Sprouty 2 protein, but not Sprouty 4, is an independent prognostic biomarker for human epithelial ovarian cancer

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Sprouty proteins are evolutionary-conserved modulators of receptor tyrosine kinase signaling, deregulation of which has been implicated in the pathophysiology of cancer. In the present study, the expression status of Spry2 and Spry4 proteins and its clinical relevance in human epithelial ovarian cancer (EOC) were investigated retrospectively. We examined the immunohistochemical expression of Spry2 and Spry4 in matched tumor and normal tissue samples from 99 patients. The expression of ERK, p-ERK, Ki67, fibroblast growth factor-2, vascular endothelial growth factor and interleukin-6 and their correlation with Sprouty homologs were also evaluated. Moreover, the correlation between Spry2 and Spry4 and the clinicopathological characteristics were analyzed along with their predictive value for overall survival (OS) and disease-free survival (DFS). Our data indicated significant downregulation of Spry2 and Spry4 in tumor tissues (p < 0.0001). A significant inverse correlation was evident between Spry2 and p-ERK/ERK (p = 0.048), Ki67 (p = 0.011), disease stage (p = 0.013), tumor grade (p = 0.003), recurrence (p < 0.001) and post-treatment ascites (p = 0.001), individually. It was found that Spry2 low-expressing patients had significantly poorer OS (p = 0.002) and DFS (p = 0.004) than those with high expression of Spry2. Multivariate analysis showed that high Spry2 (p = 0.018), low stage (p = 0.049) and no residual tumor (p = 0.006) were independent prognostic factors for a better OS. With regard to DFS, high Spry2 (p = 0.044) and low stage (p = 0.046) remained as independent predictors. In conclusion, we report for the first time significant downregulation of Spry2 and Spry4 proteins in human EOC. Spry2 expression was revealed to significantly impact tumor behavior with predictive value as an independent prognostic factor for survival and recurrence.

Epithelial ovarian cancer (EOC) is the second most common cause of gynecological cancer-related deaths worldwide. In Australia, it is the second most commonly diagnosed and the most lethal gynecological cancer, comprising 4.8% of all cancer deaths in women. Most patients with EOC have the advanced disease at diagnosis. The late presentation and widespread abdominal metastasis account for the high death rate. Despite invasive surgery and platinum-based cytotoxic chemotherapy as the standard of care for the advanced disease, episodes of recurrent disease, progressively shorter disease-free intervals and resistance to chemotherapy will develop in most cases.

Since discovery in 1998, 4 Sprouty proteins have been increasingly implicated in the multilayered, complex regulation of MAPK/ERK pathway and receptor tyrosine kinase (RTK) signaling. As such, this protein family has been shown to regulate processes central to the development, progression and dissemination of malignant conditions, including cell proliferation, migration, invasion and survival. For the past decade, deregulation of Sprouty has been investigated in a variety of cancers. However, the status and clinicopathological significance of the expressions of Sprouty 2 (Spry2) and Sprouty 4 (Spry4) proteins in EOC have not been explored before. In our initial study, we demonstrated differential expression of Spry1 and Spry2 proteins in a panel of EOC cell lines where a tendency for downregulation of Spry1 and/or Spry2 was evident. Here, we report the expression status of Spry2 and Spry4 proteins in a cohort of 99 patients with EOC studied retrospectively, as well as their association with clinicopathological features, survival and recurrence.
What’s new?

Ovarian cancer is the second most lethal gynaecological cancer. Given the need for the development of new biomarkers and the critical regulatory role of Sprouty proteins in cell biology, we explored the clinicopathological significance of the expression of Spry2 and Spry4 isoforms in ovarian cancer. Our results identify Spry2 as a prognostic biomarker of the disease and lay the basis for evaluation of this protein family in therapeutic approaches, including patient stratification in personalized therapy.

Material and Methods

Patients and clinical samples

This study was approved by South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee-Central Network (EC00135). Patients were identified through the databases of the St George Hospital (The University of New South Wales) and the St George Private Hospital, Sydney, New South Wales, Australia. Of a total of 480 cases with ovarian cancer treated between 2001 and 2012, 99 patients were selected and entered the study, after obtaining informed consent for experimentation with human subjects, based on the following inclusion criteria: (i) proven cases of primary EOC; (ii) treated with standard surgical procedure (staging laparotomy/cytoreductive surgery) and adjuvant systemic chemotherapy (paclitaxel (175 mg/m², iv over 3 hr) and carboplatin as formulated below); (iii) informative with regard to the clinicopathological characteristics studied (Table 1); (iv) with available matched, evaluable normal tissue and (v) with complete follow-up history till June 2014 (end of the study).

Adjuvant chemotherapy regimen

Paclitaxel (175 mg/m², iv over 3 hr) + carboplatin (total dose calculated by Calvert formula*, iv over 15–60 min) × six cycles

*Total carboplatin dose (mg) = Target area under concentration vs. time curve (AUC) × (GFR + 25)

Archived formalin-fixed, paraffin-embedded material from surgically resected primary EOC specimens containing tumor and the matched normal tissue (from the contralateral ovary or normal portion of the affected one) was then obtained from Department of Pathology, St George Hospital. Tissue specimens collected between 2001 and 2012 were histologically classified according to the World Health Organization (WHO) classification system. Demographic and clinicopathological data were collected from patients’ medical charts (Table 1). The demographic and clinical information gathered included age, menstrual status, date of diagnosis, staging, response to chemotherapy, recurrent disease, date of recurrence, presence of ascites at diagnosis and development of post-treatment ascites. Histopathological findings such as tumor subtype, tumor grade, lymphovascular invasion and lymph node involvement were obtained from original pathology reports. The final staging of the disease was determined on the basis of a combination of surgical and pathological findings in accord with the Federation of Gynecology and Obstetrics (FIGO) guidelines. Patient numbers may show a slight difference among factors studied because of inadequacy of cancer tissue remaining in the paraffin-embedded archival blocks at the time of study.

Immunohistochemical staining and analysis

The primary antibodies and positive control tissues used for immunohistochemical study are listed in Table 2. Formalin-fixed, paraffin-embedded tissue sections (5-μm thick) were deparaffinized with xylene and rehydrated. For antigen retrieval, sections were placed in either 10 mM Tris base, 1 mM EDTA solution at pH 9.0 for Ki-67, Spry4 and interleukin-6 (IL-6) or 10 mM sodium citrate buffer at pH 6.0 for the rest and exposed to repeated (twice) microwave heating for 10 min (or twice heating of 5 min for vascular endothelial growth factor (VEGF)) at 750 W. After 10 min incubation with 3% hydrogen peroxide for inactivation of endogenous peroxidase activity, sections were blocked with DAKO blocking buffer followed by incubation with primary antibody at 4 °C overnight. Specimens were then incubated with appropriate secondary antibody using EnVision Plus kit (Dako, Glostrup, Denmark) for 30 min and then with diaminobenzidine chromogen for 5 min. All slides were counter-stained with hematoxylin to visualize the nuclei. For negative controls, the same specimens as our positive controls for each antibody were used but the primary antibodies were replaced with the primary antibody diluents.

Under light microscope using Leica DMLB Microsystems (Leica DMLB, Leica Microsystems, Germany), staining of the epithelial cells was evaluated and scored by at least two observers blinded to patient outcome. In case of disagreement, the slides were re-examined and a consensus was reached by the observers. Representative slides were photographed using Leica DC200 digital imaging system (Leica Microsystems, Germany). Semi-quantitative scoring was performed based on the average signal intensity and the percentage of immunoreactive cells. A four-value intensity score (0, no immunoreactivity; 1, weak intensity; 2, moderate intensity and 3, strong intensity) was used as well as a four-value quantity score defined as follows: Spry2 and Spry4 (0, none; 1, 1–33%; 2, 34–66% and 3, 67–100%); ERK and p-ERK (0, none; 1, <10%; 2, 10–50% and 3, >50%); fibroblast growth factor-2 (FGF), IL-6 and VEGF (0, none; 1, 1–25%; 2,
The average intensity and quantity scores for the five cores were then multiplied yielding a 10-point immunohistochemical score ranging from 0 (no staining) to 9 (extensive, strong staining) for each case. For Ki-67, the percentage of the positively stained cells among the total number of the tumor cells in the area was scored. For p-ERK and Ki-67, the proportion of cells showing a positive nuclear stain was considered as positive staining.

Statistical analysis
All statistical analyses were performed using the statistical package SPSS, version 22 (SPSS Inc., Chicago, IL). The data were summarized using standard descriptive statistics and frequency tabulations. Wilcoxon matched-pairs signed rank test was used for comparison of the expressions of Spry2 and Spry4 between the normal and cancer tissue. Associations between the clinicopathological parameters and the expressions of Sprouty isoforms were evaluated using Spearman correlation coefficient testing. The same test was used to assess the correlation between the expressions of the Sprouty homologs and other markers studied. Overall survival (OS) and disease-free survival (DFS) analyses were carried out for the expressions of Spry2 and Spry4. OS was defined as the time from surgery to death or to the end of the study and DFS was calculated from the date of surgery to recurrence or to the end of the study. The predictive value of the Sprouty isoforms for OS and DFS was evaluated using the Kaplan–Meier method. Kaplan–Meier survival curves were constructed for patients with low and high levels of the Sprouty expression. The statistical significance between survival curves was assessed by the log-rank test. The binary cut-off points of the markers studied were identified using the Classification and Regression Tree (CART) algorithm which were near the median values. The Cox univariate and multivariate proportional hazard models with 95% confidence interval (CI) were constructed to assess the independent predictive value of Spry2 and Spry4 in the presence of other clinicopathological variables. Receiver operating characteristic (ROC) curve analysis was also performed to determine the validity of cut-off points and also the sensitivity and specificity of the markers with significant predictive values. Missing data were not included in the statistical analyses. A p value of < 0.05 was considered statistically significant for all analyses.

Results
Spry2 and Spry4 proteins are downregulated in EOC
Our immunohistochemical analysis revealed variable expressions of these proteins in both normal and cancer epithelial cells. Evaluating the Spry2 protein expression in normal epithelial cells, we observed mild (immunohistochemical score of 1–3) and moderate (immunohistochemical score of 4–6) expressions in 51% and 46% of normal tissue samples, respectively. One percent of the patients had the staining score of 0 in their normal epithelia and the remaining 2% showed high levels of expression (immunohistochemical score of 7–9). Spry2 variable expression was also seen in
cancer epithelium (Fig. 1a) where 58%, 29% and 13% of tumor samples showed mild, moderate and no staining, respectively.

Regarding the expression of Spry4 protein in normal ovarian epithelium, the majority of cases (62%) showed strong staining, 30% exhibited moderate expression and only 7% and 1% had low or no staining, respectively. When assessed in cancer epithelium (Fig. 1a), Spry4 showed mild, moderate and strong expressions in 22%, 52% and 23% of tissues, respectively, as well as no staining in 3%. When the expression levels of Spry2 and Spry4 in tumor and normal epithelium were compared, significant downregulation of Spry2 (p value < 0.0001) and Spry4 (p value < 0.0001) in tumor tissues was revealed (Fig. 1b).

Due to the variability of the protein expression in different samples, we also compared the staining scores of Spry2 and Spry4 in cancer tissue and those in matched normal tissue from the same patient for a more meaningful deduction. As a result, in 60% of samples, Spry2 staining score in cancer tissue was lower than that in matched normal tissue. However, 12% had higher expression of Spry2 in cancer tissue than in matched normal epithelium. In 28% of samples, the staining scores of cancer tissue and its matched normal tissue were similar. When the Spry4 staining score of cancerous tissue was compared with that in matched normal tissue, lower and higher score of cancer tissue was evident in 49% and 4% of samples, respectively. In 47% of cases, however, cancer tissues and matched normal tissues received similar Spry4 expression scores (Fig. 1c).

When the total of 99 tumor samples were classified by the cut-off point into Spry2 high- (>3.5) and low- (≤3.5) expressing groups, as well as into Spry4 high- (>6) and low- (≤6) expressing groups, 70 cases were identified as patients with low expression of Spry2 and 76 cases were found to be Spry4 low-expressing.

### The expression of Spry2, but not that of Spry4, correlates with the expression of p-ERK/ERK and Ki67 in EOC

Considering the role of the Sprouty isoforms in the regulation of the MAPK/ERK pathway and cellular functions, including cell proliferation, the expressions of p-ERK, ERK and Ki-67 were also evaluated (Fig. 2a). Our data revealed no significant difference between the expressions of ERK in tumor and matched normal tissue samples. However, significant upregulation of p-ERK (p < 0.0001) and, hence, a significant increase in p-ERK/ERK expression ratio (p < 0.0001) as an indicator of ERK activation in tumor tissues were evident (Fig. 2b). The expression of Ki67, known as a tumor proliferation marker, was also immunohistochemically analyzed and scored. The possible associations between the expressions of Spry2 and Spry4 and those of p-ERK/ERK and Ki-67 were subsequently analyzed. As a result, significant negative correlations of Spry2 with both p-ERK/ERK (p value: 0.048, correlation coefficient = −0.199) and Ki67 (p value: 0.011, correlation coefficient = −0.253) were found (Table 3). However, there was no correlation between Spry4 and p-ERK/ERK (p value: 0.883) or Ki-67 (p value: 0.350).

### The expression of Spry2, but not that of Spry4, correlates with clinicopathological characteristics of EOC patients

To investigate the clinical relevance of Spry2 and Spry4 expressions in EOC, we first evaluated the correlation between the expression of these two Sprouty isoforms and the clinicopathological characteristics of the EOC patients in our cohort (Table 3). Our data analysis indicated that the expression of Spry2 was inversely correlated with aggressive clinicopathological features, including the disease stage (p value: 0.013, correlation coefficient = −0.248), tumor grade (p value: 0.003, correlation coefficient = −0.297), recurrence (p value < 0.001, correlation coefficient = −0.450) and post-treatment ascites (p value: 0.001, correlation coefficient = −0.316). However, no significant correlation between the Spry4 expression and the clinicopathological characteristics of the patients was found.

### Expression of Spry2, but not that of Spry4, is associated with survival in patients with EOC

Subsequently, the influence of the expressions of Spry2 and Spry4 on OS and DFS was investigated. For this purpose, survival probabilities were estimated by the Kaplan–Meier method and differences were compared by the log-rank test. It was found that Spry2 low-expressing patients had significantly poorer OS (p value: 0.002) and DFS (p value: 0.004).
than those with high expression of Spry2 (Fig. 2c, left panel).

The median OS values for low-expressing and high-expressing groups were 2.7 and 6.8 years, respectively. The median DFS in Spry2 low-expressing patients was 24.3 months versus 78.8 months in the high-expressing group.

Outcome analysis of the Spry4 expression was next carried out. Although Spry4 low-expressing group showed lower OS (3.7 years) and DFS (26.3 months) than did the high-expressing group (OS and DFS of 6.4 years and 30 months, respectively), the results were found statistically insignificant, with $p$ values of 0.407 and 0.393 for OS and DFS, respectively (Fig. 2c, right panel).

To evaluate the predictive value of the expressions of Spry2 and Spry4, the expressions of these isoforms were then
assessed along with the clinicopathological parameters of the participants in univariate and multivariate analyses of the factors associated with survival. Our univariate (unadjusted) Cox’s proportional hazards analysis yielded different results for Spry2 and Spry4 (Table 4). High Spry2 appeared to be a significant predictor of an increased OS (HR = 0.39; 95% CI, 0.20–0.75; p value: 0.005) and a better DFS (HR = 0.41; 95% CI, 0.21–0.78; p value: 0.007). As shown in Table 4, no significant results were obtained in univariate analysis of Spry4 (p values of 0.408 and 0.395 for OS and DFS, respectively).
Factors with predictive significance in univariate analysis were then subjected to multivariate Cox’s proportional hazards analysis (Table 4). High Spry2 retained its significance for both OS (HR = 0.44; 95% CI, 0.23–0.87; p-value: 0.018) and DFS (HR = 0.50; 95% CI, 0.26–0.98; p-value: 0.044). Performing the ROC analysis with the area under the curve (AUC) of 0.694, we found that Spry2 as a prognostic biomarker has a sensitivity of 86% and a specificity of 58% for OS, giving a positive predictive value (95% CI) of 67% and a negative predictive value of 80%. The likelihood ratios of positive and negative outcomes were 2.05 and 0.24, respectively.

With regard to DFS, Spry2 showed 80% sensitivity and 66% specificity with AUC of 0.725 (Supporting Information Fig. 1). We also observed the positive and negative predictive values (95% CI) of 70% and 76%, respectively. The likelihood ratios of positive and negative outcomes were 2.35 and 0.30, respectively.

There are no correlations between the expressions of Sprouty isoforms and those of VEGF, FGF-2 and IL-6 in EOC. The immunohistochemical expression of VEGF, FGF-2 and IL-6 proteins, known as activators of MAPK/ERK, were investigated and subjected to analysis for evaluation of any possible association with the expressions of the Sprouty isoforms in a binary model. As shown in Table 5, Spry2 did not show any significant correlation with VEGF (p value: 0.655), FGF-2 (p value: 0.683) or IL-6 (p value: 0.677). Similarly, insignificant results were obtained when possible correlations of the Spry4 expression with the expressions of VEGF (p value: 0.466), FGF-2 (p value: 0.927) and IL-6 (p value: 0.118) proteins were individually analyzed (Table 5).

**Discussion**

Implication of Spry2 and Spry4 in a variety of physiological and developmental processes through the regulation of biological cell behavior has been indicated by different investigators. In this regard, Spry2 has been shown to inhibit growth factor- and/or serum-induced proliferation\textsuperscript{16–20} and migration\textsuperscript{19–21} of various normal cells. Similar physiological functions have been also ascribed to Spry4.\textsuperscript{22–25} Accordingly,
functional outcomes of the expression of Spry2 and Spry4 have been explored in a number of malignancies. However, Sprouty isoforms have proved to exert divergent effects despite functional cooperation and structural interactions. Moreover, as addressed below, the role of different Spry isoforms in cancer biology is associated with further complexity and even controversy.

Spry2 has been found to inhibit cellular functions central to malignant behavior of cancer cells, including cell proliferation, differentiation, migration and invasion, in leiomyosarcoma, osteosarcoma, hepatocellular carcinoma, neuroblastoma, B-cell lymphoma, non-small cell lung cancer (NSCLC), cervical cancer and breast cancer cells. Lo et al. for example indicated that the MCF-7 breast cancer cells transfected with a dominant-negative mutant of Spry2 proliferated faster in vitro and formed larger tumors in vivo. Similarly, Sutterluty et al. demonstrated in their in vitro and in vivo studies that Spry2 inhibits NSCLC cell proliferation and tumorigenesis via ERK-dependent and independent mechanisms. Spry4, too, has been reported to induce inhibitory effects in prostate cancer, pancreatic epithelioid carcinoma, NSCLC and breast cancer cells. Reporting downregulation of Spry4 in a variety of NSCLCs as well as in dysplastic lung cell lines, Tennis et al., for example, showed that Spry4 transfection inhibited NSCLC cell growth, migration, invasion and epithelial–mesenchymal transition. In agreement, Vanas et al. recently reported that Spry4 interferes with breast cancer cell proliferation and migration. In contrast, paradoxical regulatory functions/roles of Spry2 and Spry4 have been reported by some investigators. Holgren et al. found that Spry2 upregulation in the KRAS-mutated cell line HCT-116 significantly increased cell proliferation, accelerated cell cycle transition and enhanced cell migration and invasion, and that Spry2 transfectants formed significantly larger xenografts. These effects were attributed, at least in part, to activation of HGF/c-Met axis and its downstream effectors Akt and Erk. Reporting upregulation of Spry2 protein in the human fibrosarcoma cell lines as well as in HRAS-transformed human fibroblasts, Lito et al. provided evidence that Spry2 is necessary for their tumorigenicity with the involvement of epidermal growth factor receptor (EGFR) signaling. This effect, however, was found specific to HRAS transformation. Using expression profiling of the gastrointestinal stromal tumor (GIST) cells treated with the c-Kit inhibitor imatinib, Frolov et al. identified Spry4 as a downstream effector of the c-Kit-activated ERK which was targeted and significantly downregulated in the treated cells.

Differential expression of the Sprouty isoforms with variable clinical significance in different malignant tumors has been reported in the literature. For the first time in EOC, we explored in the present study the expression levels of Spry2 and Spry4 proteins and their clinical relevance, retrospectively. Firstly, it was found that Spry2 and Spry4 proteins

### Table 4. Univariate and multivariate Cox’s proportional hazards analysis of potential predictors of survival and recurrence in EPC

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall survival</th>
<th>Disease-free survival</th>
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</thead>
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<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value¹</td>
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<tr>
<td>Univariate</td>
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<td>Age (years) (&lt;50 vs. &gt;50)</td>
<td>0.503 (0.239–1.057)</td>
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<td>Menopause (no vs. yes)</td>
<td>0.395 (0.123–1.267)</td>
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<td>Stage (I/II vs. III/IV)</td>
<td>0.286 (0.114–0.718)</td>
<td><strong>0.008</strong></td>
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<tr>
<td>Tumor grade (I–II vs. III)</td>
<td>0.623 (0.338–1.148)</td>
<td>0.129</td>
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<tr>
<td>Tumor type (serous vs. mucinous vs. endometrioid vs. clear cell vs. others)</td>
<td>0.857 (0.386–1.903)</td>
<td>0.705</td>
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<td>Lymphovascular invasion (no vs. yes)</td>
<td>0.625 (0.317–1.230)</td>
<td>0.173</td>
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<td>Lymph node involvement (no vs. yes)</td>
<td>0.797 (0.411–1.546)</td>
<td>0.503</td>
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<td>Ascites at diagnosis (no vs. yes)</td>
<td>0.599 (0.364–0.988)</td>
<td><strong>0.045</strong></td>
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<tr>
<td>Residual tumor (no vs. &lt;1 cm vs. 1–2 cm vs. &gt;2 cm)</td>
<td>0.440 (0.230–0.844)</td>
<td><strong>0.013</strong></td>
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<td>Ki67 (&lt;10% vs. &gt;10%)</td>
<td>0.604 (0.359–1.018)</td>
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<td>Spry2 (high vs. low)</td>
<td>0.397 (0.207–0.759)</td>
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<td>Spry4 (high vs. low)</td>
<td>0.752 (0.382–1.478)</td>
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<td>Multivariate</td>
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<td>Stage (I/II vs. III/IV)</td>
<td>0.376 (0.142–0.994)</td>
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<td>Ascites at diagnosis (no vs. yes)</td>
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<td>Residual tumor (no vs. &lt;1 cm vs. 1–2 cm vs. &gt;2 cm)</td>
<td>0.393 (0.203–0.761)</td>
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<td>Spry2 (high vs. low)</td>
<td>0.448 (0.230–0.874)</td>
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HR: hazard ratio; CI: confidence interval.
¹Statistically significant values (p value <0.05) are shown in bold.
were significantly downregulated in EOC. In this regard, downregulation of Spry1 and Spry2 in breast cancer,\textsuperscript{25,41} inactivation of Spry1 and Spry2 genes in prostate cancer,\textsuperscript{42} upregulation of Spry1 and downregulation of Spry2 and Spry4 in hepatocellular carcinoma\textsuperscript{43} and upregulation of Spry1, Spry2 and Spry4 in embryonal rhabdomyosarcoma (ERMS)\textsuperscript{44} have been reported. Considering Spry2 or Spry4 individually, different reports on the expression status of the respective gene and/or protein are available, too. These mainly include deregulations of Spry2 as downregulation/inactivation in NSCLC,\textsuperscript{19} hepatocellular carcinoma (HCC),\textsuperscript{28,29} malignant peripheral nerve sheath tumor (MPNST),\textsuperscript{45} B-cell diffuse lymphoma,\textsuperscript{46} prostate\textsuperscript{47} and endometrial cancer\textsuperscript{48} as well as upregulation in fibrosarcoma.\textsuperscript{49} In the context of colorectal cancer (CRC), both upregulation\textsuperscript{38,50,51} and downregulation\textsuperscript{52} of Spry2 with different implications—as described below—have been reported. With regard to Spry4, it was found to be deactivated and downregulated in a subset of prostate cancer\textsuperscript{25} and was identified as a susceptibility gene for testicular germ cell cancer.\textsuperscript{53}

Comparing the expression scores of cancerous tissues with those of matched normal tissues, we found that 28% and 12% of tumors expressed Spry2 at equal and higher levels, respectively. As for Spry4, 47% and 4% of tumors received equal or higher scores, respectively. Therefore, although Spry2 and Spry4 proteins were expressed at significantly lower levels in a substantial fraction of EOC tumors in our cohort, decreased expressions of Spry2 or Spry4 seem to be not necessarily required in all EOC tumors.

Downregulation of Spry2 was found to be of significant clinical relevance in EOC. We also observed a negative correlation between Spry2 and p-ERK/ERK or Ki67. Since Spry2 is known as a typical inhibitor of MAPK/ERK and hence cell proliferation, increased expression of p-ERK/ERK and Ki67 as indicators of ERK activation and cell proliferation are expected to be found in association with Spry2 downregulation. In this regard, a study by Velasco et al.\textsuperscript{48} on normal endometrial and endometrial carcinoma tissues is a notable example.\textsuperscript{48} By immunohistochemical analysis of the normal tissue, including proliferative and secretory endometrium, the investigators detected the highest Ki67 in proliferative endometrium with a very significant correlation with the expression of estrogen and progesterone receptors. Accordingly, the expression of Spry2 protein was inversely correlated with the expression of hormonal receptors, and a highly significant decrease in the expression of Spry2 was seen in the proliferative phase of normal endometrium. When similar analysis was carried out with cancerous tissues, they found significant increase of the cell proliferation in tumor tissue compared to the secretory endometrium. As expected, however, the level of cell proliferation in proliferative endometrium was nearly as high as that observed in tumor samples. Finally, tumor tissues with the highest levels of Ki67 showed the lowest levels of the Spry2 immunoexpression.

Investigating the clinical significance of Spry2 downregulation in our patients, we firstly found that low expression of Spry2 was significantly correlated with such aggressive clinicopathological features of EOC as high disease stage, high tumor grade, recurrence and post-treatment ascites. In agreement, association of Spry2 downregulation and aggressive disease has been reported in some other cancers before. McKie et al.\textsuperscript{35} observed that Spry2 mRNA is downregulated in clinically high-grade prostate cancers when compared to benign prostatic hyperplasia (BPH) and well-differentiated prostate tumors.\textsuperscript{47} Taylor et al.\textsuperscript{48} later reported that while Spry2 gene inactivation was detected in 18% of the primary prostate cancers studied, it was observed in 74% of the metastatic tumors.\textsuperscript{52} In their study on endometrial carcinoma, Velasco et al.\textsuperscript{48} found that Grade III tumors expressed significantly lower levels of Spry2 protein than did Grades I and II.\textsuperscript{48} By quantitative RT-PCR on paired HCC and non-tumor liver tissues from 31 patients, Sirivatanauskorn et al.\textsuperscript{48} indicated that the expression of Spry2 was significantly lower in advanced disease and in association with angiolympathic invasion.\textsuperscript{48} In an immunohistochemical study on HCC tumor samples from 240 patients, Song et al.\textsuperscript{54} similarly reported that Spry2 downregulation accompanied highly malignant clinico-pathological features of HCC, including advanced TNM stages, poorly-differentiated tumors and those with vascular invasion.\textsuperscript{54} With regard to CRC, however, contradictory reports can be found in the literature. Feng et al.\textsuperscript{52} reported downregulation of Spry2 in association with colon cancer progression and suggested a tumor suppressor role for Spry2.\textsuperscript{52} Examining paired tumor and normal tissue samples from 67 patients with colon cancer by real-time quantitative RT-PCR, they reported that Spry2 was downregulated in 72.7% (16/22) of Stage II, 91.3% (21/23) of Stage III and 100% (22/22) of Stage IV tumors. A negative correlation was

### Table 5. Correlation of Spry2 and Spry4 expressions with VEGF, FGF-2 and IL-6

<table>
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<th>Parameter</th>
<th>Patients no.</th>
<th>Low</th>
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<td>30</td>
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</tr>
<tr>
<td>Spry4</td>
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<tr>
<td>VEGF</td>
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<tr>
<td></td>
<td>High</td>
<td>58</td>
<td>43</td>
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<tr>
<td>FGF-2</td>
<td>Low</td>
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</tr>
<tr>
<td></td>
<td>High</td>
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<tr>
<td>IL-6</td>
<td>Low</td>
<td>68</td>
<td>49</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>High</td>
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No.: number.
also evident between the expression levels of Spry2 and the microRNA miR-21, an indicator of poor survival and poor response to adjuvant chemotherapy in cancer patients. They had previously showed that the expression of Spry2 positively correlates with the sensitivity of colon cancer cells to the EGFR inhibitor gefitinib, and that Spry2 can enhance the response of colon cancer cells to gefitinib in vitro and in vivo by increasing the expression of EGFR and PTEN.\textsuperscript{35} In contrast, through immunofluorescence analysis of colon cancer biopsies quantitatively confirmed in 34 patients, Barbachano \textit{et al.} reported high levels of Spry2, along with low levels of E-cadherin, in undifferentiated, high grade tumors versus low levels of Spry2, and high levels of E-cadherin, in low grade specimens.\textsuperscript{50} In vitro, they found inverse correlation between and reciprocal regulation of Spry2 and E-cadherin in colon cancer cells whereby Spry2 was suggested to demonstrate a tumorigenic role by repressing E-cadherin. A similar role for Spry2 in CRC was suggested by Holgren \textit{et al.} where Spry2 was implicated to control metastatic potential of colon cancer cells, at least in part, by c-Met upregulation.\textsuperscript{38} KRAS mutation, however, was found later to play a critical role in CRC. Examining primary tumor samples from 113 patients with CRC, Watanabe \textit{et al.} found that Spry2 was among the 30 genes which were upregulated in the KRAS mutant CRC tumors.\textsuperscript{51} They found that the discriminating genes identified were related to not only KRas/ERK but other signaling pathways such as Wnt/\beta-catenin, NF-kappa B activation and TGF\beta signaling, thereby suggesting a crosstalk between K-Ras-mediated signaling and other pathways in colorectal cancer.

Secondly, we provided evidence that the expression of Spry2 not only is associated with survival, but might serve as an independent predictor of OS and DFS. Few studies have investigated significance of the Spry2 expression in relation to the clinical outcome of malignancies. Through their meta-analysis of the gene expression profiles of a total of 1,107 breast cancer tumors combined with a further analysis of two single datasets, Faratian \textit{et al.} identified Spry2 as an independent predictor of a more favorable outcome even for tumors with poor pathological features.\textsuperscript{41} In this study, since Spry2 gene expression was inversely correlated with that of human epidermal growth factor receptor-2 gene (HER2) and Spry2 was also shown in vitro to act synergistically with the HER2-targeting trastuzumab to reduce cell viability, the investigators then quantified the expression of Spry2 protein in a cohort of 122 trastuzumab-treated patients using the AQUA fluorescence image analysis system. Their results revealed that low Spry2 expression was associated with poor outcome and increased risk of death, thereby suggesting the usefulness of Spry2 in stratifying patients for trastuzumab therapy.\textsuperscript{41} In their immunohistochemical study on HCC addressed earlier, Song \textit{et al.} found that Spry2-negative patients had poorer survival and increased postoperative recurrence, and identified Spry2 as an independent predictor of recurrence.\textsuperscript{44} In a study by Sanchez \textit{et al.} on 55 patients with B-cell diffuse lymphoma, Spry2 promoter hypermethylation was found to be significantly associated with a lower 5-year survival rate.\textsuperscript{46} Apart from the significant downregulation in EOC, no other significant results were obtained in the present study in relation to Spry4 protein. Of the two Sprouty isoforms studied, our literature review revealed that Spry4 is the least-studied homolog with regard to cancer biology, in particular from the clinical point of view. In a study by Frolov \textit{et al.} exploring biological markers of response to the c-Kit inhibitor imatinib in GISTS, Spry4 gene was identified among imatinib-responsive genes and Spry4 protein as a downstream effector of the c-Kit-activated ERK targeted by the drug.\textsuperscript{40} In their clinical investigation on seven patients, they then found that Spry4 levels were dramatically decreased in imatinib-responsive cases and thus proposed Spry4 as a reliable marker of the imatinib-responsive treatment. This article, however, was the only report of the clinical significance of the Spry4 expression in cancer we could find in the literature. On this basis, role of Spry4 in cancer needs to be further elucidated in future research.

In conclusion, we report for the first time to our knowledge that Spry2 and Spry4 proteins are downregulated in EOC. Our findings provide the evidence that the expression of Spry2 protein impacts EOC tumor behavior and predicts the patient outcome. Spry2 is thus a promising biomarker with independent predictive value and high specificity and sensitivity for prognostication of EOC. Results from the present study also lay the basis for evaluation of this protein family in therapeutic approaches, including patient stratification in personalized therapy.

References


Spry2 and Spry4 proteins in human epithelial ovarian cancer