Original Articles

Expression of secreted frizzled-related protein 4 (SFRP4) in primary serous ovarian tumours

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Summary

Objective: Serous ovarian cancer is the most prevalent type of ovarian cancer. The majority of women present at an advanced stage and patient survival is poor. Resistance to chemotherapy is thought to relate to failure of tumours to undergo apoptosis. Secreted frizzled-related protein 4 (SFRP4) has been demonstrated to be involved in apoptosis in the ovary but not in ovarian tumours as yet. This study examined SFRP4 expression in ovarian cancers and correlated this with expression of β-catenin, a main component of the WNT-signalling pathway it inhibits. *Methods:* We examined 153 primary serous ovarian carcinomas for SFRP4 and B-catenin expression using immunohistochemistry on tissue microarrays and correlated this with clinical information. *Results:* SFRP4 expression was inversely associated with β-catenin expression in 84% of samples. However, high-level SFRP4 expression was not significantly associated with patient survival (p = 0.08). *Conclusion:* Elevated SFRP4 expression in serous ovarian tumours appears to correlate with reduced β-catenin expression but long-term survival appears unaffected by this.

Key words: Ovary; Carcinoma; Tissue microarray; SFRP4; Immunohistochemistry; β-catenin.

Introduction

Ovarian cancer is the leading cause of death from gynaecological malignancies in women from developed countries [1-3]. Despite many risk factors for ovarian cancer being identified, none have been useful as screening tools and, due to the absence of any overt symptoms, patients often present only after the disease is already at an advanced stage [4]. Once the tumour has spread from the ovaries, it is less responsive to treatment and, hence, there is poor patient survival [5-10]. Thus, a pressing need for improved detection as well as more effective treatment strategies is apparent. Research on the complex molecular pathways involved in ovarian cancer growth and, in particular, on mechanisms whereby tumour cells overcome apoptosis is needed.

Apoptosis is frequently detected in human tumours but is generally overwhelmed by the rate of cell division, allowing cell numbers to increase, selective processes to occur and, hence, the tumour to grow [11, 12]. Essentially all successful cancer treatments, such as radiation and chemotherapy, induce apoptosis in tumour cells, with the aim of these treatments being to increase the rate of apoptosis and thus slow or even reverse tumour growth [13-15]. Increasing the level of apoptosis in the tumour is therefore one of the most desired outcomes for cancer treatment.

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Molecular analysis of apoptosis in various cancer cells has established a positive relationship between the expression of secreted frizzled-related protein 4 (SFRP4) and apoptosis [16-18]. SFRP4 is upregulated in the normal rodent ovary and correlates with an increase in apoptosis [19-21], but no data exists regarding the expression and possible role of this protein in human serous ovarian cancer.

It is believed that the secreted form of sFRP4 acts to interfere with WNT protein signalling by preventing the extracellular WNT proteins from binding to their cell membrane receptors. This subsequently alters the expression of the intracellular target of WNT signalling, β-catenin, preventing it from signalling for cell survival and proliferation [22-24].

The expression of SFRP4 has been linked to apoptosis in normal human tissues [25-27] and experimental evidence suggests it can induce apoptotic cell death in cancer cells [17, 18]. Analysis of the expression pattern of SFRP4 in human endometrial and breast tumours reveals increased SFRP4 in the stromal compartment in both of these tissues, with very low levels of expression in normal control tissue [28]. Up-regulated expression of SFRP4 has also been identified in breast tumour cells [29] as well as a large proportion of prostate cancers [30].

In addition to the positive relationship that has been observed between the expression of SFRP4 and apoptosis in various cancer cells *in vitro*, an inverse relationship has been identified between apoptosis and expression of the intracellular signalling protein β-catenin [16-18, 31,

32]. In many cancer types, the expression of SFRP4 and β-catenin is inversely correlated, however, neither their expression nor association has been investigated with apoptosis in human ovarian tissue. Therefore, the aim of our study was to compare the expression of SFRP4 and β-catenin using immunohistochemistry in serous ovarian carcinoma tissue microarrays (TMA), and to correlate this with patient survival. Tissue microarrays are a good way to screen large numbers of tumours. Although the small size of the biopsies may mean that individual tumours are not well represented, the ability to look at large numbers of tumours more than compensates for this in assessing overall genetic changes in a group of tumours [33].

Materials and Methods

Tissue collection

Archived serous ovarian tumour tissue (stored as paraffin-embedded tumour blocks) were sourced from King Edward Memorial Hospital by the Western Australian Research Tissue

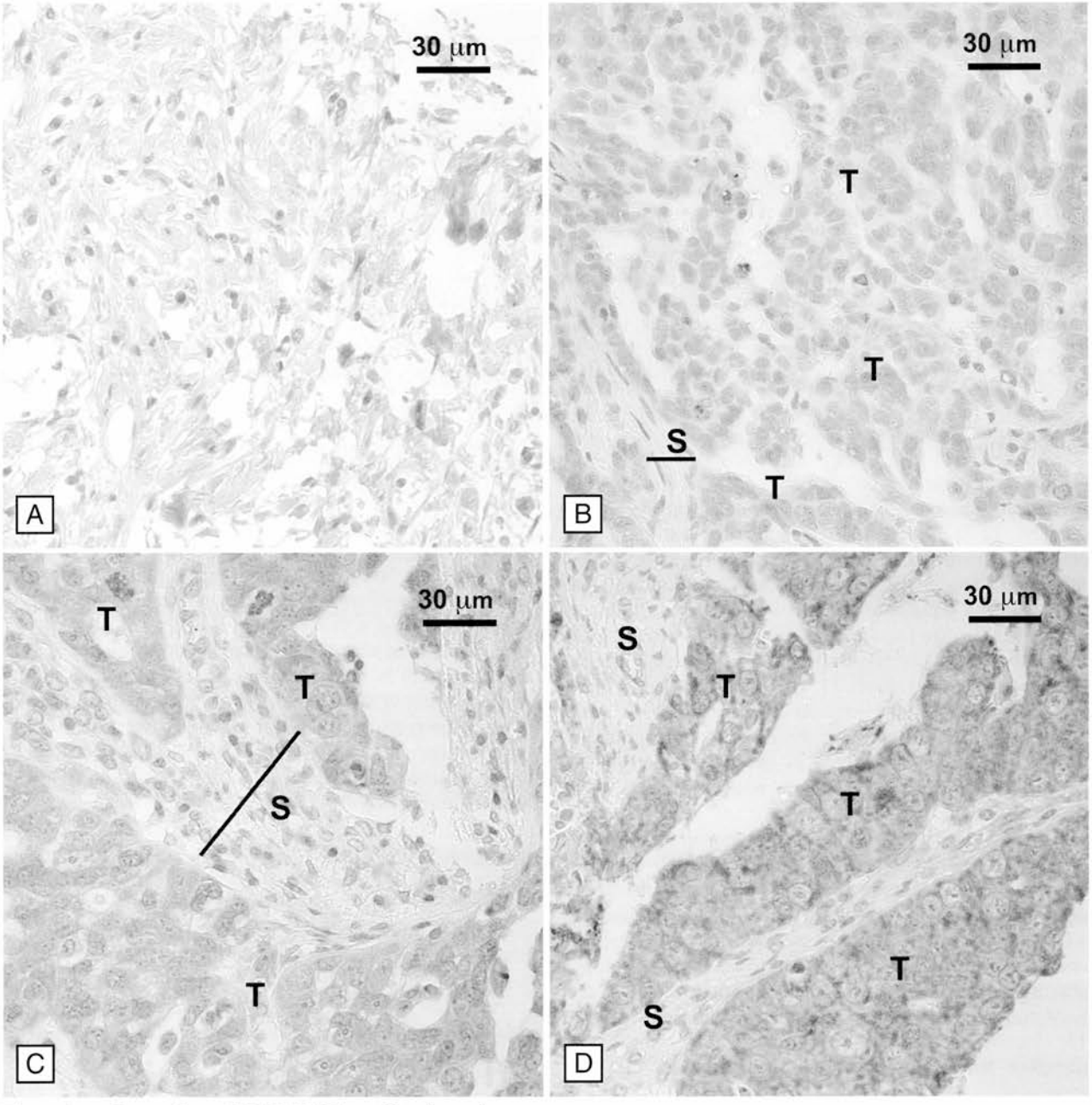

Figure 1. — Comparison of SFRP4 TMA staining intensity
Light microscope images (400 X) of ovarian tumour tissue (T) and stroma (S) from the TMA. Tissue samples expressing SFRP4 are stained brown with DAB and counterstained with haematoxylin for contrast. Images show examples of (A) negative stromal tissue (B) weak (C) moderate and (D) strong staining for SFRP4.

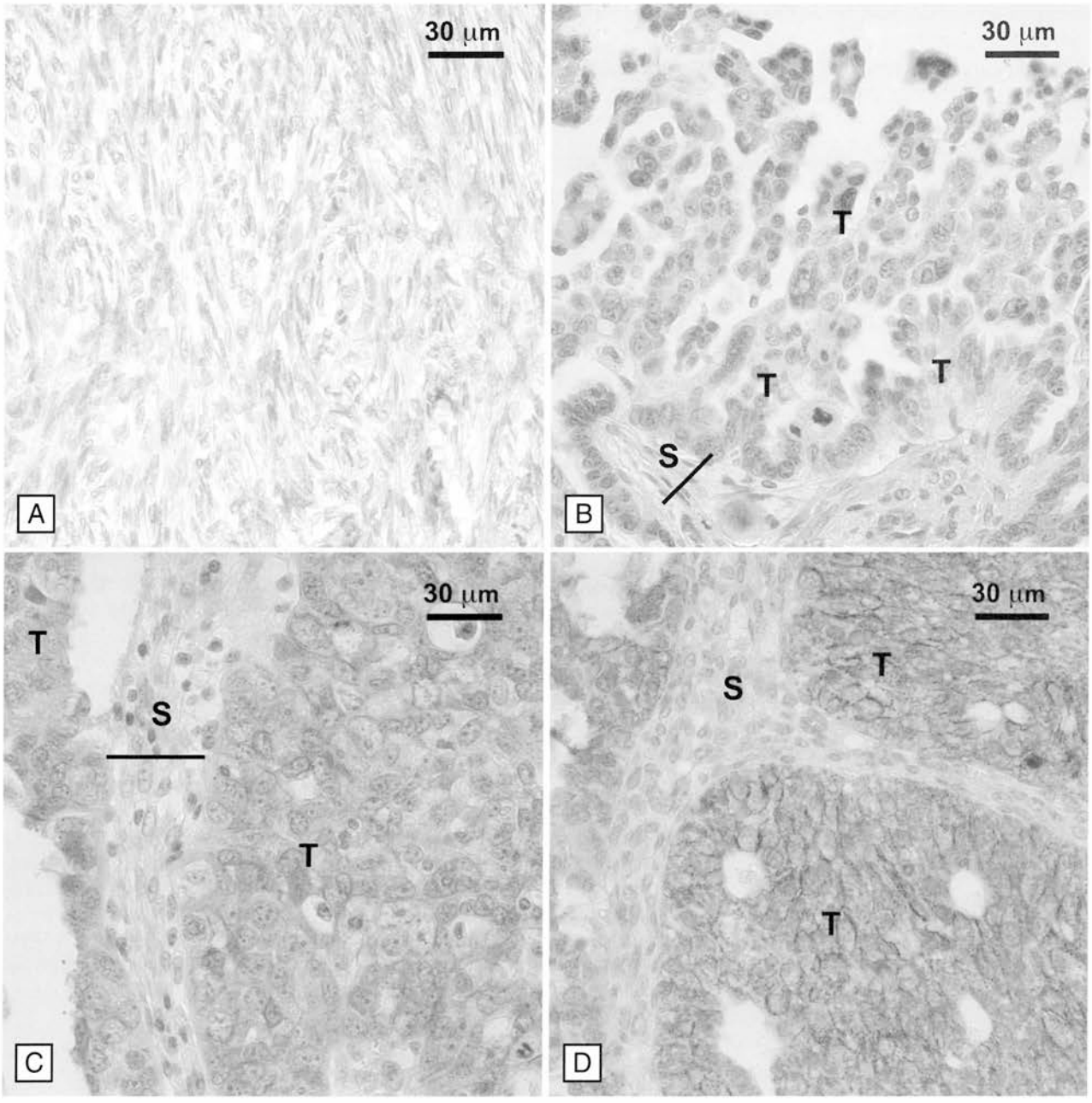

Figure 1.2. — Comparison of active β-catenin TMA staining intensity.
Light microscope images (400 X) of ovarian tumour tissue (T) and stroma (S) from the TMA. Tissue samples expressing active β-catenin are stained brown with DAB and counterstained with haematoxylin for contrast. Images show examples of (A) negative stromal tissue (B) weak (C) moderate and (D) strong staining for β-catenin.

Network. The vast majority (over 85%) of the serous ovarian cancers used in the present study were resected from patients diagnosed in an advanced stage of the disease (as defined by FIGO stage). The average age of diagnosis of the patients included in the TMAs was 64.2 years. Ethics approval for this study was granted from the Sir Charles Gairdner Hospital Human Research Ethics Committee and the Confidentiality of Health Information Committee of the West Australian Department of Health.

Constructing the TMA block

The TMA block was constructed on an MTA-1 manual tissue arrayer (Beecher Instruments, Sun Prairie, USA) as described by Kononen [34]. Tissue sample cores were 1 mm in diameter and placed in the recipient block 0.3 mm apart in a grid fashion. In addition to two tumour cores, a core of adjacent stromal tissue taken from sites adjacent to each of the tumours were included to serve as internal controls for each tumour case. A set of four control tissues was added as an extra column. Sections were cut to 5 μm thickness using a Leica R2135 microtome (Leica) and placed on silanated Starfrost A adhesive treated slides.

Immunohistochemistry to detect SFRP4 and β-catenin expression

Immunohistochemistry was performed on the TMA sections according to the protocol detailed in Han et al. [35]. Briefly, the TMA slides were dewaxed and antigen retrieval was performed with a pressure cooker in a microwave with 10 mM citrate buffer (pH 6.0). Staining was performed using the DAKO EnVision+ System (Cat#K4007) with some modifications. Peroxidase blocking solution was applied. The primary rabbit anti-SFRP4 antibody was diluted 1:200 in 1x TBS (supplemented with 10% goat serum to block non-specific binding of the antibody and 0.1% Tween) and the primary mouse anti-active-β-catenin antibody (Upstate) was diluted 1:200 in the same solution and incubated overnight at 4°C. For SFRP4 detection, the HRP-conjugated goat anti-rabbit secondary antibody (Cat#P0448, DAKO, Glostrup, Denmark) was diluted 1:450 in TBS (supplemented with 10% goat serum and 0.1% Tween) and then applied to the slides for one hour. For β-catenin detection, the kit HRP-labelled polymer was applied to the slides for one hour. The DAB chromagen was used for detection and counterstained in Meyer's haematoxylin.

Histopathologist grading of staining intensity

Tissue sections on TMA slides were analysed in conjunction with histopathologists (JW and AC). Tissue samples were examined under a Leica DMLB microscope (Leica) at 400X magnification to determine the intensity of cytoplasmic brown DAB staining for both SFRP4 and active β-catenin antibodies. Samples which had non-specific staining were excluded from subsequent analysis. Each tissue section was subjectively classified into one of four categories depending on the intensity of the cellular staining: negative, weak, moderate or strong. Weak is faint, and may represent low level production. For this study we used moderate or strong staining as positive and negative or weak as no increased production of protein.

Patient outcome statistical analysis

Patient information such as age at disease diagnosis and length of survival for each tissue sample was collected from records at the Western Australian Cancer Registry. The staining intensity value for each sample was compared to corresponding patient data fields and statistically analysed using SPSS 12.0 software package (Chicago, Illinois, USA). Relationships between categorical values were analysed using chi-square tests and logistic regression. Kaplan-Meier tests were performed to generate patient survival curves and log rank tests were used to compare between groups. A "p value" of less than 0.05 was considered to be statistically significant.

Results

SFRP4 and β-catenin protein expression were analysed in 153 serous ovarian tumours using TMAs. The level of protein expression (detected as a brown stain using DAB) was divided into one of two categories: negative or weak protein expression was classified as "low" and moderate or strong staining was classified as "high" for both SFRP4 protein and active β-catenin protein. Stromal tissue cores exhibited no expression of SFRP4 across all 153 cases investigated in this study (Figure 1.1A). The absence of stromal staining was also identified in regions adjacent to tumour samples positive for SFRP4 expression, as illustrated in Figure 1.1B-1.1D. The vast majority of stromal samples (98%) showed no expression of active β-catenin (Figure 1.2A).

In tumour samples that stained positive for SFRP4, expression of the protein was localised to the cytoplasm of cells (Figures 1.1B-1.1D), consistent with previous findings for SFRP4 protein expression [16, 29]. Unlike reports from prostate cancer, specific localisation of SFPR4 at the cell membrane was not detected in any of the tumour samples. Akin to SFRP4 expression, active β-catenin was also localised to the cytoplasm of tumour cells that expressed the protein (Figures 1.2B-1.2D) similar to previous studies, with no nuclear staining [36].

Analysis of the staining for these two proteins revealed that the majority of tumours exhibited moderate to strong expression of SFRP4 in addition to absent or weak expression of β-catenin (Figure 1.3A). In the majority of the serous ovarian tumours analysed (84%), high SFRP4 expression was associated with low β-catenin expression and low SFRP4 was associated with high β-catenin

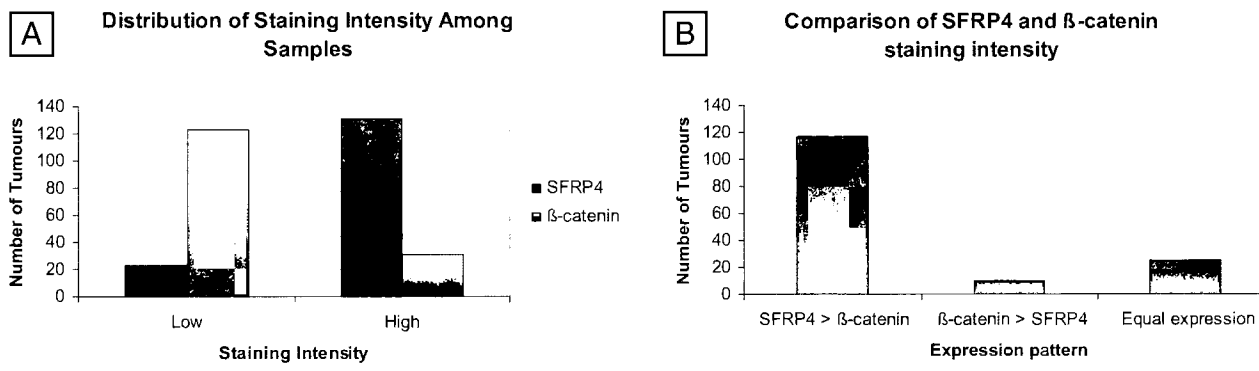

Figure 1.3. — Staining intensity statistics for SFRP4 and β-catenin
(A) A comparison of the total number of tumours present in each category of SFRP4 and β-catenin expression. (B) Graphical representation of the total number of tumours exhibiting an inverse relationship of high SFRP4 staining in conjunction with low β-catenin staining, tumours which showed low SFRP4 in combination with high β-catenin, and tumours which exhibited equivalent levels of staining intensity.

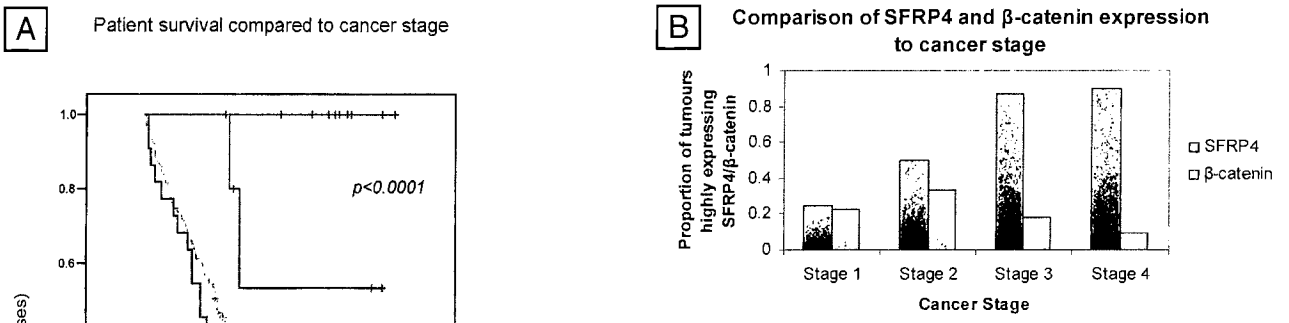

Figure 1.4. — Comparison of FIGO stage with tumour profile. (A) An analysis of patient survival over time with respect to FIGO cancer stage for the serous ovarian tumours examined in this study (Kaplan-Meier survival analysis, log rank test and chi-square analysis). (B) A comparison of the total number of tumours exhibiting high SFRP4 and β-catenin expression across each stage of ovarian cancer NOTE: high staining refers to cases graded as moderate or strong staining; low staining refers to cases graded as weak or negative.

expression. A small group of tumours (16%) exhibited comparable levels of both SFRP4 and β-catenin expression.

FIGO stage is known to be a good prognostic indicator for ovarian cancer [37] and this was also observed in the present study, with a highly significant relationship ($p < 0.0001$) between cancer stage and patient survival (Figure 1.4A). A positive trend was observed between cancer stage and the level of SFRP4 staining (Figure 1.4B), with a tendency towards increased intensity of SFRP4 expression as tumour stage increased. An inverse of this relationship was also observed for β-catenin expression, with a general trend towards decreased intensity of β-catenin staining as tumours stage increased (Figure 1.4B).

Examination of patient survival with respect to SFRP4 expression revealed that increased expression of SFRP4 tended ($p = 0.08$) to be associated with reduced patient survival in the first few years following diagnosis (Figure 1.5), although this trend was not statistically significant. The two survival curves extended past 60 months before merging at 80 months, indicating that SFRP4 staining may not be useful as a long-term prognostic marker of overall survival in serous ovarian cancer but may reflect differences in the biology of the tumours. The expression of the active form of β-catenin in certain tumours is known to be linked to poor outcome [38, 39]. However, analysis of patient survival with respect to active β-catenin expression in these serous ovarian tumours showed no significant relationship with respect to patient survival (Figure 1.6).

Discussion

Currently, screening for ovarian cancer remains very difficult and most patients are diagnosed with advanced stage disease [40-42]. Results from the present study revealed that over 85% of patients were diagnosed with serous ovarian carcinoma in an advanced stage. This delay is known to be mainly attributable to the lack of specific symptoms for ovarian cancer, with many ovarian cancer patients presenting with vague, non-specific symptoms such as abdominal pain, cramping or bloating [43]. As a consequence, the survival of patients diagnosed with advanced stage serous ovarian cancer is quite low, and results from this research revealed that the patients studied had an average five-year survival rate of 22%, consistent with the expected 20-30% five-year survival rate for such a patient cohort [8, 10, 44].

The standard classification of ovarian tumours is that of the International Federation of Gynaecologists and Obstetricians (FIGO) and is the classification system commonly used by gynaecological oncologists [45].

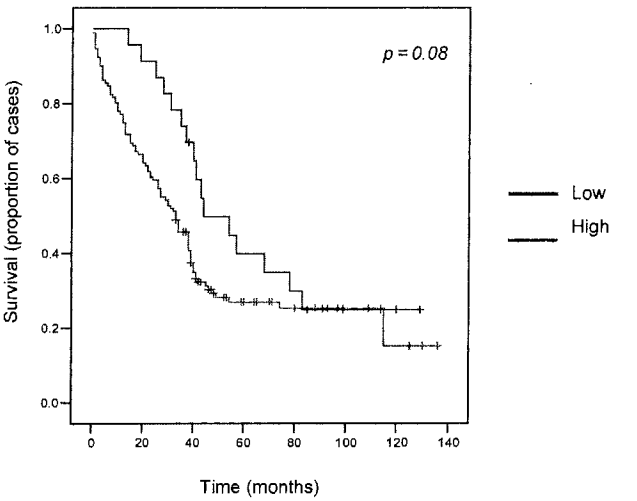

Figure 1.5. — Patient survival analysis for SFRP4 staining intensity.
A comparison of patient survival relative to the SFRP4 staining intensity of the tumour. NOTE: high staining refers to cases graded as moderate and strong staining; low staining refers to cases graded as weak and negative.

FIGO stage is recognised as the best predictor of prognosis for ovarian cancer [37, 46]. Results from this study confirm that FIGO staging is a good prognostic indicator of serous ovarian cancer survival, with a highly significant relationship ($p < 0.0001$) observed between cancer stage and patient survival.

In addition to tumour staging, much investigation in ovarian cancer is focussed on the discovery of complementary or more accurate molecular tumour markers. Unfortunately, it is unlikely that any single biological tumour factor will give accurate prognostic information for all ovarian cancer patients. Even the most promising conventional parameter for ovarian cancer, elevated CA125, has limitations and lacks specificity, as it is elevated with any form of mesothelial irritation such as pancreatitis and endometriosis, and can be absent in patients presenting with ovarian cancer at the time of diagnosis [40, 47, 48]. It is hoped that a combination of two or more independent predictive factors may yield an improved prognostic index. SFRP4 has been proposed as a possible prognostic indicator for localised prostate cancer and breast cancer due to its potential to increase cancer cell apoptosis and therefore decrease tumour volume [16, 29]. These findings suggested a similar prognostic role for SFRP4 in serous ovarian cancer which was investigated in the present study.

A positive trend was observed between cancer stage and the level of SFRP4 staining, with a trend towards increased intensity of SFRP4 expression as tumour stage increased. An inverse of this relationship was also observed for β-catenin expression, with a general trend towards decreased intensity of β-catenin staining as tumour stage increased. Data from studies on SFRP4 in cancer show a wide variation in expression levels, with the majority of endometrial and breast tumours exhibiting down-regulation of SFRP4, and up-regulation only in a small group (12%) compared to adjacent normal tissue [28, 29] whereas the majority of colon and prostate tumours show up-regulation of SFRP4 compared to normal tissue [16. 35, 49]. These findings suggest that the expression and regulation of SFRP4 is different in various tissues and cancer types.

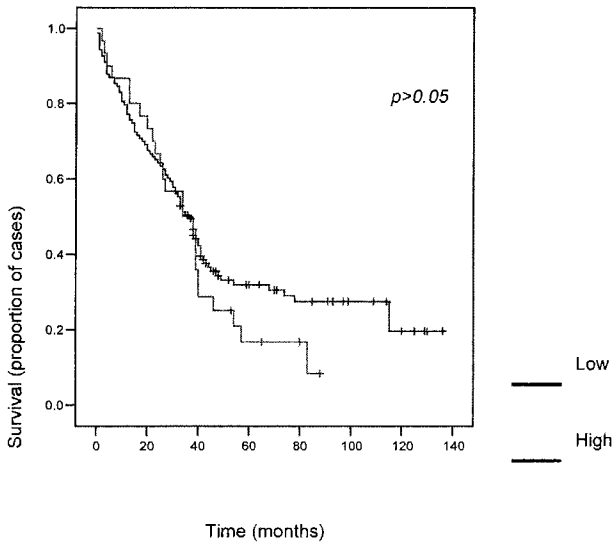

Figure 1.6. — Patient survival analysis for β-catenin staining intensity.
A comparison of patient survival relative to the β-catenin staining intensity of the tumour. NOTE: high staining refers to cases graded as moderate and strong staining; low staining refers to cases graded as weak and negative.

In the majority of the ovarian tumours analysed in this study, high SFRP4 expression was associated with low β-catenin expression and, conversely low SFRP4 was associated with high β-catenin expression. This interrelatedness of staining intensities for the two proteins suggests that SFRP4 is acting via the WNT/β-catenin signalling pathway to decrease β-catenin levels, and supports the experimental evidence from previous studies [16-18, 31, 32] that show a similar inverse relationship *in vitro*. Interestingly, the findings from this study are consistent with those described in another hormonally dependent female reproductive tissue, the endometrium [32].

The high expression of β-catenin in tumours is presumably linked to poor prognosis due its ability to bind TCF/LEF proteins and subsequently stimulate proto-oncogenes such as *c-myc*, cyclin D1 and *EGF/ras*, promoting tumour cell survival and proliferation [38, 39, 50-53]. Results from the present study indicated that the majority of ovarian tumours exhibited low levels of active β-catenin expression, and an analysis of patient survival with respect to β-catenin expression in these serous ovarian tumours showed that no significant relationship existed. Many ovarian and breast cancers studied to date show low levels of β-catenin signalling, in direct contrast to that seen in other cancers such as colon cancer [29, 54].

Mutations in the *β-catenin* gene (*CTNNB1*) that prevent the encoded protein from being degraded are common in colon cancer [51]. Additionally, *APC*, which is normally involved in the canonical WNT/β-catenin pathway and is vital for degrading β-catenin and preventing it from signalling, is also frequently inactivated by mutation in colon cancers [55]. The fact that high SFRP4 expression is observed in conjunction with low β-catenin levels in a majority of these serous ovarian cancers, as well as tumours from other hormonally regulated tissues [29, 32, 54], indicates that the canonical WNT/β-catenin signalling pathway remains intact in these tumours and suggests that β-catenin may not play as important a role in the progression of these cancers as in colon cancer.

Examination of patient survival with respect to SFRP4 expression revealed that increased expression of SFRP4 was linked to reduced patient survival in the first few years following diagnosis. This observed trend between the survival curves was approaching statistical significance ($p = 0.08$) but despite extending past the clinically significant five year survival time period, the curves overlapped at 80 months, indicating that SFRP4 staining may not be useful as a long-term prognostic marker for serous ovarian cancer survival.

The trend towards decreased patient survival with high SFRP4 expression (in the short term) is consistent with data from breast cancer, which shows that patients with tumours expressing higher levels of SFRP4 have a significantly higher mortality rate than those with lower levels of SFRP4 expression [29]. If SFRP4 expression is linked to apoptosis and reduced proliferation in serous ovarian cancer as it is in other cancer types *in vitro* [16-18], the results of this study suggest additional, overriding factors are influencing tumour progression. The demonstration of patients with reduced short-term survival, in spite of exhibiting high SFRP4 expression, indicates that, in these cases, SFRP4 is upregulated as part of an ineffective homeostatic mechanism to curb the excessive proliferation of these more aggressive tumours. However, additional research with a greater sample cohort is needed to further substantiate this hypothesis.

In the present study, expression of SFRP4 in serous ovarian tumours was localised to the cytoplasm, in accordance with the pattern of intracellular staining seen previously in breast ductal carcinomas [29]. However, the cytoplasmic localisation of SFPR4 seen in these ovarian tumours contrasts with that of the prostate, where SFRP4 expression was localised exclusively in the cytoplasm in normal prostate, but localised to both the cytoplasm and cell membrane in prostate cancer samples [16]. Gene transfer experiments have revealed that tissue-specific enhancer and promoter elements exist for many genes, allowing the same gene from different tissues to be affected in variety of ways by external stimuli [56]. This is also true for SFRP4, with the different splice variants of SFRP4 described by Yam and colleagues [57] having different promoters. Because this is a common method for regulating gene expression in a tissue-dependant manner [58], there is a possibility that *SFRP4* is being regulated by different stimuli in these two organs, which could account for the paradoxical localisation of the protein observed in these different tissues.

The SFRP4 antibody used in the present study has previously been shown to detect different localisation patterns of SFRP4 in human skin, where cytoplasmic and/or nuclear expression have been observed in different epidermal cell types. The differing staining patterns seen in tumours of the ovary compared to the prostate may be due to the varying localisation of different isoforms of SFRP4, formed from different mRNA splice variants as described by Yam *et al.* [57].

It is possible for different isoforms of a protein to show different expression patterns with immunohistochemical staining, as exemplified by the three isoforms of leptin, which exhibit localisation to different areas of the cell, such as the cytoplasm or nucleus, depending on the isoform of protein being expressed. The regulation of these leptin isoforms is believed to be under hormonal control, with expression profiles shown to alter across the estrus cycle of the rat. There is evidence to suggest that SFRP4 expression is also under hormonal control [28, 59], and as the hormonal regulation of the prostate is different to the ovary, this may offer a potential explanation for the disparity seen between the localisation of SFRP4 in these two tissues.

Additionally, if the various isoforms of SFRP4 are differentially expressed in the prostate, breast and ovary, this may explain the contradictory results seen between survival trends in cancers of these tissues, with high SFRP4 expression in the male linked to increased survival for prostate cancer [16], compared to high SFRP4 expression in the female linked to decreased survival for tumours of the breast [29] and ovary. Alternatively there may be more influential forces driving the imbalance between proliferation and apoptosis in hormonally regulated tissue or possibly acting to disrupt the SFRP4/Wnt/β-catenin pathway downstream. Other SFRP family members, SFRP1 and SFRP2, are known to have paradoxical functions in different tissues [62-66]. So, the possibility exists that different isoforms of protein translated from various SFRP4 splice variants have assorted functions, which may offer a potential explanation for the paradoxical expression of SFRP4 seen in these different tissues. However, at present this hypothesis remains untested, and further work is necessary to examine whether any link exists between the potential isoforms of SFRP4 and the seemingly different functional outcomes seen in these different cancers.

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