Knockdown of splicing factor SRp20 causes apoptosis in ovarian cancer cells and its expression is associated with malignancy of epithelial ovarian cancer

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Abstract

Our previous study revealed that two splicing factors, polypyrimidine tract-binding protein (PTB) and SRp20, were up-regulated in epithelial ovarian cancer (EOC) and knockdown of PTB expression inhibited ovarian tumor cell growth and transformation properties. In this report, we show that knockdown of SRp20 expression in ovarian cancer cells also causes substantial inhibition of tumor cell growth and colony formation in soft agar and the extent of such inhibition appeared to correlate with the extent of suppression of SRp20. Massive knockdown of SRp20 expression triggered remarkable apoptosis in these cells. These results suggest that overexpression of SRp20 is required for ovarian tumor cell growth and survival. Immunohistochemical staining for PTB and SRp20 of two specialized tissue microarrays (TMAs), one containing benign ovarian tumors, borderline/low malignant potential (LMP) ovarian tumors as well as invasive EOC and the other containing invasive EOC ranging from stage I to stage IV disease, reveals that PTB and SRp20 are both expressed differentially between benign tumors and invasive EOC, and between borderline/LMP tumors and invasive EOC. There were more all-negative or mixed staining cases (at least two evaluable section cores per case) in benign tumors than in invasive EOC while there were more all positive staining cases in invasive EOC than in the other two disease classifications. Among invasive EOC, the great majority of cases were stained all-positive for both PTB and SRp20 and there were no significant differences in average staining or frequency of positive cancer cells between any of the tumor stages. Therefore, the expression of PTB and SRp20 is associated with malignancy of ovarian tumors but not with stage of invasive EOC.

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Keywords
splicing factors; polypyrimidine tract-binding protein; SRp20; siRNA; tissue microarray; epithelial ovarian cancer

Introduction

Ovarian cancer (OC) is the most lethal gynecological malignancy and was estimated to cause 14,600 deaths in the United States in 2009 (Jemal et al., 2009). Despite considerable advances in the treatment of this disease, the mortality of OC has not decreased significantly over the past decades (Ries et al., 2007), and the majority of patients with OC, especially those at advanced stages, still succumb to it. This dismal outcome is a consequence, in part, of the absence of reliable biomarkers for early detection as well as the lack of effective therapies for advanced and relapsed disease, both of which, in turn, reflect our poor understanding of the underlying biology of OC. Therefore, to improve patient survival, more work is needed to identify the molecular events that are associated with ovarian oncogenesis in order for new biomarkers and new therapeutic targets to be discovered.

Defects in pre-mRNA splicing have been shown to be causes of a variety of human diseases, including cancer (Caceres & Kornblihtt, 2002; Stoilov et al., 2002). Mounting evidence has revealed that altered splicing is associated with and possibly involved in tumor progression and/or metastasis (Bartel et al., 2002; De Marzo et al., 1998; Feltes et al., 2002; Gunther et al., 1991; Lukas et al., 2001; Persengiev et al., 2004; Sanchez Lockhart et al., 2001; Schroder et al., 1999; Silberstein et al., 1997; Stickeler et al., 1999; Wielenga et al., 1993; Xie et al., 2003). Out of the most studied examples is the correlation between CD44 splice variants (SVs) and tumor progression. CD44 SVs are aberrantly expressed in many human tumors including breast and ovarian cancers (De Marzo et al., 1998; Sanchez Lockhart et al., 2001; Schroder et al., 1999; Wielenga et al., 1993). Some CD44 variants are associated with the metastatic potential of cells, and their expression levels are an indicator of poor prognosis (De Marzo et al., 1998; Wielenga et al., 1993). Recent computational analyses based on alignments of expressed sequence tags (EST) to human RefSeq mRNAs or human genomic DNA showed that many alternative splicing (AS) forms were significantly associated with cancer, and the majority of these genes have functions related to cancer (Wang et al., 2003; Xu & Lee, 2003). These results suggest that altered splicing might be widespread, and tumor-specific SVs may play a functional role in human tumors.

In general, the direct causes behind the alteration of pre-mRNA splicing can be divided into two categories: mutations in cis-elements and changes in trans-acting factors. At present, it is not clear whether mutations make significant contributions to aberrant splicing found in tumors. As for CD44, its misregulated splicing is not the result of gene mutation; rather, altered splicing patterns are very likely due to changes in trans-acting splicing factors. Indeed, in a mouse model of mammary gland tumorigenesis, expression of some serine/arginine-rich (SR) protein family members was found to be altered during tumor progression, and alternative splicing of CD44 correlated with the expression of these SR proteins (Stickeler et al., 1999). It was also reported that activation of oncogenic
Ras/Raf/MEK/ERK signaling pathway controlled the inclusion of variable exon 5 (v5) of CD44 (Konig et al., 1998; Matter et al., 2000; Weg-Remers et al., 2001). We observed that two splicing factors, polypyrimidine tract-binding protein (PTB) and SRp20, were highly expressed in human ovarian tumors, compared with matched normal ovarian tissues (He et al., 2004). Knockdown of PTB expression substantially impaired the growth and transformation properties of an ovarian tumor cell line (He et al., 2007).

As a negative regulator of pre-mRNA splicing, PTB blocks the inclusion of a variety of alternative exons into mRNA (Black, 2003; Wagner & Garcia-Blanco, 2001). It is also involved in the regulation of polyadenylation (Castelo-Branco et al., 2004; Lou et al., 1999), mRNA stability (Hamilton et al., 2003; Knoch et al., 2004; Kosinski et al., 2003), mRNA export from the nucleus (Zang et al., 2001) and mRNA localization in the cytoplasm (Cote et al., 1999). Another important function of PTB is to modulate the internal ribosomal entry site (IRES)-mediated translation (Cornelis et al., 2005; Mitchell et al., 2001). Besides ovarian tumors, PTB was also found overexpressed in glioblastomas (Jin et al., 2003; Jin et al., 2000).

SRp20 belongs to the serine-arginine (SR) rich protein family, a group of essential splicing factors required at different steps of spliceosome assembly (Graveley, 2000). It has been shown that SRp20 regulates the splicing of fibronectin (de la Mata & Kornblitt, 2006), tau (Yu et al., 2004), insulin receptor (Sen et al., 2009), CD44 (Galiana-Arnoux et al., 2003) and itself (Jumaa & Nielsen, 2000). It also plays roles in mRNA polyadenylation (Lou et al., 1998) and export (Huang & Steitz, 2001). A knock-out study indicated that SRp20 was essential for mouse development (Jumaa & Nielsen, 1999).

In the present study, we hypothesize that overexpressed SRp20, like overexpressed PTB, is required for ovarian tumor cell growth and maintenance of transformation properties. Therefore, we examined herein the effects of SRp20 knockdown in ovarian cancer cells and found that this manipulation impairs ovarian tumor cell growth and malignant properties, similar to what we have demonstrated with PTB (He et al., 2007). Moreover, we found that substantial suppression of SRp20 expression caused apoptosis in ovarian cancer cells. Because of these effects of PTB and SRp20 on ovarian tumor growth and transformation properties in cell culture, we asked whether the expression of PTB and SRp20 is related to the malignancy and stage of ovarian tumors from patients. By immunohistochemical staining of epithelial ovarian tumor tissue microarrays (TMAs) for PTB and SRp20, we found that the expression of these two splicing factors is associated with malignancy of epithelial OC (EOC) but not with tumor stage.

Results

SRp20 knockdown inhibits ovarian cancer cell growth

We previously reported that splicing factors PTB and SRp20 were overexpressed in human ovarian tumors compared to normal ovarian tissues (He et al., 2004), and subsequently showed that suppression of PTB expression by siRNA substantially inhibited ovarian cancer cell growth and malignant properties (He et al., 2007). However, what role, if any, SRp20 may have in ovarian cancer remained unanswered, due to challenges in manipulating SRp20.
expression by siRNA which were recently overcome. After testing over 15 siRNAs (designed by ourselves or by commercial companies) targeting various regions of human SRp20 mRNA, we have now identified two effective siRNAs, SRp20si1 and SRp20si2, which can knockdown SRp20 expression by about 50% and about 90%, respectively, in A2780 cells. A Dox-inducible lentiviral system was constructed to deliver and express SRp20si1 or SRp20si2 in the cell as we had done for siRNAs against PTB (He et al., 2007). Following lentiviral infection, A2780 sublines A2780/SRp20si1 and A2780/SRp20si2 were established that expressed SRp20si1 and SRp20si2, respectively, under the control of Dox. The Dox-induced suppression of SRp20 expression is shown in Fig 1A. The effects of SRp20 knockdown on cell growth was determined by cell growth curve. As shown in Fig 1B, the growth of A2780/SRp20si1 and A2780/SRp20si2 cells was inhibited in the presence of Dox compared to the growth without Dox, but the growth of control subline A2780/LUCsi, which expresses Dox-induced luciferase siRNA, was not affected by Dox treatment. The growth inhibition was detectable by day 4, corresponding to the time when SRp20 started to decrease, which occurred by day 3 (data not shown). In addition, the extent of growth inhibition appeared to correlate with the extent of suppression of SRp20 expression. Growth inhibition by SRp20 knockdown was also observed in two other ovarian cancer cell lines, SKOV3 and IGROV1, as shown in Fig s1B and Fig s2B, respectively, in the Supplemental data.

SRp20 knockdown inhibits anchorage-independent growth (AIG) of ovarian cancer cells

AIG is a characteristic phenotype of transformed cells and is measured by colony formation assay in soft agar. To determine whether SRp20 overexpression is required to maintain AIG of ovarian cancer cells, we examined the effect of SRp20 knockdown on A2780 cells' capability to form colonies in soft agar. As shown in Fig 2, A2780/SRp20si1 and A2780/SRp20si2 cells formed substantially fewer and smaller colonies when they were grown in the presence of Dox (i.e. SRp20 expression was suppressed) than when they were grown in the absence of Dox. Moreover, the degree of AIG inhibition correlated with the extent of SRp20 suppression by siRNA. In A2780/SRp20si1 cells, Dox treatment induced knockdown of SRp20 expression about 50% (Fig 1A), which subsequently led to inhibition of colony formation -- fewer and smaller colonies (Fig 2). By contrast, in A2780/SRp20si2 cells, SRp20 expression was suppressed more than 90% by Dox induction (Fig 1A), which resulted in almost complete abolition of colony formation (Fig 1B).

SRp20 knockdown induces apoptosis in ovarian cancer cells

To determine whether the inhibition of cell growth by SRp20 knockdown was due to increased apoptosis, we stained A2780 sublines treated with or without Dox for five days with Hoechst 33342 and counted apoptotic cells. As shown in Fig 3, the percentage of apoptotic cells in A2780/SRp20si2 subline cells was remarkably increased after Dox treatment, which induced substantial suppression of SRp20 by more than 90% (see Fig 1A). By contrast, moderate suppression by Dox treatment of SRp20 expression (~ 50% reduction, see Fig 1A) in the A2780/SRp20si1 subline did not trigger apoptosis, which was maintained at similarly low levels in both Dox-treated and untreated cells. Consistent with these results, we also observed substantial increases in apoptosis in the SKOV3/SRp20si2
and IGROV1/SRp20si2 sublines after Dox treatment but not in those sublines expressing SRp20si1 or control siRNA (Fig s1C and Fig s2C in the Supplemental Data).

**SRp20 knockdown activates intrinsic apoptotic pathway**

Apoptosis is triggered through three major cellular pathways, i.e. the extrinsic pathway, the intrinsic pathway and the PIDDosome-mediated pathway (Li & Yuan, 2008). Caspase-8, caspase-9 and caspase-2 are corresponding initiator caspases in these pathways. To determine which pathway is activated in SRp20 knockdown-induced apoptosis, we examined the activation of caspase-8, caspase-9 and caspase-2 by Western Blotting. The antibodies against caspases-2 and-8 can recognize procaspases as well as cleaved fragments and the antibody against caspase-9 detects only cleaved caspase-9. As shown in Fig 4A, cleaved caspase-9 was detected in Dox-treated A2780/SRp20si2 cells, which showed substantial increase in apoptosis (Fig 3), but not detected in Dox-treated A2780/SRp20si1 cells and in untreated cells. In contrast, no cleaved caspases-2 and -8 but procaspase-2 and -8 were detected in any of these cells. We further examined the activation of downstream effector caspase-3, -6 and -7 using antibodies against the cleaved fragments of these caspases and found that caspase-3 and caspase-7 were activated in Dox-treated A2780/SRp20si2 cells but not in other cells (Fig 4B). We failed to detect the cleaved caspase 6 in any of these cells (data not shown). These results indicate that the intrinsic apoptotic pathway is activated in SRp20 massively knocked-down A2780 cells. Since Bcl-2 is a major anti-apoptotic regulator of the intrinsic pathway (Youle & Strasser, 2008), we asked whether the apoptosis induced by SRp20 knockdown is mediated through its action on Bcl-2. Therefore, we examined the expression of Bcl-2 in A2780/SRp20si1 and A2780/SRp20si2 cells by Western Blotting. As shown in Fig 4C, Bcl-2 is indeed down-regulated in Dox-treated A2780/SRp20si2 cells but not in other cells.

**Expression of PTB and SRp20 is associated with malignancy of human ovarian tumors**

Our above and previously reported results (He et al., 2007) indicate that overexpression of PTB and SRp20 is required for growth and maintenance of transformed properties of ovarian tumor cells. To further assess the clinical significance of these two splicing factors, we studied the expression of PTB and SRp20 in non-malignant epithelial ovarian tumors in comparison with malignant tumors by immunohistochemical staining of ovarian disease status TMAs, which contain benign, borderline/LMP and invasive ovarian tumors. After staining, we had 133 valid cases for analysis of PTB expression and 117 valid cases for analysis of SRp20 expression. Our rule for valid cases was that there were a minimum of 2 satisfactory cores for each case. Unsatisfactory cases were those with missing core(s), scant/insufficient tumor cells, increased background or folded/wrinkled/torn sections. The distribution of these valid cases is summarized in Table s1 of Supplemental Data. Representative staining for PTB and SRp20 in benign tumor, borderline/LMP tumor and invasive EOC are shown in Fig 5. We categorized the average staining for each case into three groups: all negative (all evaluable cores were negative), all positive (all evaluable cores were positive), and mixed (at least one evaluable core negative and one evaluable core positive). Based on this categorization, the results of staining for PTB and SRp20 are summarized in Fig 5B.
As shown in the figure, the percentage of cases that stained all positive increased while the percentage of cases stained all negative or mixed decreased in the order of benign tumor, borderline/LMP tumor and invasive EOC. Approximately 85% and 97% of invasive EOC stained all positive for PTB and SRp20, respectively, whereas a great majority of benign ovarian tumors stained all negative or mixed for PTB with only 17.6% stained all positive. The percentages of borderline/LMP ovarian tumors that stained all positive, all negative, or mixed fell between those of benign and invasive tumors. Statistical analyses indicated that the differences in PTB staining among benign, borderline/LMP and invasive ovarian tumors were significant in both overall comparison and all pair-wise comparisons (p<0.01 for all comparisons). SRp20 staining varied significantly between benign and invasive tumors (p<0.01) and between borderline/LMP and invasive tumors (p<0.01) but not between benign and borderline/LMP tumors.

Further analysis focusing on mucinous tumors generated results consistent with the above conclusion, i.e. both PTB and SRp20 were expressed differentially between benign and invasive tumors (p<0.05) and between borderline/LMP and invasive tumors (p<0.05) but not between benign and borderline/LMP tumors. The staining results in this group of tumors are summarized in Table S2 of Supplemental Data. Other subtypes on this TMA could not be further analyzed because of the limited number of valid cases retained after staining.

Among cases with at least one positive core, we calculated the average frequency of positive cancer cells for each case. When this average frequency was categorized into low (<50% positive tumor cells) or high (≥50% positive tumor cells), a significant association was observed between disease status and categorized staining frequency for PTB (p<0.001 for the overall comparison between the three groups, p<0.01 for the pairwise comparison between benign and invasive tumors and between borderline/LMP and invasive tumors) or SRp20 (p<0.01 for the overall comparison between the three groups, p<0.01 for benign vs. invasive tumors, and p<0.05 for borderline/LMP vs. invasive tumors), as shown in Fig 5C.

Staining intensity for each case with at least one positive core was categorized as low (weak to light brown staining; score<2.0) or high (moderate to intense dark brown staining; score ≥2.0). The results are shown in Fig 5D. There was statistical evidence of an association between disease status and categorized staining intensity for PTB (p<0.05 for the overall comparison between the three groups and p<0.05 for the pairwise comparison between benign and invasive tumors), but not for SRp20 (p>0.05).

In summary, both PTB and SRp20 were differentially expressed between benign tumors and invasive EOC, and between borderline/LMP tumors and invasive EOC, but the modest differences between benign and borderline/LMP tumors were not always statistically significant.

**Expression of PTB and SRp20 is not associated with stage of human EOC**

We also asked whether the expression of PTB and/or SRp20 is correlated with the stage of EOC. Therefore, we conducted immunohistochemical staining of ovarian cancer stage TMAs that contain EOC specimens ranging from stage I to stage IV. After staining, there were 169 and 161 cases, respectively, that were judged to be valid for analysis of PTB and
SRp20 expression. The rule for valid cases was the same as above. The major subtypes of EOC on this TMA were serous and endometrioid. The distribution of valid cases on this TMA is summarized in Table s3 of Supplemental Data. Representative staining of stages I to IV serous and endometrioid EOC for PTB and SRp20 is shown in Fig 6A. The results of average staining, categorized as all negative, all positive and mixed, are summarized in Fig 6B. As seen in the figure, no case was stained all negative and the great majority of cases were stained all positive for both PTB and SRp20, which was consistent with what we observed in staining of the disease status TMA described above. Statistical analysis indicated that there was no statistical evidence of a significant difference in average staining or frequency of positive cancer cells for either PTB or SRp20 among the four stages or between any two stages. However, the intensity of staining for PTB or SRp20, when categorized as low (score <2.5) or high (score ≥2.5), as shown in Table s4 of the Supplemental Data, was associated with categorized stages, i.e. advanced stage (stages III and IV) exhibited greater intensity than early stage (stages I and II) (p=0.019 for PTB staining and p=0.002 for SRp20 staining).

Discussion

We previously reported that overexpressed PTB played an important role in maintaining ovarian tumor cell growth and transformation properties (He et al., 2007). Here we provide the first evidence to show that overexpressed SRp20 is also required for ovarian tumor cell growth and survival. Moderate knockdown of SRp20 expression caused substantial inhibition of tumor cell growth and colony formation in soft agar while nearly complete suppression of this protein triggered substantial apoptosis in these cells. The mechanism(s) mediating SRp20's role in ovarian tumor cells remains to be elucidated. Based on its known molecular functions, SRp20 may exert its role directly by itself and/or indirectly via other molecules regulated by its activities. A recent report has demonstrated that SRp20, as well as SF2/ASF, associates with chromatin before and after mitosis but is excluded from chromatin during mitosis (Loomis et al., 2009). This newly identified interaction between SR proteins and the chromosome implies that such proteins may be involved in the regulation of chromatin structure and function and thus may play a role in the control of cell cycle progression. Therefore, the depletion of SRp20 could directly disrupt the cell cycle process, which subsequently causes growth inhibition and apoptosis. Nonetheless, it is still possible that the effects of SRp20 knockdown are mediated by other proteins under its regulation. For example, we showed that SRp20 knockdown-triggered apoptosis was mediated by down-regulation of Bcl-2 (Fig 4C), which subsequently activates the intrinsic apoptotic pathway. Microarray analysis has revealed that the expression and splicing of hundreds of genes are altered in SRp20 knockdown cells (X He, AD Arsalan, TT Ho, WT Beck, unpublished results).

Aberrant splicing is a common phenomenon found in human tumors (Venables, 2004). Our results presented herein and those of others (Watermann et al., 2006) suggest that abnormal regulation of splicing factor expression could be responsible, in part, for this phenomenon. Given the importance of alternative splicing in the generation of proteomic complexity (Matlin et al., 2005), it is conceivable that up- or down-regulation of splicing factors may be an indispensable component of the process of tumorigenesis that is involved in mediating
the effects of transformation. Thus, controlling splicing factor expression may become a novel and effective way to inhibit tumor cell growth; that is, certain splicing factors may be good therapeutic targets. Our data reported here showing the effects of SRp20 knockdown as well as our previous studies (He et al., 2007) support this idea. Our preliminary experiment with a mouse xenograft model provided further evidence that ovarian tumor growth can be inhibited by suppression of PTB expression (X He, AD Arsalan, TT Ho and WT Beck, unpublished results).

In the present study, we also examined the expression of PTB and SRp20 in ovarian tumors by immunohistochemical staining of two specialized ovarian tumor TMAs - one focusing on tumor progression and the other focusing on cancer stages. Our results reveal that PTB and SRp20 are differentially expressed among benign ovarian tumors, borderline/LMP ovarian tumors and invasive EOC, with benign tumors having the lowest percentage of all positive cases and the highest percentage of all negative cases and invasive EOC having the highest percentage of all positive cases and the lowest percentage of all negative cases. By contrast, we found no significant differences among invasive EOC and both splicing factors were highly expressed in ovarian tumors of early stages as well as late stages. Together, these results suggest that the expression of PTB and SRp20 is associated with malignancy of ovarian tumors. This observation is consistent with our previous finding that overexpression of PTB and SRp20 is an early event in the ovarian tumorigenesis (He et al., 2007). Because we did not have access to follow-up information of the cases on the TMAs, we could not assess whether there was a correlation between the expression of these two splicing factors and patient clinical outcome. It merits further investigation to determine whether there are differences in clinical outcome between negatively stained cases and positively stained cases.

Overexpression of PTB and SRp20 in invasive EOC also raises a question of how other splicing factors are changed in this disease. Our previous and current studies revealed that the overexpression occurred only in certain splicing factors, because we found that two other splicing factors, SF2/ASF and U2AF65, were expressed at similar levels in both normal ovarian epithelia and tumor ovarian cells (He et al., 2007). Therefore, to have a better understanding of the roles of alternative splicing and splicing factors in ovarian tumorigenesis, we will need to examine the expression profile of all splicing factors in normal and transformed ovarian epithelial cells as well as in ovarian tumor cells and correlate these changes with ovarian tumor progression and response to therapy. According to some proteomic analyses (Jurica & Moore, 2003), there are approximately 336 proteins identified as components of splicing machinery. It will be clinically important to know whether there exist any splicing factor signatures for ovarian tumors or their subtypes.

Materials and Methods

Cell lines and culture conditions

Ovarian cancer cell lines A2780, IGROV1 and SKOV3 were obtained from the National Cancer Institute and were maintained in DMEM (A2780 and SKOV3) or RPMI1640 (IGROV1) supplemented with 10% FBS under a humid environment at 37°C, 5% CO2.
Doxycycline (Dox)-induced RNA interference (RNAi)

We used the lentiviral system developed in Dr. Didier Trono’s lab (Wiznerowicz & Trono, 2003) to achieve Dox-induced siRNA expression in the cell. The sequences of two oligonucleotides whose transcripts can be processed into effective SRp20-targeted siRNAs, SRp20si1 and SRp20si2, respectively, are 5′- CGCGCCGGCGAGAGCTAGATGGAAGAACA CTGAGTTCTTCCATCTACTAGCT CTCGTTTTT-3′ and 5′- CGCGCCGGGACGGAATTGGAACGGGCTTT TCAGAGGAGCCCGTTCAATTC CGTCTTTTTT-3′ with SRp20si1 and SRp20si2 sequences in bold. The procedure for cloning the sequences into the lentiviral vector and preparation of lentiviruses is described in the Supplemental Materials and Methods.

Western blot analysis

Described in the Supplemental Materials and Methods.

Cell growth curve

Described in the Supplemental Materials and Methods.

Colony formation assay in soft agar

Described in the Supplemental Materials and Methods.

Apoptosis assay

Described in the Supplemental Materials and Methods.

Tissue microarray (TMA)

Two types of TMA were used in this study: one was an ovarian disease status TMA and the other was an ovarian cancer stage TMA. The detailed description of these TMAs is provided in the Supplemental Materials and Methods.

Immunohistochemical staining

Unstained slides were first deparaffinized three times, 5 min each, in xylene, and then rehydrated sequentially in 100%, 95% and 80% ethanol two times, respectively, 5 min each. To quench any endogenous peroxidase, slides were incubated in 0.1% H2O2 for 30 min. After antigen retrieval in boiling 10 mM sodium citrate for 10 min, the TMAs were blocked in 1.5% normal horse serum for 2 h, followed by incubation overnight with 1:5 diluted primary antibody PTB (Ab-1) (Oncogene Research Products, San Diego, CA) or 1:5 diluted SRp20 antibody (7B4) (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:500 diluted SF2/ASF antibody (Zymed Laboratories Inc, South San Francisco, CA) and then with biotinylated secondary antibody for 2 h. The antibody binding was detected by Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) and visualized with peroxidase substrate 3, 3′-diaminobenzidine (DAB) solution. Afterwards, tissue sections were counterstained in hematoxylin solution, Gill No. 1 (Sigma, St. Lois, MO). A positive and a negative tissue staining controls were included in every staining run to assure staining quality.
Evaluation of PTB and SRp20 Expression in tissue sections

Stained slides were evaluated independently by two pathologists (M.P. and J.C.). The evaluation included an assessment of staining consistency of replicate tissue cores for each case (all negative, all positive, or mixed, meaning at least one core was positive and one negative). A core was scored positive if at least 10% of the tumor cells expressed the marker in question. The frequency of positive cells and staining intensity were also categorized, as explained in detail in the Results section. The staining categories assigned for each case were approximately 95% concordant for multiple viewings of the same core by each pathologist and 90% concordant between the 2 pathologists evaluating them. Discrepant categories were resolved by consensus using a double headed microscope.

Statistical Analysis

Biomarker and pathological data for this study were analyzed using SPSS version 10.1 (SPSS Inc., Chicago, IL) or SAS version 8.2 (SAS Institute Inc., Cary, NC). Fisher's exact test was used to test the hypothesis of independence between categorical variables in 2 × 2 tables (Fisher, 1922). The Mehta and Patel version of Fisher's exact test was used to test categorical variables in r × c tables (Mehta & Patel, 1983). Student's t-test was used in comparisons between samples treated with or without Dox. All tests were two-sided and p-values<0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.
SRp20 knockdown inhibits ovarian cancer cell growth. A: Western blot showing the suppression of SRp20 expression by Dox induction of SRp20 siRNAs in A2780 subline cells. B: Cell growth curve. Shown are the results of three independent experiments. Error bars represent standard error. A2780/LUCsi, A2780/SRp20si1 and A2780/SRp20si2 are A2780 sublines stably expressing the Dox-induced luciferase siRNA, SRp20 siRNA1 and SRp20 siRNA2, respectively.
Fig 2.
SRp20 knockdown inhibits anchorage-independent growth of ovarian cancer cells. A: Sample pictures showing colonies formed in soft agar. B: Average ratios (expressed in percentage) of colony numbers formed in the presence vs in the absence of Dox (n=3). Error bar: standard error.
Fig 3.
SRp20 knockdown induces apoptosis in ovarian cancer cells. A: Sample micrographs of Hoechst 33342-stained nuclei of A2780 subline cells. Arrow indicates the typical apoptotic cells. B: Percentage of apoptotic cells. Shown are averages of three independent experiments. Error bar: standard error.
Fig 4.
SRp20 knockdown activates the intrinsic apoptotic pathway. 
A: Western blot of initiator caspases, caspase-9, -2 and -8. The antibody against caspase-9 detects only cleaved form and the antibodies against caspase-2 and -8 detect both procaspases and cleaved fragments.
B: Western blot of effector caspases, caspase-3 and -7. The antibodies against caspase-3 and -7 detect only cleaved fragments. 
C: Western blot of Bcl-2.
Fig 5.
Expression of PTB and SRp20 is associated with malignancy of human ovarian tumors. A. Sample micrographs of staining for PTB or SRp20 of ovarian disease status TMA. Shown are cases of mucinous ovarian tumors. Magnification: 400×. B. Summary of overall PTB and SRp20 staining categorized into all negative, mixed and all positive. For PTB staining, p<0.01 for all pair-wise comparisons; For SRp20 staining, p<0.01 for benign vs invasive tumors and borderline/LMP vs invasive tumors; P>0.05 for benign vs borderline/LMP tumors. C. Frequencies of PTB and SRp20 staining in Ovarian Disease Status TMAs. For
PTB staining, p<0.01 for benign vs invasive tumors and borderline/LMP vs invasive tumors. For SRp20 staining, p<0.01 for benign vs invasive tumors; p<0.05 for borderline/LMP vs invasive tumors. D. Intensity of PTB and SRp20 staining in Ovarian Disease Status TMAs. For PTB staining, p<0.05 for benign vs invasive tumors.
Fig 6.
Expression of PTB and SRp20 is not associated with ovarian cancer stage. A. Sample micrographs of staining for PTB or SRp20 of Ovarian Cancer Stage TMA. Magnification: 400×. B. Summary of overall PTB and SRp20 staining categorized into all negative, mixed and all positive. No case was all negative.