added to each well to stop the color development. The absorbance of wells was read at 450 nm and used to calculate the concentration of the GASP-1. Ideally, the absorbance reading for a control well (without a competing peptide) should be around 1.5. Slightly higher or lower readings are acceptable.

Results

Previously, we have identified a cancer biomarker, G-protein coupled receptor-associated sorting protein 1 (GASP-1), that is involved in breast cancer progression [14, 15]. To further demonstrate that GASP-1 overexpression is required for cancer progression, we have now analyzed biopsy tissue samples from normal and four different stages of breast cancers from hyperplasia, benign, malignant to highly metastatic cancer via immunohistochemistry (IHC) staining with an anti-GASP-1 polyclonal antibody. Additionally, we show that GASP-1 is a highly predictive and sensitive breast cancer serum marker.

GASP-1 is involved in the transformation of a normal cell to a tumor cell

Figure 2 shows that no detectable GASP-1 staining is observed in the luminal epithelial cells of normal breast tissue (left panel). GASP-1 is known to regulate the availability of a number of G protein coupled receptors [16]. While the normal ductal breast cells are pretty much devoid of staining, we cannot rule out the possibility that some very low-level GASP-1 synthesis below the threshold of detection may be occurring. The fact that normal cells do not pick up stain or are only minimally stained provides a clean background for the appearance of cytoplasmic GASP-1 during cancer progression. The H-scoring system was used to quantify the expression of GASP-1. The normal cell has a H-number at or close to 0. However, when normal cells are transformed into abnormal tumor cells, GASP-1 starts to appear in the cytosol of the luminal epithelial cells (Figure 2, right panel). The cells are abnormal because the shape of their nuclei is different from that of normal cells and becomes much more elongated. It is not known what causes the overexpression of GASP-1 or whether GASP-1 initiates this transformation process. GASP-1 apparently is synthesized on endoplasmic reticulum as evidenced by the appearance of nuclear membrane staining in the new tumor cells. Because GASP-1 is subsequently released to cytoplasm, the appearance of nuclear membrane staining is transient. Cytoplasmic GASP-1 expression, on the other hand, can be used for monitoring tumor cell progression.

Based on GASP-1 expression, we can clearly identify tumor cells amongst normal cells at the earliest stage of cancer (Figure 2, right panel). Also, GASP-1 appears to form an invasion front that helps guide the production of more layers of tumor cells toward the lumen leading tumor to the hyperplasia stage (Figure 2, right panel). Essentially, at this stage, tumor cell progression has started even though they could still be morphologically considered as "normal" under current tumor grading systems. It should be pointed out that at early stage cancer, cytoplasmic GASP-1 appears as powdery granular form and the H-number is low.


Normal   Hyperplasia
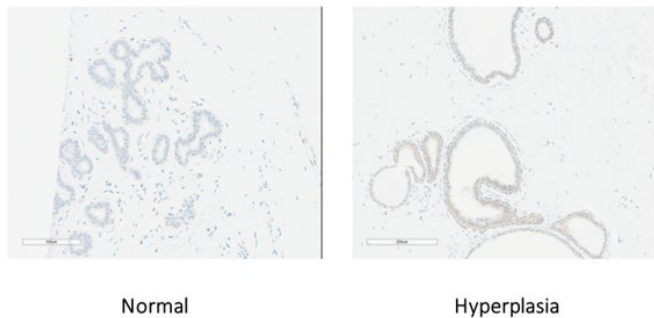
Figure 2. Expression of GASP-1 in normal individual (left panel, H=0) and early hyperplastic patient (right panel, H=20).

Involvement of GASP-1 in breast cancer invasion

Continuing production of GASP-1 and expansion of tumor cells eventually leads to ductal carcinoma in situ (DCIS) stage. Figure 3 shows that in DCIS, GASP-1 stays as powdery granular form in the cytosol and appears to be rather homogeneous (Figure 3, left panel). Due to the presence of powdery GASP-1 granules, the H-number is increased to around 100. We found that the cancer invasion process appears to be triggered by a substantial further increase in GASP-1 production in DCIS which appears to cause the aggregation powdery GASP-1 granules to become fine granules. As in the case of tumor cell expansion during hyperplasia leading to DCIS, GASP-1 appears to act as a proliferation factor in tumor cell expansion. Consequently, the size of tumor becomes bigger, takes up more space and creates pressure on surrounding tissues. When this happens, tumor cells begin to bulge and form invasive fronts such as tumor buds (Figure 3, right panel).

Figure 4 (left panel) shows that at an early cancer invasion stage, in addition to an increase in GASP-1 sizes from powdery to fine granules, many of the fine GASP-1 granules start to attach to cell membranes. Attachment of large number of GASP-1 granules to cancer cell membrane is apparently critical to the cancer invasion process because it could cause distortion of cell membrane structure and weaken cell-cell interactions that hold the tumor cells together. Figure 4 (right panel) also shows that as cancer invasion process advances further, more GASP-1 granules are produced resulting in an increase in the H-numbers. Attachment of many fine GASP-1 granules to cell membranes (some with more than 20 granules attached) would further disrupt cell-cell interactions and may have caused tumor to release invasive clusters of varying sizes. GASP-1 granules are also abundantly present inside the cancer cells. It is interesting to note that some invasive clusters appear to contain pointed edges although we cannot rule out that some of them were caused by processing of

biopsy tissue samples. Invasive clusters with sharper edges have been reported and they may facilitate the malignant process [17], although invasive clusters enriched in GASP-1 granules have not been documented before.

DCIS   Early Invasive Cancer
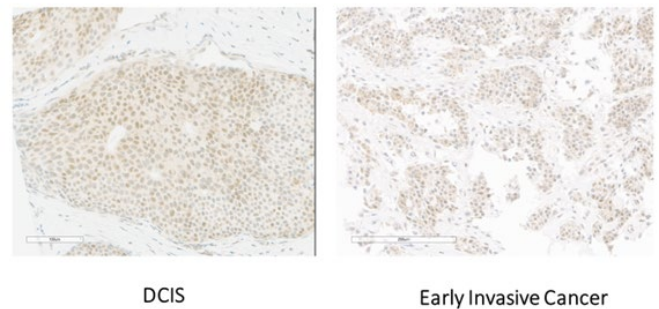
Figure 3. Presence of GASP-1 powdery granules in DCIS (left panel, H=100) and in early stage invasive cancer (right panel, H=110).

Invasive   Late Invasive
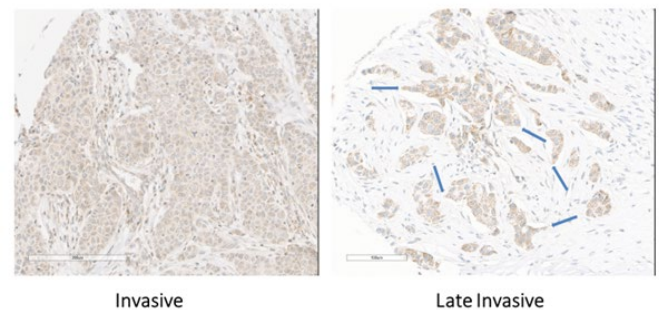
Figure 4. Example of invasive breast cancer with fine GASP-1 granules and their attachment to cell membrane (left panel, H=150). Appearance of invasive clusters in invasive breast cancer (right panel). Arrows indicate accumulation of GASP-1 granules at edges (H=180).

Increased GASP-1 granule size is associated with advance of invasive cancer to metastatic cancer

Figure 5 (left panel) shows that when cancer reaches the metastatic stage, GASP-1 continues to be produced and some of the fine GASP-1 granules have increased their size to become coarse granules, resulting in an increase of H-number to around 200 or higher. In many metastatic cancer biopsy samples we analyzed, we found that their cell membranes are loaded with GASP-1 granules with some membranes containing 40 or more granules. Attachment of so many GASP-1 granules to a cancer cell membrane could further weaken the cell-cell interactions causing cancer cells inside the invasive clusters to have even less affinity for neighboring cells and become more easily released. Additionally, some of the coarse granules are now found to attach to the nuclear membranes. We do not know the significance of this attachment except that it occurred in metastatic cancer and could be used as one of the indicators of metastatic cancer. Smaller invasive clusters of varying sizes and shapes are also produced. Some invasive clusters appear to have rather sharp tip-like structures (see arrows). It is possible that cells at the invasive front may have undergone epithelial mesenchymal transition (EMT) and attain a locomotor phenotype for conducting invasion [17]. At the invasive front/edge, tumor cells have also been

reported to be highly proliferative [17]. This could explain the reason for the presence of GASP-1 granules at the invasive front. For metastasis to occur, intravasation or penetration of the blood vessels appears to be one of the limiting steps. The invasive clusters with sharp GASP-1 granules at their tips could facilitate this important step. Once in circulation, these GASP-1 granules could more easily penetrate the blood vessel (extravasation) and enter the cells at secondary sites, followed by proliferation there to complete the metastatic process. We believe the accumulation of coarse GASP-1 granules and their attachment to cell membranes are important indicators of a metastatic cancer. An example of a highly metastatic cancer (H=300) is shown in Figure 5 (right panel). The cancer cells are covered with GASP-1 granules on their cell membranes and again, some nuclei have GASP-1 coarse granules concentrated on their nuclear membranes. Furthermore, attachment of so many granules to cell membranes appear to cause lysis of some cancer cells resulting in the release of GASP-1 granules into the surrounding environment and into the circulation. The released GASP-1 containing granules would be detected by our ELISA assay.
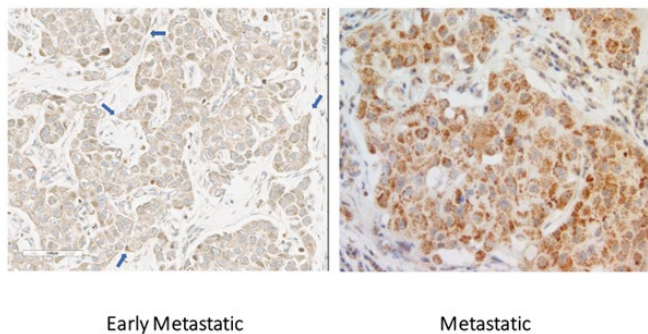
Figure 5. Binding of GASP-1 granules to cell membranes and nuclear membranes in early metastatic cancer (left panel, H=210) and metastatic cancer (right panel, H=300).

The above results demonstrated for the first time that overexpression of GASP-1 in DCIS appears to initiate the cancer invasion process. Aggregation of overexpressed GASP-1 results in at least three different granular sizes: powdery, fine and coarse granules. As cancer invasion progresses, more and more GASP-1 are produced and the size of GASP-1 granules also increased from powdery to fine and later to coarse granules. The size and number of GASP-1 granules attached to cancer cell membranes appear to correlate with cancer severity, with the cell membranes of metastatic cancers containing predominantly the coarse GASP-1 granules. Thus, GASP-1 IHC can be used to detect early cancer invasion, assess cancer severity and cancer metastasis. It can also be used to assess the likelihood of a tumor such as DCIS becoming malignant and/or metastatic.

H-scoring system for monitoring cancer progression

We have used the H-number system to monitor cancer progression from normal to benign, malignant, and metastatic cancer. As shown in Figure 6, the normal breast ductal cells have an averaged H-number of 9. Although technically, the number should be very close to or at 0, we found that some of the so-called normal biopsy samples we obtained already had tumor

cells in them. Hyperplastic lesions have an average H-number of 65 indicating that most of the overproduced GASP-1 are in a powdery granule form. Due to production of more powdery GASP-1 granules, the H-number increases slightly when the hyperplasia stage is advanced to the DCIS stage. In DCIS, the tumor cells are rather uniformly filled with the powdery GASP-1 granules with H-numbers close to or around 100.

Figure 6 also shows that there is about a 10-fold increase in GASP-1 expression when normal ductal cells progress to benign (DCIS) tumors, and a 23-fold difference between normal and metastatic cancers. An H-number of 100 appears to be a good cutoff point separating non-invasive (hyperplasia, benign and DCIS) tumors from malignant cancers. Currently, there is no method for assessing the likelihood of tumor cells becoming malignant. It would appear that H-numbers between 100 and 150 would be a good indicator for identifying early stage malignant cancers.

It is also interesting to note that we can divide the invasive cancer biopsy samples into two distinct groups, upper and lower groups, based on their H-numbers. The lower group consisting about 60% of total malignant biopsy samples, has H-numbers below 200 whereas the upper group has H-numbers above 200. For metastatic cancers (Figure 6, last column), they can also be divided into upper and lower groups using the same cutoff point except that two-thirds of the metastatic cancers are in the upper group with H-numbers over 200. The overlapping of H numbers between malignant and metastatic breast cancers could be due to deficiency in current breast cancer grading system which is based primarily on morphological changes rather than using a protein biomarker. Our GASP-1 H-scoring is more sensitive and could be used to detect early stage invasive cancer and early metastasis. Thus, in our H-number grading system, the invasive cancers with H-number above 200 may be considered to have started the metastatic process but have not been identified by current grading and detection systems.
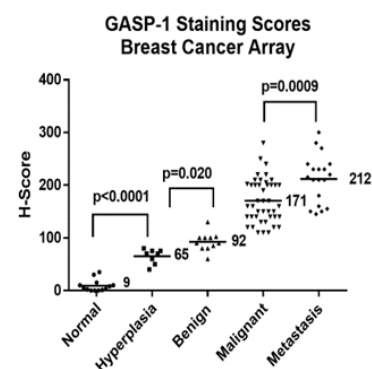
Figure 6. H-score numbers for different stages of breast cancer.

Development of GASP-1 ELISA

Although biopsy samples are readily available in breast cancer for conducting IHC analysis, we have also developed an ELISA assay to facilitate cancer detection. To be useful, new blood tests for cancer must have very high specificity; otherwise, too many healthy individuals will receive positive test results, leading to unnecessary follow-up procedures and anxiety. Ideally, for a cancer test to be of practical use, the sensitivity should be at about 90% or higher so that the false positive rate is less than

10%. Because some of the overexpressed GASP-1 and GASP-1 granules in cancer cells are likely to be released into circulation as GASP-1 containing exosomes, oncosomes, or other microvesicles, a highly sensitive competitive ELISA procedure was developed to quantify GASP-1 levels in serum samples.

In one test, we used this ELISA to quantity GASP-1 levels in serum samples from 18 symptom-free (normal) individuals and 37 breast cancer patients. As shown in Figure 7 (left panel), the ELISA has a detection limit of about 0.02 ng/ml. The average serum GASP-1 concentration for breast cancer patients was 3.5 ng/ml, which was about 13 times higher than the average serum GASP-1 concentration for normal individuals, which was only 0.27 ng/ml (Figure 7, right panel). Because there was a complete separation of serum GASP-1 levels between cancer patients and normal individuals, when a ROC (receiver operating characteristic) curve was constructed, the AUC (area under the curve) value was 1.0. An AUC value of 1.0 is a perfect number indicating 100% sensitivity and 100% specificity for the GASP-1 ELISA test. In a separate test involving 23 patient breast cancer patients and 13 normal individuals, we also found a clear separation between cancer patients and normal individuals (figure not shown). Again, an AUC analysis shows 100% sensitivity and 100% specificity for this analysis. Some normal individuals had

Figure 7. Standard curve for GASP-1 ELISA (left panel). Analysis of Sera from Normal individuals and Breast Cancer Patients (right panel).

Normal	Inflammatory Breast CA	Stage IV Invasive Breast CA
0.208	1.0	34
0.248	1.8	23

Figure 8. GASP-1 concentrations in serum samples from symptom-free (normal) individuals, patients with inflammatory breast cancer or patients with Stage IV invasive breast cancer.

very low serum GASP-1 levels and reached the detection limit of 0.02 ng/ml for the assay. As indicated earlier, the normal individuals do not overproduce GASP-1, therefore one would expect little or no GASP-1 to be released into circulation from them.

Figure 8 shows that metastatic invasive breast cancer (Stage 4) patients had extremely high GASP-1 levels, which are 90 times or more when compared to those from normal individuals. Interestingly, another form of breast cancer, inflammatory breast cancer, which appears to be caused by bacterial infection, does not overproduce GASP-1.

Discussion

About 20 to 30% of DCIS will eventually become invasive and metastatic. Even with extensive research and efforts, it is currently unknown as to what causes DCIS to become a malignant cancer and eventually to metastatic cancer. Metastasis is one of the leading causes of death globally. As the metastasis cascade progresses, the number of viable cancer cells which survive and successfully complete each metastasis stage decreases precipitously. Given the dynamic, multi-step nature of metastasis, it would appear that certain cancer cell subpopulations may perform some steps of metastasis much more efficiently than others [5]. Invasive clusters highly enriched in GASP-1 granules could be one of the subgroups.

We have identified a key step in transformation of DCIS into invasive cancer. We believe that continuous production of GASP-1 and the aggregation of powdery GASP-1 granules to fine GASP-1 granules may be one trigger for starting this cancer invasion process. Due to strong affinity of GASP-1 fine granules for cell membranes, the attachment of too many granules to cell membrane could disrupt and weaken cell-cell interactions. As a result, invasive clusters of varying sizes and cell numbers are released from the tumor (see Figures 4 and 5). The abundance and size of these granules can be correlated with tumor progression. A refined cancer grading system was used for analyzing cancer progression and invasiveness. The H-number system quantifies the amounts and size of cytoplasmic GASP-1 granules. In general, the color intensity of our H-number correlates with GASP-1 granular sizes, with H-number of about 100 for powdery GASP-1 granules, around 200 for fine GASP-1 granules, and reaching 300 for coarse GASP-1 granules. We found that when the H-number is at 100 or below, indicating that the tumors are expressing mostly powdery GASP-1 granules, the tumor is benign. Early invasive cancers have H-numbers between 100 and 150 caused by aggregation of some powdery GASP-1 granules into fine granules. As cancer progresses during the invasive stage, many more of fine GASP-1 granules are produced and are attached to cell membranes. This causes their H-numbers to increase to around 200. In highly metastatic cancer, many coarse GASP-1 granules are present and the H-number could reach a maximum number of 300. Therefore, the H-scoring system can be used to assess cancer severity.

In an era dominated by complex analysis of DNA mutations in cancer cells and so far achieved only limited success in detecting early cancers, it is refreshing that a single biomarker, GASP-1, is found to be associated with the transformation of a normal cell to a tumor cell and can be used for early cancer detection using well-established technologies such as IHC. Early
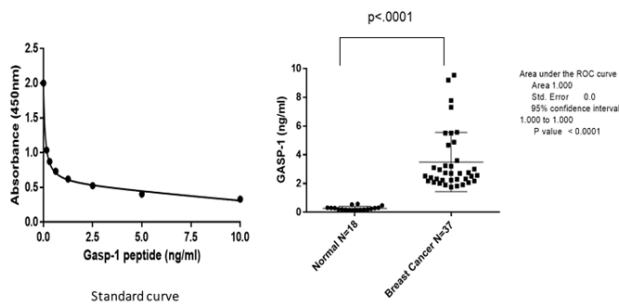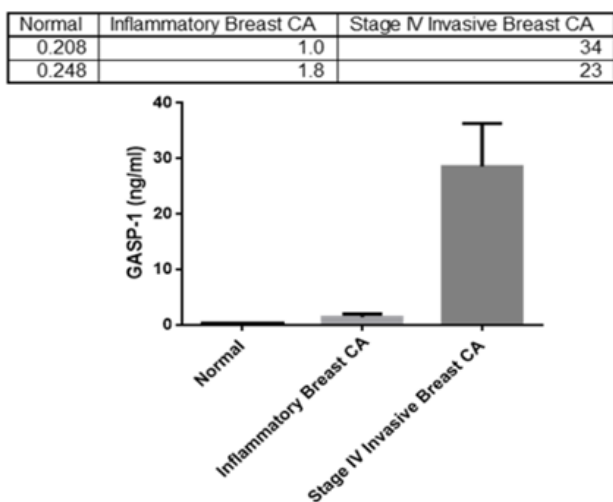
cancer detection is by far the most effective way to save lives, and the estimated cost-savings from early diagnosis could add up to over $26 billion a year, more than any other new approach can promise [10].

The use of GASP-1 as a cancer proliferation factor has not been reported before. We have also found that the B cells of germinal centers are actively producing GASP-1 and over-expression of GASP-1 may have contributed to their very high proliferation rate (unpublished observations). It would appear that cancer cells may have utilized the mechanism of increasing cell proliferation by overexpressing GASP-1. We postulate that by weakening the cell-cell interactions, GASP-1 could cause the release of cancer cells loaded with GASP-1 granules into circulation. Furthermore, it could also cause lysis of cancer cells resulting in the release of GASP-1 granules and free GASP-1 from inside the cancer cells for spreading cancer. The GASP-1 granules attached to membrane could also be released as onco-somes, microvesicles, or small membrane fragments containing GASP-1 granules. Attachment of coarse granules to nuclear membranes (in addition to cancer cell membranes) in metastat-ic cancer and their release into circulation after cell lysis could represent a new way for spreading cancer. The consequence of such release is that in metastatic cancer there will be numerous GASP-1 granules circulating in the blood. It is therefore possible that inactivation of circulatory GASP-1 granules, for example, by therapeutic agents such as monoclonal antibodies could rep-resent a new approach for treating cancers.

The failure in predicting which lesions will become in-vasive in breast cancer has led to DCIS being overtreated due to greater screening. The false-positive mammograms and overdi-agnosis create health-care expenditures estimated to approach $4 billion annually—and that much of this financial "burden" comes from treating DCIS specifically [10].

The absence of reliable biomarkers for predicting DCIS becoming invasive has led to the development of "multigene as-says" to obtain a gene-expression profile to identify the function of specific genes in cancer cells. Several tests are commercially available and one in particular, OncoType DX, samples 21 dif-ferent gene arrays to gauge risk of early stage, favorable progno-sis, tumors coming back later and spreading to other parts of the body ("distant recurrence"), and to help patients and their doctors decide if chemotherapy benefits will outweigh its risks [18, 19]. The test results produce a recurrence score that tells how likely cancer will come back. The score ranges from 0 to 100. If a re-currence score is 17 and under, the chance that cancer returns is low. A medium recurrence score is 18 to 30 indicating the chance that the cancer will return is in the medium range. The benefits of chemotherapy, however, are uncertain. The patient will have to discuss with the doctor to decide the course of action. A high recurrence score of 31 or over indicating the chance of cancer re-turning is somewhat high. Adding chemotherapy to cancer treat-ment may help keep the cancer from coming back. However, a high recurrence score does not mean that the cancer will definite-ly come back. For patients with OncoType DX numbers that fall between 18 and 30, or even a higher number, we suggest using our GASP-1 IHC to assess tumor severity and provide a second opinion in deciding whether chemotherapy is required. GASP-1 IHC can assess tumor severity at a much lower cost and can also

be used as a stand-alone test. Furthermore, we have found that GASP-1 is also highly over-expressed in all other cancers we studied including lung, pancreatic, ovarian, liver, colon, prostate, bladder, renal, stomach, melanoma and glioblastoma. Therefore, GASP-1 IHC can also be used to assess severity of these cancers as well. We believe that GASP-1 is a universal cancer biomarker required for proliferation of many, if not all, cancers.

An initiative to develop a "universal cancer test" which could detect dozens of different diseases before they become deadly was started in England in 2016 [20]. They hope such a test could detect cancer early, before it has spread, and when it can be cured quickly with surgery or drugs. Patients flagged by the test could then undergo procedures like imaging or endos-copies to identify the specific nature of their cancer. For the test to be effective, it would need to perform with AUC of around 0.95. An AUC of 0.95 would indicate sensitivity and specificity close to the 90 percent range. Few, if any, commercially avail-able proteomic tests offer this level of performance, even though they typically have far narrower uses than the pan-cancer ear-ly detection they are pursuing. With our GASP-1 ELISA assay having AUC values of over 0.95 for breast and other cancers, it would appear that GASP-1 is an ideal marker for developing such a universal cancer biomarker. Our GASP-1 ELISA can de-tect breast and many other cancers (unpublished observations) and can be used as a universal cancer biomarker they are looking for.

Conclusions

GASP-1 overexpression starts when abnormal (tumor) cells first appeared and GASP-1 appears to guide the tumor cells to reach the hyperplasia stage. More GASP-1 is produced when hyperplasia stage is advanced to become DCIS. The over-expressed GASP-1 in the cytosol is present in three different granular sizes: powdery, fine and coarse granules. In DCIS, the GASP-1 is predominantly present in a powdery granular form. Continuing overexpression of GASP-1 leads to aggregation of GASP-1 powdery granules to become fine granules. Attachment of many fine GASP-1 granules to tumor cell membrane is appar-ently associated with DCIS becoming malignant cancer. In met-astatic cancer, GASP-1 is even more highly overexpressed and is present mostly in coarse granular form. Because GASP-1 is required for cancer invasion and currently there is no method for assessing the likelihood of DCIS becoming invasive cancer, we suggest GASP-1 granules as a valid cancer invasion biomarker. To semi-quantify the extent of GASP-1 overexpression during cancer progression, we have developed a refined tumor grading system (the H-score system). A highly sensitive ELISA assay to quantify serum GASP-1 levels in patient sera was also devel-oped.

Consent for Publication

Not applicable.

Competing Interests

There are no financial disclosures or competing interests for this study.

References

1. Kietzman W, Riegel AT, Ory V. Early stage progression of breast cancer. Breast cancer, From Biology to Medicine. Edited by Phuc Van Pham. 2017; ISBN: 978-953-51-3000-0.

2. MacMillan A. 20 Things to Know About DCIS, or 'Stage 0' Breast Cancer. Health. 2017.

3. Walker C, Mojarea E, del Rio Hernandez A. Role of Extracellular Matrix in Development and Cancer Progression. Int J Mol Sci. 2018; 19: 3028-3046.

4. Khalil AA, Ahmad MR. integrins located on cell surfaces form the anchors that hold the cells in place. Int J Mol Sci. 2015; 16: 18149-18184.

5. Hapach LA, Mosier JA, Wang W, et al. Engineered models to parse apart the metastatic cascade. Npj Precision Oncol. 2019; 3: Article number: 20.

6. Sauter ER. Reliable Biomarkers to Identify New and Recurrent Cancer. Eur J Breast Health. 2017; 13: 162-167.

7. He ZY, Li, X, Chen QS, et al. Elevated serum carcinoembryonic antigen and CA15-3 levels and the risk of site-specific metastases in metastatic breast cancer. Transl Cancer Res. 2016; 5 No. 5.

8. Cancer antigen 15-3 (CA15-3) test - Canadian Cancer Society. www.cancer.ca.cancer antigen.15-3.

9. Volik S, Alcaide M, Morin RD, et al. Cell-free DNA (cfDNA): Clinical significance and utility in cancer shaped by emerging technologies. Mol Cancer Res. 2016; 14: 898-908.

10. Raza A. Cancer is still beating us- we need a new start. Cited in Wall Street J. 2019.

11. Raza A. The First Cell: And the Human Costs of Pursuing Cancer to the Last. 2019; ISBN-13: 978-1541699526.

12. McFarland CD, Yaglom JA, Wojtkowiak JW, et al. The Damaging Effect of Passenger Mutations on Cancer Progression. Cancer Res. 2017; 77: 4763-4772.

13. Cohen JD, Li L, Wang Y, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science. 2018; 359: 926-930.

14. Tuszynski GP, Rothman VL, Zheng X, et al. G-protein coupled receptor-associated sorting protein 1 (GASP-1), a potential biomarker in breast cancer. Exptl Mol Pathol. 2011; 91: 608-613.

15. Chang FN, Duong PT, Tuszynski GP. Serum markers associated with early and other stages of breast cancer. 2015; US 9,140,704.

16. Moser E, Kargl J, Whistler JL, et al. G Protein-Coupled Receptor-Associated Sorting Protein 1 Regulates the Postendocytic Sorting of Seven-Transmembrane-Spanning G Protein-Coupled Receptors. Pharmacology. 2010; 86: 22-29.

17. Gerashchenko TS, Novikov NM, Krakhmal NK, Zolotaryova SY, Zavyalova MV et al. Markers of Cancer Cell Invasion: Are They Good Enough? J. Clin. Med. 2019; 8:1092-1110.

18. McVeigh TP and Kerin MJ. Clinical use of the Oncotype DX genomic test to guide treatment decisions for patients with invasive breast cancer. Breast Cancer. 2017; 9: 393-400.

19. Wang SY, Chen T, Dang W, et al. Incorporating tumor characteristics to maximize 21-gene assay utility: A cost effectiveness analysis. J Natl Compr Canc. 2019; 17: 39-46.

20. Universal cancer test to pick up 95% of disease on horizon, www.telegraph.co.uk., 2016: July 30.

To cite this article: Chang FN, Tuszynski GP. G Protein Coupled Receptor-Associated Sorting Protein 1 (GASP-1) Granule as a Cancer Invasion and Progression Biomarker. British Journal of Cancer Research. 2020; 3:1.