Characterisation and protein expression profiling of annexins in colorectal cancer

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The annexins are family of calcium-regulated phospholipid-binding proteins with diverse roles in cell biology. Individual annexins have been implicated in tumour development and progression, and in this investigation a range of annexins have been studied in colorectal cancer. Annexins A1, A2, A4 and A11 were identified by comparative proteomic analysis to be overexpressed in colorectal cancer. Annexins A1, A2, A4 and A11 were further studied by immunohistochemistry with a colorectal cancer tissue microarray containing primary and metastatic colorectal cancer and also normal colon. There was significant increase in expression in annexins A1 (P = 0.01), A2 (P < 0.001), A4 (P < 0.001) and A11 (P < 0.001) in primary tumours compared with normal colon. There was increasing expression of annexins A2 (P = 0.001), A4 (P = 0.03) and A11 (P = 0.006) with increasing tumour stage. An annexin expression profile was identified by k-means cluster analysis, and the annexin profile was associated with tumour stage (P = 0.01) and also patient survival. Patients in annexin cluster group 1 (low annexin expression) had a better survival (log rank = 5.33, P = 0.02) than patients in cluster group 2 (high annexins A4 and A11 expression). In conclusion, this study has shown that individual annexins are present in colorectal cancer, specific annexins are overexpressed in colorectal cancer and the annexin expression profile is associated with survival.

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All annexins share an ability to bind to negatively charged phospholipid membranes in a calcium-dependent manner. This property is found within the annexin core motif where the calcium- and membrane-binding sites are located. Annexins bind to the cytosolic surface of the plasma membrane and to organelle membranes such as the Golgi apparatus. This binding can be reversed by the removal of calcium, freeing the annexin from the phospholipid membrane. However, the functional significance of their reversible membrane-binding ability remains unknown in many annexins, although in some it is thought to be important for vesicle aggregation and membrane organisation (Liemann and Huber, 1997; Rand, 2000; Hayes and Moss, 2004; Rescher and Gerke, 2004; Lim and Pervaiz, 2007). Although all annexins share this binding property, there is variation in calcium sensitivity and phospholipid specificity between individual annexins. For example, within one cell there can be differences in the distribution of annexins, with annexin A1 having an endosomal localisation, A2 to be found in cytosol and A4 being associated with the plasma membrane (Liemann and Huber, 1997).

Some annexins are capable of calcium-independent binding and several have roles in vesicle aggregation. Annexins A1, A2 and A11 function in cooperation with other calcium-binding proteins to form complexes while annexins A1, A2 and A5 interact with cytoskeletal proteins. Many annexins are involved in exocytic and endocytic pathways and some have roles in ion channel regulation (Gerke and Moss, 2003). Extracellularly, annexin A1 has a role in controlling the inflammatory response while annexin A2 is present on the external surface of endothelial cells, where it may act as a receptor for ligands, including plasminogen and tissue plasminogen.
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MATERIALS AND METHODS

Proteomics

Two-dimensional gel electrophoresis and matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) on normal colon and colorectal cancer were performed as previously described (Lawrie et al., 2004; Dundas et al., 2005; Coglin et al., 2006). Proteins were solubilised from Dukes C adenocarcinoma tissue samples and patient-matched morphologically normal colorectal mucosa. The annexins A1, A2, A4 and A11 were identified as four such cancer, compared with morphologically normal colorectal mucosa (Zimmermann et al., 2004b), prostate adenocarcinoma (Patton et al., 2005), breast cancer (Shen et al., 2006) and B-cell non-Hodgkin’s lymphoma (Vishwanatha et al., 2004).

Other annexins have also been implicated in tumorigenesis. Overexpression of annexin A2 has been found in renal cell cancer, where it is associated with tumour stage (Zimmermann et al., 2004a), invasive breast cancer (Sharma et al., 2006) and sarcomas, including both soft tissue sarcomas (Sayed et al., 2007) and osteosarcomas (Gillette et al., 2004). There is increased expression of annexin A4 in renal clear cell carcinoma (Zimmermann et al., 2004b). In prostate cancer, decreased expression of annexin A4 has been shown to correlate with worsen pathological stage (Xin et al., 2003), and loss of annexin A7 has been associated with metastatic and local recurrences of hormone refractory prostate cancer (Srivastava et al., 2001).

However, the annexins have received no significant study in colorectal cancer and in this study we used comparative proteomic analysis to identify proteins that are overexpressed in colorectal cancer, compared with morphologically normal colorectal mucosa. The annexins A1, A2, A4 and A11 were identified as four such proteins. In order to further define the roles played by these proteins in colorectal neoplasia, their expression and cellular localisation was studied by immunohistochemistry in a large series of colorectal cancers represented within a colorectal cancer tissue microarray.

Table 1 Clinicopathological data of the patients in this study

| Characteristic | Number (%) |
|---------------|------------|
| Gender        |            |
| Male          | 135 (50.4%)|
| Female        | 133 (49.6%)|
| Age (years)   |            |
| Mean          | 68         |
| Range         | 33–92      |
| <70           | 127 (47.4%)|
| >70           | 141 (52.6%)|
| Dukes stage   |            |
| A             | 53 (19.8%)  |
| B             | 104 (38.8%) |
| C             | 111 (41.4%) |
| Tumour site   |            |
| Proximal colon| 95 (35.4%)  |
| Distal colon  | 97 (36.2%)  |
| Rectum        | 76 (28.4%)  |
| Tumour differentiation | | |
| Well          | 10 (3.7%)   |
| Moderate      | 228 (85.1%) |
| Poor          | 30 (11.2%)  |
Department of Pathology, University of Aberdeen for diagnosis. The tumour excision specimens were fixed in formalin, representative blocks were embedded in wax and sections were stained with haematoxylin and eosin. Permission for this study was obtained from the Grampian Research Ethics Committee.

A colorectal cancer tissue microarray was constructed as described (Dundas et al, 2005; Kumarakulasingham et al, 2005). The tumours within the array were representative of the distribution of anatomical locations and the Dukes stages found in colorectal cancers within this population. The tissue microarray contained primary colorectal cancer (Dukes A = 53, Dukes B = 104 and Dukes C = 111). In addition, it contained lymph node metastases and morphologically normal colonic mucosal samples. The lymph node metastases were from the corresponding Dukes C cases (n = 111). Each normal sample (n = 52) was acquired from at least 10 cm distant from the tumour as previously described (Kumarakulasingham et al, 2005). Using a steel Menghini needle, a representative 1.6 mm core of tissue was taken from each donor block and arrayed into the recipient wax block. In order to check the histopathological diagnosis and the adequacy of tissue sampling, a section from each microarray was stained with haematoxylin and eosin and examined by light microscopy.

Immunohistochemistry

Annexin immunohistochemistry was carried out using a Dako autostainer (Dako, Ely, UK) as previously described (Dundas et al, 2005; Coghlin et al, 2006). Sections (4 μm) of the tissue microarray were dewaxed, rehydrated and an antigen retrieval step performed when required. The antigen retrieval step consisted of microwave heating the sections in 0.01 M citrate buffer at pH 6.0 for 20 min in an 800 W microwave oven operated at full power. The sections were then allowed to cool to room temperature. Primary antibody was appropriately diluted (Table 2) in antibody diluent (Dako) was applied for 60 min at room temperature, washed with buffer (Dako) followed by peroxidase blocking for 5 min (Dako), followed by a single 2-min buffer wash. Prediluted peroxidase polymer-labelled goat anti-mouse/rabbit secondary antibody (Envision™, Dako) was applied for 30 min at room temperature, followed by further washing with buffer to remove unbound antibody. Sites of peroxidase activity were then demonstrated with dianinobenzidine as the chromogen applied for three successive 5 min periods. Finally, sections were washed in water, lightly counterstained with haematoxylin, dehydrated and mounted. Omitting the primary antibody from the immunohistochemical procedure and replacing it with antibody diluent or non-immune rabbit serum served as negative controls.

The sections were evaluated by light microscopic examination, and cellular localisation and intensity (negative = 0, weak = 1, moderate = 2, strong = 3) of immunostaining in each section were assessed by two observers (RD and GIM).

Table 2 Details of annexin antibodies used in this study

| Antibody | Source      | Type     | Antigen retrieval | Dilution |
|----------|-------------|----------|-------------------|----------|
| Annexin A1 | BD Bioscience | Monoclonal | Yes               | 1/100    |
| Annexin A2 | BD Bioscience | Monoclonal | Yes               | 1/100    |
| Annexin A4 | Own laboratory | Polyclonal | Yes               | 1/500    |
| Annexin A7 | BD Bioscience | Monoclonal | Yes               | 1/100    |
| Annexin A11 | BD Bioscience | Monoclonal | Yes              | 1/400    |

RESULTS

Proteomics

Comparative proteomic analysis using two-dimensional gel electrophoresis identified certain protein spots that were represented in the colorectal cancer samples but not in the normal colorectal mucosal samples (Figure 1). Protein spots of interest were digested and the masses of the tryptic fragments were determined using MALDI-TOF MS. These masses were entered into MS-Fit, which identified annexins A1, A2, A4 and A11 with a high degree of significance. The MOWSE score (a measure of the identity of the protein) for annexin A1 was 1.68e+003, annexin A2 was 6.53e+005, annexin A4 was 1.52e+008 and annexin A11 was 6.67e+005.

Immunohistochemistry

Primary colorectal cancer The annexins with the exception of annexin A7 showed increased immunostaining in primary tumours in comparison to normal colon (Figures 2 and 3). Weak staining was seen for all annexins varying from 0.4 to 25.9% of tumours. Moderate and strong tumour cell staining was seen in the

Mann–Whitney U-test. The chi-square (χ²) test was used to compare annexin expression with tumour stage while the annexin expression profile was determined by k-means cluster analysis. The relationship of patient survival and annexin expression was determined using the method of Kaplan–Meier and the log-rank test. Cox-multivariate analysis was used to determine the relative significance of individual clinicopathological factors, annexin expression and patient survival. All the statistical analyses were performed using SPSS v15 for Windows XP™ (SPSS UK Ltd, Woking, UK).

Figure 1 Two-dimensional gels of (A) colorectal cancer and (B) normal colon mucosa. The circles labelled A1, A2, A4 and A11 correspond to the protein spots annexins A1, A2, A4 and A11, respectively (Coomassie blue-stained two-dimensional electrophoresis gel).
Figure 2 The immunohistochemical localisation of annexins in normal colon and colorectal cancer. Normal colon (A, C, E, G and I) and colorectal cancer (B, D, F, H and J). Annexin A1 (A and B), annexin A2 (C and D), annexin A4 (E and F), annexin A7 (G and H) and annexin A11 (I and J).
Annexins A1, A2, A4 and A11. More tumours showed strong staining for A1, A2 and A4 than showed moderate staining. The highest percentage of strong staining was seen in annexin A4, with 64.9% of tumours showing strong staining. However, for annexin A11, moderate staining was observed in 33.3% of tumours compared to 31.1% of tumours that showed strong staining (Figures 2 and 3). There was significant increase in expression in annexins A1 ($P = 0.01$), A2 ($P < 0.001$), A4 ($P < 0.001$) and A11 ($P < 0.001$) in primary tumours compared with normal colon (Figure 3). There was increased expression of annexins A2, A4 and A11 with increasing tumour stage (Table 3).

**Lymph node metastasis**  The annexins except annexin A7 showed immunoreactivity in lymph node metastasis. Annexins A2 and A11 showed the greatest percentage of weak staining at 20.7 and 29%, respectively, with less tumours showing moderate and strong staining for these annexins. As in normal and primary tumours, annexin A4 showed the greatest percentage of strong staining at 62.3% of tumours. Comparing the expression of annexins in lymph node metastasis with the corresponding primary tumours showed that there was a significant decrease in expression of annexin A11 ($P = 0.01$) in lymph node metastasis compared with corresponding primary colorectal cancers (Figure 4).

**Annexin expression profile and clinicopathological factors**

To further dissect the role of annexin expression in colorectal cancer, the annexin expression profile was determined. To identify the relationship of the overall annexin profile within tumours, $k$-means cluster analysis was performed and this identified four clusters or groups with distinct annexin profiles (Table 4). The annexin profile was associated with Dukes stage ($\chi^2 = 16.76$, $P = 0.01$). The annexin expression profile was also associated with survival. Patients in annexin cluster group 1 (low annexin expression) had a better survival (log rank $= 5.33$, $P = 0.02$; Figure 5) than patients in cluster group 2 (high annexin A4 and A11 expression). The mean survival in group 1 was 96 months (95% CI: 80 – 113 months) and in group 2 was 72 months (95% CI: 62 – 82 months). However, there was no relationship between the expression of individual annexins and patient survival and also the annexin profile was not an independent marker of prognosis following multivariate analysis.

**DISCUSSION**

The annexins are a multigene family of calcium-dependent phospholipid-binding proteins (Gerke and Moss, 2003; Hayes and Moss, 2004; Gerke et al, 2005). There are 12 human annexins each of which shows a cell- and tissue-type-specific pattern of expression. Some of the annexins have been well characterised while less is known about the biology of some of the other

### Table 3  The relationship of annexin expression and tumour (Dukes) stage

| Annexin | $\chi^2$ | $P$-value | Interpretation |
|---------|----------|-----------|---------------|
| A1      | 5.48     | 0.484     | There is no relationship of annexin A1 expression and tumour stage |
| A2      | 23.6     | 0.001     | There is increased expression of annexin A2 with increasing tumour stage |
| A4      | 7.1      | 0.029     | There is increased expression of annexin A4 with increasing tumour stage |
| A7      | NA       | NA        | NA |
| A11     | 10.2     | 0.006     | There is increased expression of annexin A11 with increasing tumour stage |

NA, not available. The expression of individual annexins as determined by immunohistochemistry in each tumour stage (Dukes A vs Dukes B vs Dukes C) was compared to assess the trend in annexin expression (i.e., increase, decrease, no change) with advancing tumour stage.
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Annexins and colorectal cancer (Falini et al, 2004). In oesophageal adenocarcinoma, the tumour cell expression of annexin A1 has been associated with poor prognosis (Wang et al, 2006). Furthermore, annexin A1 has been found to be overexpressed in immortalised colorectal cell lines (Guzman-Aranguez et al, 2005). This annexin has also a potential role in tumour invasion and metastasis, as inhibition of annexin A1 using siRNA resulted in a significant reduction of cell invasion using an in vitro assay on an immortalised colorectal cancer cell line (Babbini et al, 2006).

Annexin A2 shows increased expression in several type of cancer, including renal cell cancer (Zimmermann et al, 2004a), breast cancer (Sharma et al, 2006) and sarcomas (Gillette et al, 2004; Syed et al, 2007), and there are several possible mechanisms by which annexin A2 may be involved in tumour progression. Annexin A2 interacts with tissue-type plasminogen activator and disruption of this interaction resulted in decreased tumour cell invasion (Rand, 2000; Diaz et al, 2004; Sharma et al, 2006). Annexin A2 is also known to form a complex with cathepsin B that can initiate proteolytic cascades and degrade extracellular matrix proteins. These functions may enhance tumour cell detachment, invasion and motility and thus promote tumour invasion and metastasis (Mai et al, 2000). Cell-surface annexin A2 also acts as a receptor for tenasin C, a key extracellular matrix protein involved in epithelial–stromal interactions, and increased annexin A2 expression is associated with progression in pancreatic neoplasia from pancreatic intraepithelial neoplasia through to invasive pancreatic carcinoma (Esposito et al, 2006). Recently, it has also been shown that the production of matrix metalloproteinase 1, a key enzyme promoting colorectal cancer invasion (Murray et al, 1996), can be mediated by annexin A2. Inhibition of annexin A2 was associated with loss of production of this matrix-degrading enzyme (Zhang et al, 2007).

Renal clear cell carcinoma also shows overexpression of annexin A4 and this seems to be related to the metastatic potential of this type of tumour (Zimmermann et al, 2004b). Annexin A4 has a distinct subcellular localisation in tumour cells and this was linked to loss of cell-to-cell adhesion and increased tumour cell dissemination (Zimmermann et al, 2004b). Additionally, it has been demonstrated that overexpressed annexin A4 promotes cell migration in a model tumour system (Zimmermann et al, 2004b), which correlates with our observation that annexin A4 expression increased as tumour stage progressed, such findings are indicative that annexin A4 is implicated in tumour spread. Annexin A4 is known to form complexes with protein kinase C, and there are 10 isoforms of protein kinase C that have roles in cancer progression (metastasis) and some of these isoforms have been shown to be overexpressed in colorectal cancer (Gokmen-Polar et al, 2001). It could be through association with protein kinase C isoforms that annexin A4 has an effect on the pathogenesis of colorectal cancer. Annexin A4 has also been shown to be overexpressed in a paclitaxel-resistant cell line and, moreover, overexpression of annexin A4 in this cell line resulted in a four-fold increase in paclitaxel resistance also indicating a role for annexins in anticancer drug resistance (Han et al, 2000).

Annexin A11 was overexpressed in colorectal cancer and increased expression correlated with more advanced tumour stage. Annexin A11 is implicated as being involved in cell growth (Farnaes and Ditzel, 2003) and a reduction in annexin A11 expression using RNAi stops cell division (Tomas et al, 2004). However, annexin A11 expression was decreased in metastasis, suggesting further dysregulation of this protein with tumour progression and possibly indicating that the tumour microenvironment plays a role in regulating annexin A11, although the specific mechanisms regulating this annexin remain to be elucidated.

Annexin A7 expression was not detected in either normal colon or colorectal cancer, whereas annexin A7 has been proposed as a putative tumour suppressor gene in prostate cancer (Srivastava et al, 2001) and that high expression of annexin A7 is associated
with poor prognosis in breast cancer (Srivastava et al., 2004), thus providing further evidence that there is tumour-type-specific regulation and expression of individual annexins.

In conclusion, this study has shown that annexins A1, A2, A4 and A11 are significantly overexpressed in colorectal cancer and that the overexpression of annexins A2, A4 and A11 showed a significant correlation with increasing tumour stage. The overall expression profile of annexins was associated with survival in colorectal cancer, indicating collectively that annexin expression may contribute to outcome and this would be consistent with the putative roles of annexins in some of the cellular processes that led to tumour invasion. These annexins may also represent tumour biomarkers and potential therapeutic targets (Oh et al., 2004; Falsey et al., 2006; Wozney et al., 2007).

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Conflict of interest

GIM and CT are named inventors on a patent application made by the University of Aberdeen and Auvation Ltd to exploit the overexpression in colorectal cancer of proteins, including annexin A4, as diagnostic markers and therapeutic targets.
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