A pan-cancer analysis of secreted Frizzled-related proteins: re-examining their proposed tumour suppressive function

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Secreted frizzled-related proteins (SFRPs), containing five family members (SFRPs 1–5) are putative extracellular Wnt inhibitors. Given their abilities to inhibit Wnt signalling, as well as the loss of SFRP1 in many cancers, this family is generally considered to be tumour suppressive. In this study we analyzed gene expression, promoter methylation and survival data from over 8000 tumour and normal samples from 29 cancers in order to map the context-specific associations of SFRPs 1–5 with patient survival, gene silencing and gene expression signatures. We show that only SFRP1 associates consistently with tumour suppressive functions, and that SFRP2 and SFRP4 typically associate with a poor prognosis concomitant with the expression of genes associated with epithelial-to-mesenchymal transition. Moreover, our results indicate that while SFRP1 is lost in cancer cells via the process of DNA methylation, SFRP2 and 4 are likely derived from the tumour stroma, and thus tend to increase in tumours as compared to normal tissues. This in-depth analysis highlights the need to study each SFRP as a separate entity and suggests that SFRP2 and SFRP4 should be approached as complex matricellular proteins with functions that extend far beyond their putative Wnt antagonistic ability.

Secreted Frizzled-related proteins (SFRPs) were initially described as tumour suppressor genes when SFRP1 was found to be downregulated by loss of heterozygosity or promoter methylation in breast and colorectal cancer cell lines1,2. Given their crucial role in Wnt signalling, and in development, the SFRP gene family was quickly recognized for its potential to modulate tumourigenic behaviour.

SFRPs 1–5 are secreted glycoproteins of ~300 amino acids in length, which fold into two independent domains: (1) a N-terminal cysteine-rich domain (CRD), and (2) a C-terminal netrin-like domain (NTR)3. The cysteine-rich domain shares considerable sequence homology with Fzd receptors, and due to this molecular mimicry, SFRPs were immediately recognized for their potential to sequester Wnt ligands away from receptor complexes and ultimately antagonize Wnt signalling4. Wnt signalling pathways have been shown to play central roles in cell survival, proliferation, fate determination, polarity, and tissue patterning. Unsurprisingly, dysregulation of Wnt-associated pathways is a key event in the development of many types of cancer. In general, constitutive activation of Wnt signalling (eg. through stabilizing beta-catenin mutations) is recognized to contribute to tumourigenesis4. Thus, due to their ability to antagonize Wnt signalling, and their frequent epigenetic silencing, SFRPs were initially designated as tumour suppressor genes and many studies have gone on to support this proposed functional role (reviewed in ref. 7).

However, accumulating evidence suggests that they may also promote tumourigenesis in certain contexts. One instance is canine mammary gland tumours, where SFRP2 was found to be overexpressed and induces cancerous transformation in normal mammary epithelial cells. In this case, SFRP2 associated with a fibronectin-integrin extracellular matrix protein complex, and this interaction mediated cell adhesion and blocked apoptosis8–10. In metastatic renal cell carcinoma, SFRP1 was found to be upregulated, concomitant with a hypomethylated promoter region. Functionally, knocking down SFRP1 resulted in increased apoptosis and decreased invasive potential11. Furthermore, in renal cancer, SFRP2 was also shown to have oncogenic potential; SFRP2 promoted both in vitro

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but has also been observed at the genetic level, through loss of heterozygosity\(^1,2,15,16\). We investigated these possibilities in tumour tissue, indicating that they do not undergo the same silencing process as other genes in over 8,000 primary tumours and 780 associated normal tissues from 29 different cancers (Fig. 2, Supplementary Tables 1 and 2). SFRP1 and 5 consistently have lower expression in primary tumour tissue compared to normal tissue. However, expression of SFRP2, SFRP3 and SFRP4 were often unchanged or even increased in tumour tissue, indicating that they do not undergo the same silencing process as SFRP1 and 5.

Regulation of SFRP gene expression is commonly observed at the epigenetic level, through DNA methylation, but has also been observed at the genetic level, through loss of heterozygosity\(^1,2,15,16\). We investigated these possibilities in breast cancer by looking at how copy number events and CpG site methylation correlates with cellular proliferation and in vivo tumour growth\(^2,12\). Deciphering the complex effects of SFRPs on tumour progression is likely complicated by the local context of Wnt signalling components, differences between SFRP family members and the unknown impact of NTR domain interactions.

Recent transcriptional and genomic profiling of thousands of patient tumour samples by The Cancer Genome Atlas (TCGA) has enabled a thorough investigation of the functions of the SFRP gene family across different types of cancers. In this study, we investigate the context-specific associations of SFRP1–5 expression in over 8,000 tumour and normal samples from 29 different cancers. We show that the putative tumour suppressor function is not consistent between members of the SFRP family and that specific SFRPs behave in a cancer type-dependent manner. We found that SFRP1 is the only family member whose expression is consistently decreased in primary cancer samples as compared to associated normal tissues. Moreover, this loss of SFRP1 expression correlates with gene promoter methylation. Despite these abstruse associations, SFRP2 and 4 expression consistently clusters together, suggesting a common gene program. We found that SFRP2 and 4 expression is tightly correlated to stromal content, and forms part of a common epithelial-to-mesenchymal transition (EMT) gene program that is expressed in a multitude of different cancers.

### Results

**Association of SFRPs with patient survival reveals strong correlations, but inconsistency between family members and cancer type.** We were first interested in determining if SFRP expression was associated with favourable patient outcomes, as suggested by their proposed tumour suppressor function. We looked at primary tumours from fifteen different cancer types (Supplementary Fig. 1) and dichotomized SFRP expression into high and low expressors by ROC curve. Univariate Cox regression analysis was conducted and Kaplan–Meier curves were constructed based on overall survival (Fig. 1, Supplementary Fig. 2). To determine the robustness of these associations, re-sampling analysis was conducted (Supplementary Fig. 3). In general, we found that SFRP1 expression was frequently significantly associated with patient outcomes. However, the direction of that association varied with regards to the particular SFRP isoform queried and the cancer type. Despite this, we observed select consistent cancer-specific or gene-specific effects: For example, high expression of any SFRP was associated with poor prognosis in stomach cancer; and high expression of SFRP4 only associated with poor outcomes (\(p < 0.05\)). In colorectal cancer, where promoter SFRP methylation and functional studies in cell lines have implicated their role as tumour suppressive\(^15,14\), we found that high expression of SFRP2 and SFRP4 is associated with poor patient outcomes (\(SFRP2: HR = 2.14 [1.27–3.58], p = 0.004; SFRP4: HR = 2.76 [1.25–6.08], p = 0.01\)).

**Expression of SFRP1 and 5, but not other SFRPs, is lost in primary tumours.** One of the defining features of SFRP expression during tumourigenesis is a decrease in gene expression. While much of this work has been conducted on normal and cancer cell lines, we investigated the expression levels of all five genes in over 8,000 primary tumours, and 780 associated normal tissues from 29 different cancers (Fig. 2, Supplementary Tables 1 and 2). SFRP1 and 5 consistently have lower expression in primary tumour tissue compared to normal tissue. However, expression of SFRP2, SFRP3 and SFRP4 were often unchanged or even increased in tumour tissue, indicating that they do not undergo the same silencing process as SFRP1 and 5.

Figure 1. Association of SFRP expression with patient survival across different cancer types. Hazard ratios and 95% confidence intervals for overall survival, by cancer type. The forest plot shows the overall survival advantage or disadvantage of increased SFRP expression (high versus low as stratified by ROC curve) by cancer type, unadjusted for other covariates. The vertical line represents a hazard ratio of one, where there are no survival differences between the two groups.
Figure 2. Expression levels of SFRPs in normal and cancerous tissue types. Expression levels of (a) SFRP1, (b) SFRP2, (c) SFRP3, (d) SFRP4, and (e) SFRP5 in patient samples. Each data point represents the SFRP expression levels (log2[RSEM normalized values relative to TBP]) of one tumor or normal sample. Horizontal bars indicate median expression values for normal (blue) or primary tumor (red) samples. Zero value SFRP expressors are plotted at the bottom of the y axis. Comparisons between the normal and tumor expression values were performed using the Mann-Whitney U test to determine significance (*p < 0.05).
SFRP2 and 4 expression in tumours is likely contributed by stroma. A possible alternative mechanism governing alterations in SFRP expression in tumours is that SFRPs are differentially expressed by various cell types in the tumour microenvironment. Several studies have indicated that SFRPs may be expressed by tissue stroma17–19 and we investigated that possibility by correlating SFRP expression to tumour-specific Stromal Scores (as determined by the ESTIMATE algorithm, Fig. 4). We found that SFRP2 and SFRP4 strongly correlated with Stromal Scores in the fourteen cancer types investigated, with an average Spearman's correlation coefficient of 0.67 and 0.66, respectively. Furthermore, single cell RNA-sequencing in breast cancer suggests that SFRPs are expressed by tumour stroma17–19 and we investigated that possibility by correlating SFRP expression to tumour-specific Stromal Scores. We found that SFRP2 and SFRP4 expression is restricted to cells identified as stromal (Supplementary Fig. 5). By contrast, expression of SFRP1, 3, and 5 only weakly or conditionally associate with Stromal Scores: for example, SFRP1 expression strongly correlates with Stromal Scores in colorectal cancer, a cancer where SFRP1 promoter methylation has been demonstrated to occur in both cell lines and patient samples15,14.

Common pan-cancer gene program associated with SFRP2 and 4 expression. When expression levels of the various SFRPs were correlated to each other (Supplementary Fig. 6) and averaged across cancers, we found SFRP2 and 4 expression to be tightly correlated (Fig. 5a). This suggests that those two SFRPs share a common gene program. Furthermore, the pattern of correlation of SFRP2 and 4 expression to gene set enrichments are highly concordant, with tight correlations to EMT and angiogenesis gene sets (Supplementary Fig. 7, Fig. 5b). Correlation network analysis of SFRP2 and 4 reveals a common gene program of 180 genes that are tightly correlated (r > 0.5) across multiple cancers (Fig. 5c). This program includes previously identified key EMT proteins such as ZEB1, ZEB2, VIM, and MMP2. Gene ontology reveals an enrichment of extracellular matrix constituents and metallopeptidase activity ontologies in the identified SFRP2/4 gene set (Fig. 5D). This correlation network analysis and gene ontology strengthens the notion that SFRP2 and 4 have stromal functions — distinct from their proposed Wnt antagonistic activity, and that they may promote processes such as cellular invasion and metastasis.

Discussion
This is the first study of its kind to systematically investigate the survival associations and context-specific interactions of secreted Frizzled-related protein family members. Our results contradict the notion that SFRPs are tumour suppressive across all cancers. Indeed, in many cancers, high expression is associated with poor patient outcomes. We show that while SFRP1 is frequently silenced in many cancers through methylation, the other SFRPs do not undergo this silencing event. In fact, SFRP2 and SFRP4 expression is often increased in tumours and are tightly correlated to Stromal Scores, suggesting that their expression is produced by the tumour stroma. Gene ontology strengthens this observation, with both genes contributing to a common pan-cancer gene set that is tightly associated with EMT. Based on these discoveries, we anticipate that SFRP2 and SFRP4 function as complex matricellular proteins, and that they may have functions that extend far beyond the regulation of Wnt signalling.
SFRP1 was the first of the gene family found to be altered during tumourigenesis. It was discovered in this manner when it was found to be silenced by methylation or loss of heterozygosity in colorectal and breast cancers\textsuperscript{1,2}. Following these initial reports, others went on to show similar trends and to tie the effects of SFRP1 loss to increased Wnt-related signalling in a variety of other cancers. We further validated the essence of these studies with our pancancer analysis by showing that in 17 different cancers, SFRP1 expression is significantly downregulated in tumourous tissues compared to normal counterparts. In addition, we confirmed that in breast cancer the downregulation of SFRP1 associated with promoter methylation. Several groups have proposed the use of SFRP1 promoter methylation as a cancer biomarker, and our study provides further support for this concept\textsuperscript{20–24}. However, our investigation into the other SFRPs did not lead to the same conclusions. We found that high expression of SFRP2 and 4 most often was associated with poor patient prognoses. Furthermore, expression of SFRP2 and 4 often increased in primary tumours compared to their normal counterparts, and this expression tightly correlated to tumour stromal content. Given that SFRPs are secreted proteins, expression from any cell type has the potential to modulate the tumour microenvironment, and affect tumourigenesis. Since many of the initial studies into SFRP2 and 4 were done using cancer cell lines, which are composed solely of tumour cells, this nuance has likely been largely overlooked. The effects of stromal-derived SFRPs could be completely different from tumour cell-derived SFRPs. For example, only tumour cell-derived, not stromal-derived, MMP-2 and MMP-13 correlate with poor patient outcomes and aggressive tumour phenotypes in ovarian or breast cancer, respectively\textsuperscript{25,26}. This is in line with multiple studies that are beginning to appreciate and characterize the complexity involved in biomarker generation\textsuperscript{27–29}. Moreover, the effects of SFRPs on three-dimensional tumours composed of a plethora of cell types is likely entirely distinct from their effects on cancer cell lines. This theory is strengthened by a recent study of SFRP2 in melanoma\textsuperscript{30}. Kaur et al. found that SFRP2 expressed by aged fibroblasts drove melanoma angiogenesis and metastasis. This study was unique in that much of the work was conducted in models that incorporated alternative cell types, such as skin reconstructions or transfer of conditioned media. We suggest that future studies investigating the role of SFRP2 or 4 in tumourigenesis consider possible contribution by the tumour microenvironment and incorporate this into model choice.

Despite production by tumour stroma, SFRP2 or 4 promoter gene methylation may still show promise as a cancer biomarker. Kalmar et al. found that despite an increase in SFRP2 expression in colorectal cancer compared to associated normals, the SFRP2 promoter region became hypermethylated in the cancerous tissues\textsuperscript{31}. They used laser-capture microdissection of colonic epithelial cells to show that this increase was not due to expression within the tumour cells themselves. Moreover, the tumour cells had hypermethylation in the SFRP2 promoter region. This has also been observed in various cell lines, where the SFRP2 promoter region of normal cell line derivatives are unmethylated, but that region is hypermethylated in tumour cell lines\textsuperscript{16,32,33}. Therefore, despite the stromal contribution of SFRP2 and/or SFRP4, hypermethylation within the tumour cell compartment may still show utility as a clinical biomarker. Given that the robustness of cancer biomarkers is often an issue, the utilization of SFRPs with other biomarkers (individual or signature-based) would likely improve accuracy.

In all solid cancers investigated, SFRP2 and 4 showed high concordance in terms of associations with survival, correlation to one another and association with enriched gene sets. The exception to this was brain cancers, where these two proteins appear to act independently of one another. In glioma, high SFRP4 expression is strongly
associated with poor outcomes (HR = 3.93 [2.27–6.81], p < 0.0001), while high SFRP2 expression is strongly associated with favourable outcomes (HR = 0.16 [0.09–0.28], p < 0.0001). In addition, in glioma, SFRP4 is strongly associated with EMT and angiogenesis gene sets, like in all other cancers. On the other hand, SFRP2 in glioma is an exception: It is the only cancer where SFRP2 expression negatively correlates with EMT and angiogenesis gene sets. This disparity may be driven by tissue of origin effects — SFRP2 has been shown to be highly expressed in the developing neural system whereas SFRP4 is not expressed in those structures. Further studies are needed to clarify the details of this divergence. However, it may provide a unique opportunity to elucidate some of the distinct molecular mechanisms of these two proteins.

Conclusions
This study is the first of its kind to systematically analyze the SFRP gene family in multiple cancers. We focused on gene expression and patient survival data and sought to identify if all SFRPs are associated with tumour suppression. We determined that SFRP1, the prototypical family member, is downregulated during tumour formation, silenced by methylation and likely follows much of the dogma surrounding this gene family. On the other hand, SFRP2 and 4 expression levels often increase during tumourigenesis, likely as a result of increased production by the tumour stroma. These two SFRPs, which are often associated with poor patient outcomes, form part of a common gene program that is expressed across many cancers and is associated with EMT. We anticipate that as more studies are conducted, new functions will be discovered for these complex matricellular proteins that extend far beyond their putative Wnt antagonistic ability.
Methods

Datasets. RNA-seq expression, patient clinical and methylation data were retrieved from The Cancer Genome Atlas Data Matrix on 17 August 2014. For gene expression analysis of normals and primaries, RNASeqV2 (HiSeq) data was used for ACC, BLCA, BRCa, CESC, COAD, DLBC, GBM, HNSC, KICH, KIRC, KIRP, LAML, LGG, LIHC, LUAD, LUSC, MESO, OV, PAAD, PCPG, PRAD, READ, SARC, SKCM, THCA, and UCS. RNASeqV2 (GA) data was used for UCEC. RNASeq (HiSeq) level data was used for ESCA and STAD cancers. SFRP expression values (RSEM or RPKM) were normalized to the expression of TBP for each tumour sample. Comparisons between the normal and tumour RNA-seq-derived expression values were performed using the Mann–Whitney U test to determine significance. Breast cancer (BRCA) SFRP copy number status was accessed using the cBioPortal on 17 May 2015 (http://www.cbioportal.org/). Single cell RNA-sequencing data was accessed on 7 December 2015 for 51 breast cancer cells from tumour BCO2 profiled by the Gene Expression Omnibus (GEO) series, GSE75688.

Survival Analysis. To include the most recently released patient samples, additional RNASeqV2 (HiSeq) and clinical data were downloaded on 20 August 2015 for COAD, LIHC, BLCA, STAD, and ESCA cancers. These data were used in downstream survival analyses. SFRP expression values (RSEM normalized values) were dichotomized by receiver operating characteristics (ROC) curves and the Youden index J method was used to determine the optimal cutoff. Survival curves for overall survival (OS) were constructed using the Kaplan–Meier method and significance was determined by log-rank test. The associations between SFRP expression (high versus low) and OS were tested in univariable Cox regression models. Re-sampling Cox regression analysis was conducted by calculating hazard ratios on SFRP expression on 70% of the dataset and re-sampled 500 times.

Methylation analysis. Breast cancer sample associations between individual CpG site methylation (beta values) and SFRP expression (log2(RSEM + 1)) were calculated using Pearson’s correlation in normal, primary and metastatic samples.

Tumour purity. Stromal Scores were defined for tumours through the use of the ESTIMATE (Estimation of STromal and Immune cells in MAignant Tumour tissues using Expression data; original publication7) algorithm using RNASeqV2 data or accessed through the MD Anderson Bioinformatics Portal on 15 May 2015 (http://bioinformatics.mdanderson.org/estimate/). Spearman’s correlation coefficient was used to calculate the association of specific genes to Stromal Scores.

Gene set enrichment analysis. Enrichment for SFRP associated gene sets was conducted using Generally Applicable Gene-set Enrichment (GAGE, v2.12.3). Hallmark gene sets were downloaded from the Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb) v5.0 on 10 August 201538. Enrichment was calculated against a formulated sample composed of the mean expression values for each gene and sample-specific test statistics were correlated to SFRP expression values (log2(RSEM + 1)) using Spearman’s rank correlation on a cancer-specific level.

Correlation network analysis. Correlation network analysis was conducted to determine the context in which SFRPs are expressed. Pearson’s correlation coefficient were determined for SFRPs (log2(RSEM + 1)) to all genes. Correlations were determined for cancers where SFRP2 is associated with poor prognosis (BLCA, COAD, HNSC, KIRC, LIHC, LUSC, PAAD, and STAD) and for cancers where SFRP4 is associated with poor prognosis (BLCA, COAD, HNSC, KIRC, LGG, and STAD). Genes were included in the network if their expression values (log2(RSEM + 1)) were strongly correlated (Pearson’s correlation coefficient >0.5) in at least 50% of the cancers analyzed for correlation with SFRP2 and 50% of the cancers analyzed for correlation with SFRP4. Cytoscape (v3.2.1) was used to visualize the constructed network80. Edges depict an average gene-gene expression correlation coefficient >0.8 in the STAD dataset.

Gene ontology. The identified SFRP2/4 gene set (n = 180) was classified using the PANTHER Classification System (http://www.pantherdb.org, version 10.0, released 2015-05-15)40. The genes were classified based on their molecular function and a Statistical Overrepresentation Test was performed on these genes to examine enrichment of GO terms.

Statistical analysis. We conducted all analyses and visualizations in the RStudio programming environment (v0.98.501). R/Bioconductor packages ggplot2, corrplot, plyr, gplots, matrixStats, survival, and GAGE were used where appropriate.

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Author Contributions
K.M.V. and L.-M.P. conceived of the analysis. K.M.V. prepared the data and performed all data analysis. All authors participated in interpreting results. K.M.V. wrote the manuscript. L.-M.P. participated in revising the manuscript. All authors have read and approve the final manuscript.

Additional Information
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