# Expression of splicing factors in human ovarian cancer

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**Abstract.** Alternative splicing represents an important nuclear mechanism in the post-transcriptional regulation of gene expression, which is frequently altered during tumorigenesis. Previously, we have described marked changes in alternative splicing of the CD44 gene in ovarian and breast cancer. In the latter one we described also a specific induction of splicing factors during tumor development. Now we have focussed our studies on the expression profiles of splicing factors, including classical SR proteins, Tra2 and YB-1 in physiological and malignant ovarian tissues by RT-PCR and Western blot analysis. We detected changed expression pattern with higher levels of phosphorylated 30 kDa SR proteins as well as relatively high concentrations of hyperphosphorylated Tra2 protein isoforms in ovarian cancer. RT-PCR analysis revealed a marked induction of SC35 and ASF/SF2 as well as mRNA levels in malignant ovarian tissue. These results suggest gene-specific alterations of expression rather than a general induction of the splicing machinery. Together with previously performed functional studies of CD44 splicing these findings implicate that altered expression profiles of SR proteins, Tra2ß and YB-1 might be responsible for the known changes of alternative CD44 splicing in ovarian cancer.

## Introduction

Alternative pre-mRNA processing represents an important mechanism in the regulation of gene expression. Due to the effort of the Human Genome Project it is known that as many as 40-60% of human genes utilize alternative RNA processing leading to a diversity of gene products (1-4). Splicing has been more recently quantified. For humans, it was shown that 59% of the genes present on chromosome 22 are alternatively spliced (5). The comparison of expressed sequence tags with the human genome sequence indicated that 47% of all human genes are alternatively spliced (6).

Besides the physiological importance of this process, alternative splicing is affected during tumor development, progression and metastasis (7-10). Substantial genes encoding for cell-surface receptors, transcription factors and signal transduction proteins are known to be alternatively spliced, suggesting pronounced changes in behavior of tumor cells. However, little is known about the expression of splicing factors in normal and malignant tissues leading to altered expression pattern of splicing factors, which might affect the RNA processing.

SR proteins represent a family of essential splicing factors (11,12), which are characterized by extensively phosphorylated serine-arginine-rich (SR) domains (13). When phosphorylated, these domains are recognized by the murine monoclonal antibody mAb 104. In HeLa cells, this mAb recognizes the major SR proteins SRp20, ASF/SF2, SC35, 9G8, SRp30c, SRp40, SRp55, and SRp75 (14,15). SR proteins recognize splice sites and exonic splicing enhancers. Depending on their relative concentrations they are able to influence alternative RNA processing *in vivo* and *in vitro* (11,16-18). In addition, individual members of this nuclear protein family exhibit substrate-specific binding activity and show distinct tissue distribution (19,20). The tissue distribution was shown by Hanamura *et al* (21). We were previously able to show marked and specific alterations in the expression patterns of SR proteins during breast cancer development using an established mouse model of mammary tumorigenesis. These changes were accompanied by an altered pattern of alternative spliced CD44 proteins (22).

Furthermore, we have investigated splicing changes of the CD44 gene in ovarian cancer and observed a complex expression pattern of the variable protein isoforms CD44v5, v6 and v7 in ovarian cancer cell lines as well as abnormal retention of the CD44 intron 9 in 60% of all cell lines expressing CD44. In contrast, physiological ovarian tissue was found to be negative for intron retention (23,24). These results characterized *de novo* expression of certain CD44 isoforms and abnormal RNA processing with retention of non-coding CD44 intron sequences as common for established ovarian cancer. Whether these events are crucial during development of ovarian cancer remains to be elucidated.

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Table I. Sequence of primers used for PCR of splicing factors and size for expected amplicons.

| Gene | Primers | Amplicon size (bp) |
|---|---|---|
| SRp20 | Sense: 5'-GCCGTGTAAGAGTGG-3'<br>Antisense: 5'-AAGCTTCTCCTTCTTGGA-3' | 142 |
| SC35 | Sense: 5'-AGGGAATCCAAATCCAGGTC-3'<br>Antisense: 5'-CTGCTACACAACTGCGCCTA-3' | 693 |
| ASF/SF2 | Sense: 5'-TACCTCCAGACATCCGAACC-3'<br>Antisense: 5'-TGCCATCTCGGTAAACATCA-3' | 403 |
| SRp40 | Sense: 5'-CATCATGAGTGGCTGTCCGGTATT-3'<br>Antisense: 5'-ATTTAATTTAGGTCGGTGTGATC-3' | 443 |
| SRp55 | Sense: 5'-CGTTCGACAACCAGCCCTTG-3'<br>Antisense: 5'-AGTCCTCGAACTCCACGAAG-3' | 173 |
| Tra2α | Sense: 5'-CGAGGTCAAGGAGACATTCTCAT-3'<br>Antisense: 5'-TATCATAGTAAGAATCTCGACGT-3' | 455 |
| Tra2ß | Sense: 5'-CATAGACGATCACGTAGCAGGT-3'<br>Antisense: 5'-GAGAGCTGCCATAGGTAGGTC-3' | 420 |
| YB-1 | Sense: 5'-TCGCAGTGTAGGAGATGGAGAGACT-3'<br>Antisense: 5'-CGGTACCGACGTTGAGGTGGCT-3' | 544 |
| ß-actin | Sense: 5'-TGACGGGGTCACCCACACTGTCCCCATCTA-3'<br>Antisense: 5'-CTAGAAGCATTGCGGTGGACGATCGAGGG-3' | 661 |

Functional studies with respect to the specific regulation of CD44 splicing revealed distinct effects of SR proteins, the human YB-1 protein and human Tra2 (25).

Here we report on a pilot study focussing on the expression pattern of classical SR proteins, Tra2 and YB-1 in physiological and malignant ovarian tissue. Ovarian cancer is characterized by an increased expression of distinct SR proteins, Tra2ß and YB-1. Just a subset of SR proteins were induced in tumors, suggesting gene-specific alterations rather than an unspecific boost of the general RNA processing machinery. Together with the functional analysis of CD44 splicing these findings support the hypothesis that alterations in the expression of SR proteins and induction of YB-1 are causing changes in CD44 splicing. Moreover, this might be a crucial step during tumorigenesis and manifestation of serous ovarian cancer.

## Materials and methods

*Tissues.* Archived samples of the Department of Pathology from normal ovary (n=4) and ovarian cancer (n=5) were submitted to analysis of the splicing factor profile. Samples were initially taken during cytoreductive surgery (serous ovarian cancer FIGO III-IV) or during combined ovarectomy and hysterectomy for benign disease. In all cases, diagnosis of normal or malignant tissue was performed by the local pathologist. All specimen were frozen in liquid nitrogen and stored at -80°C until further analysis.

*RNA isolation and RT-PCR.* Tissues were minced on dry ice before treatment with a tissue homogenizer (Polytron, Littau, Switzerland) in TRIzol solution (1 ml per 100 mg tissue, 3 times 10 sec; Gibco-BRL, Gaithersburg, MD). After 5 min of incubation at room temperature (RT), chloroform (0.2 ml per 1 ml of TRIzol) was added, the solution was shaken vigorously and incubated (RT, 5 min). After centrifugation (12,000 x g, 15 min, 4°C) total RNA was precipitated by addition of isopropanyl alcohol to the aqueous phase (0.5 ml per 1 ml of TRIzol, 10 min RT). The RNA was pelleted by centrifugation (12,000 x g, 10 min, 4°C), washed with 75% ethanol (1 ml of ethanol per 1 ml of TRIzol reagent used for the initial homogenization), recovered by centrifugation (7,500 x g, 5 min, 4°C), finally dissolved in RNAse-free water and stored at -80°C for further analysis.

Five micrograms of RNA, as determined by optical densitometry, were used for cDNA synthesis using M-MuLV reverse transcriptase (Perkin Elmer, Branchburg, NJ) and oligo-dT primers. For amplification of cDNA, primers specific for different SR proteins, YB-1 and ß-actin (housekeeping gene) were used (Table I). Each tube contained 1 µl of sense and antisense primer, respectively, dNTps, buffer, Taq polymerase in a total volume of 50 µl. Amplification was for 25 cycles consisting of denaturing at 94°C (1 min), annealing at 59°C (1 min) and extension at 72°C (1.5 min).

For visualization of amplified cDNA, 10 µl from each reaction were submitted to electrophoresis on an agarose gel (2%). PeqGold DNA Sizer XI or XII (Peqlab Biotech, Erlangen) were used for size determination.

*Protein isolation and Western blot.* Total cellular protein was isolated from the interphase and phenol phase from the initial homogenate after precipitation of the DNA (0.3 ml 100% of ethanol per 1 ml of TRIzol) as described previously (22).

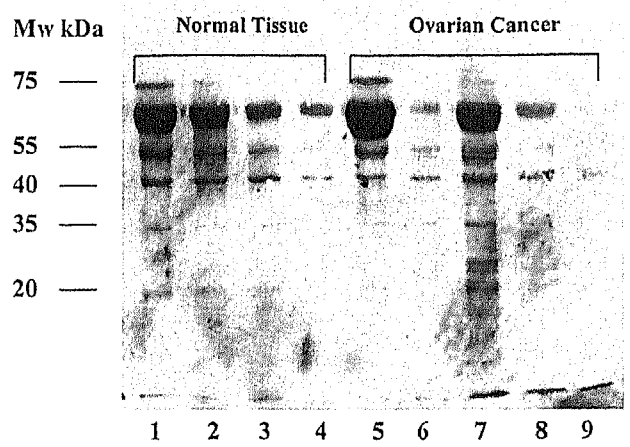


Figure 1. Expression of SR proteins in normal ovarian tissue and ovarian cancer as detected by Western blot analysis using mAb 104 specific for a phosphorylated SR epitope. Lanes 1-4, physiological ovarian tissue; lanes 5-9 ovarian cancer.

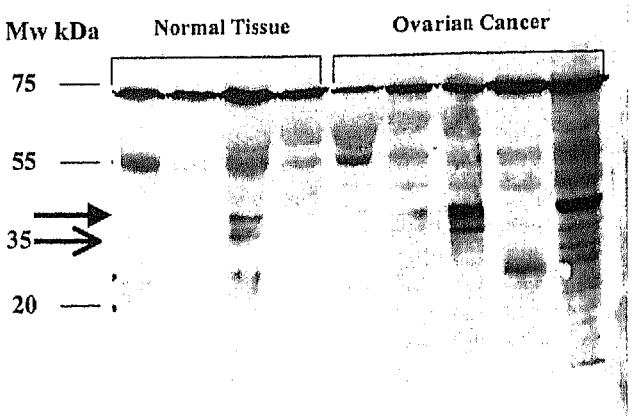


Figure 2. Expression of Tra2 protein isoforms in normal ovarian tissue and ovarian cancer as detected by Western blot analysis using polyclonal antibody 568 directed against an epitope common to all Tra2 proteins. Lanes 1-4, physiological ovarian tissue; lanes 5-9, ovarian cancer. A closed arrow indicates the hyperphosphorylated form, an open arrow the hypophosphorylated form.

Briefly, proteins dissolved in the phenol-ethanol supernatant were precipitated with isopropanyl alcohol (1.5 ml per 1 ml TRIzol), pelleted (12,000 x g, 4°C, 10 min) and washed three times with 0.3 M guanidine hydrochloride in 95% ethanol. Finally, the pellets were resuspended in 1% SDS solution and incubated at 50°C for complete dissolution. Insoluble material was removed by centrifugation (10,000 x g, 10 min, 4°C) and the supernatants were stored at -80°C until further analysis. For SDS-PAGE (10% gel), 20 µg of total protein were applied per lane and separated according to the protocol described by Laemmli (26). Proteins were electroblotted onto a PVDF membrane (PolyScreen, NEN Life Science, Boston, MA) using the method described by Towbin *et al* (27) at 100 V for 1.0 h at 4°C.

Unspecific binding sites of the membrane were blocked with 5% Blotto/PBST (mAb 104, recognizing the SR proteins) or 5% BSA/TBS (polyclonal rabbit-anti-Tra2-antibody) prior to incubation with the specific antibodies diluted appropriately in blocking solution. Bound antibodies were visualized using horseradish-peroxidase labeled secondary antibodies in conjunction with a chemiluminescence detection system (NEN Life Science, Ort, Germany). Secondary antibodies were for mAB 104 a peroxidase-conjugated goat anti-mouse-IgM-antibody (Pierce, Rockford, USA) or a peroxidase-conjugated goat anti-rabbit Ig antiserum (Dako Diagnostica, Hamburg, Germany).

## Results

For this study, samples from physiological ovarian tissue specimen (n=4) and serous ovarian cancers (n=5) were used. The characterization of the tissue samples was in accordance with the local pathologist.

*Differential alterations in SR protein expression profiles in ovarian tumorigenesis.* To examine the expression profiles of the phosphorylated SR-splicing factor family in ovarian cancer, we first analyzed SR proteins in normal and malignant ovarian tissues by Western blotting using the monoclonal antibody mAb 104. In HeLa cells, mAb 104 recognizes approximately equal amounts of the major SR proteins (SRp20, ASF/SF2, SC35, 9G8, SRp30c, SRp40, SRp55, and SRp75) with molecular weights between 20 and 75 kDa (14).

Physiological ovarian tissue showed constant expression of SRp75 and SRp40 at different levels (Fig. 1, lanes 1-4). The 30 kDa class of SR proteins and SRp55 were heterogeneously expressed with very low levels especially for the 30 kDa class of proteins (Fig. 1, lanes 1-4). For SRp20 the analysis revealed low but constant expression in physiological tissues.

In the serous adenocarcinomas of the ovary, heterogeneous levels of SR proteins were detected (Fig. 1). SRp75, SRp55 and SRp40 expression seemed not to be increased. Western blot analysis with mAb 104 revealed an increasing amount of the 30 kDa class of SR proteins in some tumors while SRp20 expression remained constantly low (Fig. 1, lanes 5-9). Thus, the pattern of phosphorylated SR protein expression changed specifically with higher levels of phosphorylated 30 kDa SR proteins in some tumors and constant concentrations of phosphorylated SRp75, SRp55, SRp40 and SRp20.

We also evaluated the expression of human Tra2, another member of the extended family of SR splicing factors. For this purpose, the polyclonal rabbit Tra2 antibody (28), which recognizes an epitope common to all Tra2 isoforms, was used. The detected proteins migrate as doublet at a molecular weight of 40 kDa. The slower migrating band represents a hyperphosphorylated form (29). The hypophosphorylated form is present at moderate levels in most normal tissues (Fig. 2, lanes 2 and 3). Only in one of the physiological ovarian tissues expression of hyperphosphorylated Tra2 protein isoforms was detected at higher levels (Fig. 2, lane 3). In contrast, in all ovarian cancer specimen Tra2 isoforms were detected with two samples containing relatively high concentrations of hyperphosphorylated protein (Fig. 2, lanes 5-9).

*The RNA expression levels of splicing factors change during ovarian tumorigenesis.* To provide more sensitive detection

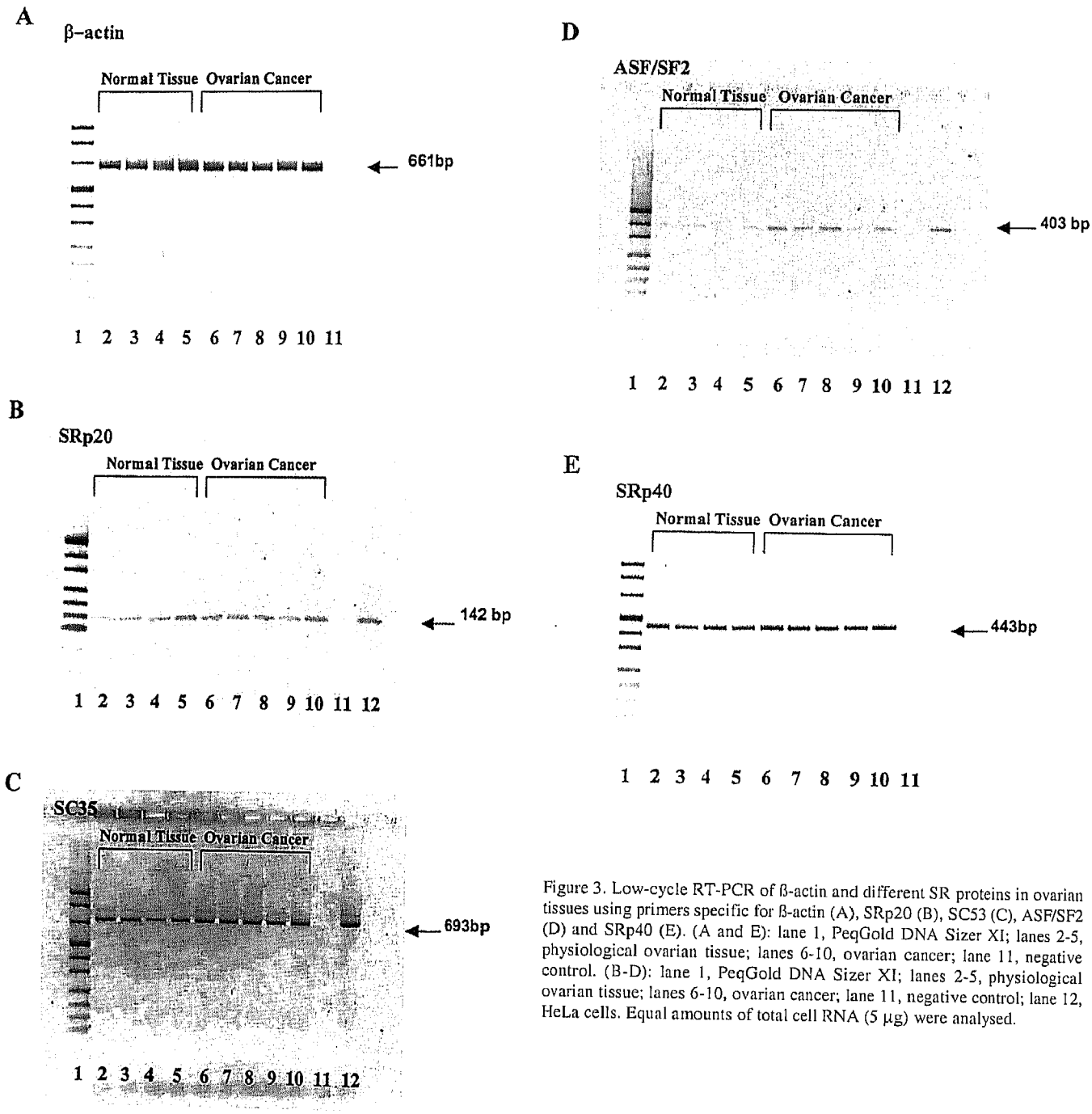


Figure 3. Low-cycle RT-PCR of ß-actin and different SR proteins in ovarian tissues using primers specific for ß-actin (A), SRp20 (B), SC53 (C), ASF/SF2 (D) and SRp40 (E). (A and E): lane 1, PeqGold DNA Sizer XI; lanes 2-5, physiological ovarian tissue; lanes 6-10, ovarian cancer; lane 11, negative control. (B-D): lane 1, PeqGold DNA Sizer XI; lanes 2-5, physiological ovarian tissue; lanes 6-10, ovarian cancer; lane 11, negative control; lane 12, HeLa cells. Equal amounts of total cell RNA (5 μg) were analysed.

of SR proteins and Tra2 isoforms, we monitored mRNA levels of these proteins using RT-PCR (Fig. 3A-D). While RT-PCR for ß-actin revealed equal signals for physiological and cancerous tissue (Fig. 3A), SRp20 mRNA levels were slightly increased in tumors. The constant protein levels of SRp20 seen in tumors by Western blotting indicate an increased mRNA synthesis rather than increased phosphorylation of an existing protein population (Fig. 3B).

RT-PCR indicated a marked induction of SC35 and ASF/SF2 (Fig. 3C and D) mRNA levels in ovarian cancer. These findings are in accordance with the results for Western blotting, where an induction of the 30 kDa class of SR proteins was also recognized. In contrast, mRNA concentrations of SRp40 and SRp55 remained constant over the tested paradigm (Fig. 3E and data not shown).

Additionally, we investigated the expression of the two Tra2 isoforms, Tra2α and ß, and the YB-1 protein, all three known to be specific effectors of alternative splicing events *in vivo* and *in vitro*. Western blotting indicated a general increase of Tra2 isoform expression in ovarian cancer. RT-PCR analysis was able to relate this observation to specific induction of Tra2ß in ovarian cancers, while Tra2α mRNA levels remained constant (Fig. 4A). The RT-PCR for YB-1 showed similar alteration with a marked induction of YB-1 mRNA concentrations in ovarian cancer specimens (Fig. 4B).

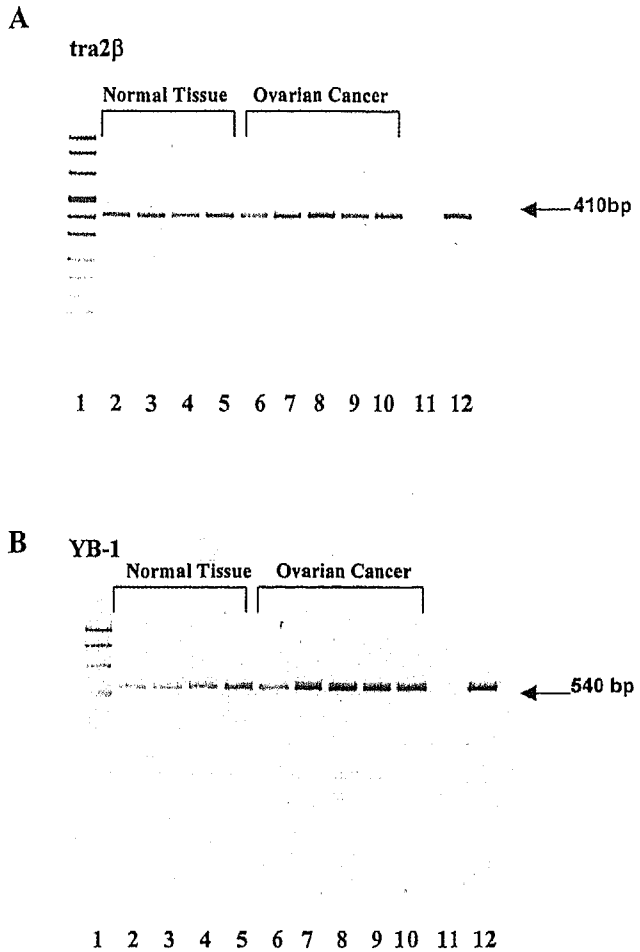


Figure 4. Induction of Ta2ß and YB-1 expression in ovarian cancer. Low-cycle RT-PCR of ß-actin and different SR proteins in ovarian tissues using primers specific for Ta2ß (A) and YB-1 (B). (A and B): lane 1, PeqGold DNA Sizer XI; lanes 2-5, physiological ovarian tissue; lanes 6-10, ovarian cancer; lane 11, negative control; lane 12, HeLa cells. Equal amounts of total cell RNA (5 µg) were analysed.

## Discussion

A growing number of genes involved in cell signaling, migration and adhesion are known to be alternatively spliced and this processing appears to be altered during tumor development and progression. Alterations in alternative pre-mRNA processing during tumorigenesis is observed for a multitude of human malignancies and the underlying molecular mechanism are currently a focus of scientific interest, mostly because these changes can have pronounced effects on cellular behavior.

SR proteins are splicing factors, which are essential in early substrate recognition by the spliceosome (15,19) and binding to splicing enhancer sequences of alternative exons (16,18,30).

Furthermore, individual SR proteins have distinct tissue distributions and relative changes in the concentration of SR proteins are known to alter alternative splicing decisions (12,31). Therefore, this nuclear protein family is an interesting target for the analysis of molecular changes causing differential alternative splicing.

In a mouse model of mammary tumorigenesis we demonstrated marked changes of SR protein splicing factor expression accompanying breast cancer development. This leads potentially to changes in alternative splicing.

The aim of the presented study was to examine the expression pattern of different nuclear splicing factors in the physiological human ovary and to compare these with human ovarian cancers. SR proteins are known to be potential regulators of alternative pre-mRNA processing thereby significantly influencing cellular behavior. Interestingly, ovarian carcinomas showed an increased expression of distinct members of the SR splicing factor family. Whereas a marked increase in the expression of SC35 and ASF/SF2 was seen, the concentrations of SRp40, SRp55 and SRp75 remained constant. For SRp20, the smallest member of the SR protein family, we detected increased mRNA concentrations in tumors in comparison to physiological tissue but constant protein levels when utilizing mAb 104. These findings indicate an induction of SRp20 mRNA synthesis without consecutively increased phosphorylation of the SRp20 protein.

Thus, in comparison to physiological ovarian tissue, the relative abundance of individual SR proteins is changed in serous ovarian cancer. Knowing the wide distribution of genes whose alternative mRNA processing is influenced by the relative concentration of SR proteins (16-18), our observation imply changes of alternative pre-mRNA splicing in ovarian tumorigenesis. This hypothesis is supported by our findings of significant changes of alternative CD44 splicing in ovarian cancer. We were able to demonstrate the occurrence of aberrant CD44 mRNA species and over-expression of several alternatively spliced CD44 isoforms using a tissue culture model for serous ovarian cancer (24). Recently, we were able to show that altered expression profiles of SR proteins are highly specific and not the result of a general induction of the splicing machinery during neoplastic development (22).

Western blot analysis revealed a marked induction of Tra2 isoform expression in ovarian cancer when compared to physiological tissue. Given the specificity of the anti Tra2 antibody, we were not able to discriminate between Tra2 isoforms. RT-PCR analysis unveiled that the increased expression was rather due to an induction of the Tra2ß than of Tra2α. These results support the hypothesis of specific changes of splicing factor expression patterns during ovarian tumorigenesis as described for the classic SR proteins recognized by mAb 104.

YB-1, a specific regulator of alternative mRNA splicing, was also strongly upregulated in ovarian tumors. YB-1 was originally identified as a transcription factor that binds single-stranded DNA (32,33). Recently, YB-1 was identified as an RNA binding protein (25,34,35). YB-1 is an abundant protein in both the nucleus and the cytoplasm of many cell types (36,37). Like the SR proteins, YB-1 is highly phosphorylated, which is required for binding to RNA (38,39).

Here we described changes in the expression profiles of splicing factors in serous ovarian cancer. These changes are most likely also present during tumorigenesis. Different families of splicing factors, which exhibit different mechanisms of RNA-protein interaction are affected. The differential induction of SR proteins, Tra2 isoforms and YB-1 suggest a specificity of the observed alterations. SR proteins are known

to interact with GAR-rich exon enhancer elements of the pre-mRNA, YB-1 unfolds its effect through recognition of C/A-rich exon enhancer sequences. Thus, alternative splicing of multiple target genes, regulated by either SR proteins, Tra2 and/or YB-1 is possibly affected by the changed expression profiles.

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