SRSF2 mutations in epithelial ovarian cancer

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Abstract
Resistance to platinum chemotherapy regimens represents a major obstacle in the successful treatment of epithelial ovarian cancer (EOC) patients. Among the molecular mechanism responsible for resistance to platinum, alternative splicing, which is induced upon platinum treatment, can control apoptosis by regulating the expression of apoptotic protein variants with opposite functions. Alterations in alternative splicing are found in tumors and can hinder apoptotic response. In the present study we sequenced SRSF2, a splicing factor that regulates Caspase-8 and Caspase-9 variants, in search of mutations that could possibly explain alternative mechanisms of platinum resistant in EOC.

Key words: epithelial ovarian cancer, platinum treatment, alternative splicing, SRSF2

Introduction

Epithelial ovarian cancer (EOC) is a rare but dreadful disease often diagnosed at advanced stages and represents the fourth cause of cancer death in women. Radical surgery together with platinum-taxol chemotherapy is the standard of care for advanced EOC. Even when radically resected, EOC invariably tends to recur, with the development during successive lines of chemotherapies of a platinum-resistant disease, which predicts poor prognosis [1].

The mechanisms underlying platinum resistance are several and include abnormalities in the apoptotic pathway [2]. One particular mechanism of altered apoptotic response can be found in the alternative splicing of apoptotic regulators, due to alterations in splicing factor proteins [3]. Alternative pre-mRNA splicing represents an important nuclear mechanism in the posttranscriptional regulation of gene expression. It is involved in several cellular processes including cell proliferation, differentiation, and cell apoptosis. During alternative splicing, splicing factor proteins (i.e., serine/arginine SR-proteins and heterogeneous ribonuclear proteins-hnRNP) control the removal of introns and the joining of selected exons to produce multiple transcripts, and thus multiple protein isoforms, from a single gene. Interestingly, often these different gene variant products exert opposite effects in the cellular process in which they are involved [3, 4].

Not surprisingly alternative splicing is frequently altered during tumorigenesis; one example is somatic mutations occurring in the SRSF2 splicing factor, which contribute to myelodysplastic syndrome [5] and to chronic myelomonocytic leukemia [6].

In EOC, a marked and specific induction of different splicing factors was observed in primary tumors and in their metastatic sites [7], including SRSF2 [8], and the increased expression of SRSF3 has been associated with the malignant potential of EOC by regulation of the apoptotic pathway [9]. Similarly, SRSF2 was shown to regulate levels of anti-apoptotic-caspases in response to platinum treatment [10, 11], and we recently showed that SRSF2 silencing increased the survival of EOC cells during exposure to platinum [12].

Thus, the aim of the present study was to see whether EOC samples carry SRSF2 mutations, which might explain a possible novel route to the development of platinum-resistant EOC.

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Materials and methods

Patient sample collection

CRO-Aviano's institutional Biobank collected samples and obtained informed consent from all patients. The Internal Review Board approved this study (#IRB-06/2011). One hundred and thirty EOC samples were collected at CRO-Aviano National Cancer Institute between 2010 and 2016 from women undergoing primary or secondary surgery for suspected gynecological malignancies. For all samples, clinical information including histology, stage, and platinum response was compiled.

SRSF2 sequencing

Genomic DNA was extracted from fresh frozen tissue samples by Maxwel 16 DNA purification kit (Promega). 50 ng of genomic DNA was amplified with TruSeq Custom Amplicon kit (TSCA, Illumina) specially designed for the targeted sequencing of SRSF2 (5 amplicons) among others. Libraries were run in an Illumina MiSeq instrument achieving a median coverage >1000 reads. Data were aligned to human reference genome hg18 and analyzed, after quality control, using Variant Studio and IGV program, reporting only variants with a mutant allelic frequency (MAF) greater than 5%. We considered not only variants inside the coding sequence, but also the ones inside the 5'-, 3'-untranslated regions (UTRs) and inside splicing regulatory elements [13].

Cell lines and treatments

MDAH 2774 and 293FT cell lines were respectively maintained in RPMI-1640 and DMEM supplemented with 10% heat-inactivated FBS (all from Sigma-Aldrich). Sh-RNAs lentiviral particles were produced by transfecting 293FT cells with pLP1, pLP2, pVSV-G and pLKO vectors specific for SRSF2 (Sigma). MDAH 2774 cells were then transduced with sh-RNAs lentiviral particles, and 48 hours later cells were treated with cisplatin 50 µM (TEVA) as indicated. Cells were then collected, and total RNAs were extracted.

RNA extraction and PCR

Total RNA was isolated using TRIzol reagent (Life Technologies). 2 µg of total RNA were reverse transcribed using AMV transcriptase (Promega) according to the provider's instructions. For Caspase-8 and Caspase-9 primer sequences, as well as for polymerase chain reaction (PCR) conditions refer to [11]. 100 ng of cDNA was used for PCR amplification using pol2A as an internal control. PCR products were run on a 1.5% agarose gel and visualized by ethidium bromide staining.

Results

Data were available for 130 patients. The median follow-up was 32 months, with median survival for censored patients of 38 months (Table 1). Thirty-five patients were lost to follow-up.

Platinum treatment induces alternative splicing of Caspase-8 and Caspase-9 via SRSF2 in ovarian cancer cells

It has been shown that platinum treatment can induce alternative splicing of Caspase-8 and Caspase-9 favoring the expression of their pro-apoptotic isoforms (Caspase-8a and Caspase-9a, respectively) [10]. In particular, this relies on the increase of activity or of total levels of SRSF2, in models of lung [10, 11, 14] and kidney cancer [15]. With this evidence in mind and given the cen-

Table 1. Clinical characteristics of patients with epithelial ovarian cancer (EOC) included in the study (n=130).

	Histotype	HGSOC (n=108)				ENDOM (n=10)				CCOC (n=7)				MUCINOUS (n=5)				Median time to first progression (months)	
	Stage	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV		No. pts
	No. pts	7	6	72	23	3	1	4	2	3	1	3	0	4	0	0	1	12.8	130
Platinum response	SENSITIVE	5	3	26	7	2	1	1	–	3	1	2	–	1	–	–	–	24.0	52
	PARTIALLY*	–	–	10	5	–	–	2	–	–	–	–	–	–	–	–	–	11.4	17
	RESISTANT†	–	–	13	5	–	–	–	1	–	–	–	–	–	–	–	1	4.0	20
	Not available	2	3	23	6	1	–	1	1	–	–	1	–	3	–	–	–	–	41
Median age (years)		63				52				48				63					

*Partially sensitive. †Also includes two refractory cases. Median age was 62 years overall.
CCOC: clear-cell EOC; ENDOM: endometrioid EOC; HGSOC: high-grade serous EOC (also included two undifferentiated cases).

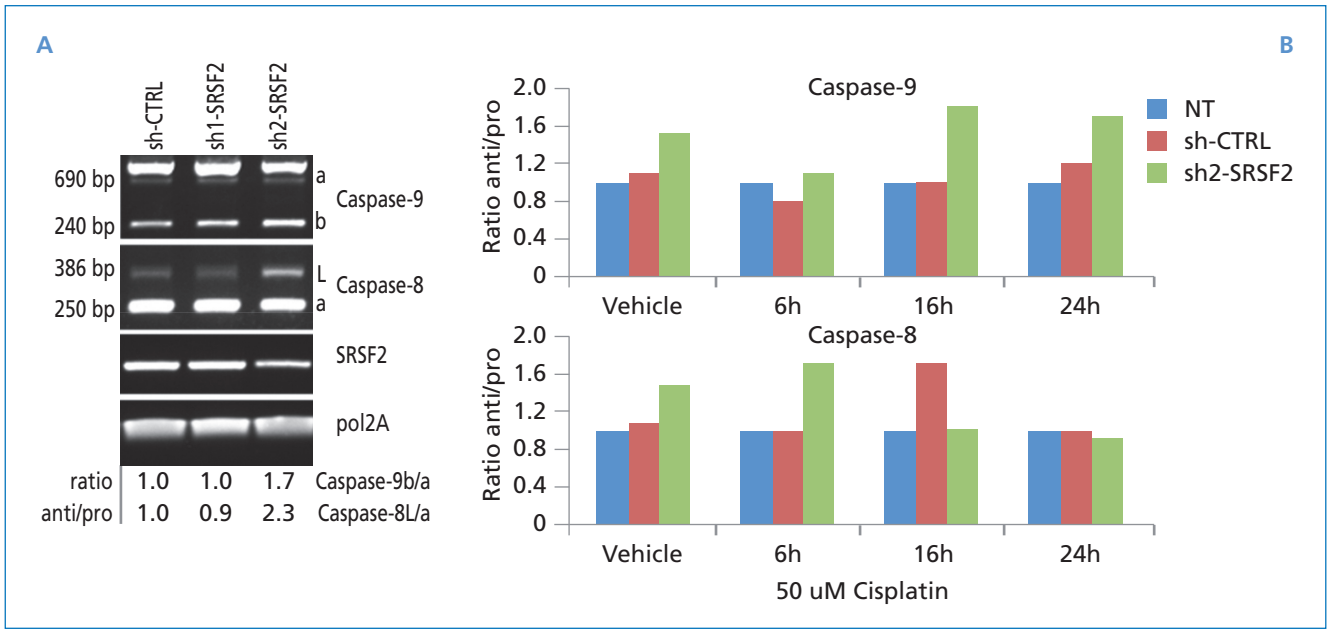

Fig. 1. Cisplatin treatment induces alternative splicing of Caspase-8 and Caspase-9 via SRSF2 in MDAH 2774 EOC cells. **A)** Expression levels of SRSF2, Caspase-8a, -8L and -9a, 9b isoforms by polymerase chain reaction (PCR) in sh-CTRL, sh1-SRSF2 and sh2-SRSF2 transduced cells. **B)** Ratio between anti/and proapoptotic Caspase-9 (upper graph) and Caspase-8 (lower graph) RNA variant levels, as detected by PCR, in non-transduced (NT), sh-CTRL and sh2-SRSF2 transduced cells, following exposure to 50 μM cisplatin or vehicle control, for the indicated time-points.

tral role of platinum-based therapies in EOC treatment, we wanted to verify whether platinum-induced apoptosis also relies on alternative splicing of Caspase-8 and Caspase-9 in EOC cells, and whether this was due to SRSF2. To this aim, we used a MDAH 2774 EOC cell line transiently silenced for SRSF2 by lentiviral SRSF2-sh-RNA transduction. As shown in Figure 1A, only one of the two SRSF2-sh-RNA vectors used was able to efficiently silence SRSF2 expression in MDAH 2774 cells. Most importantly, the reduction of total SRSF2 levels affected the ratio between anti- and pro-apoptotic variants of Caspase-8 and Caspase-9, with a net increase of the anti-apoptotic forms (i.e., the less expressed forms of the two caspases, Caspase-8L and -9b) (Figure 1A). Consistently, downregulation of SRSF2 in EOC cells has an anti-apoptotic effect, as we have recently demonstrated in [12]. Furthermore, when we exposed parental, sh-control and SRSF2 silenced MDAH 2774 cells to 50 µM cisplatin for up to 24 hours (Figure 1B), we observed, on one hand, that platinum treatment in all cell lines reduced the expression of the isoforms of the anti-apoptotic caspases, thus sustaining in part its apoptotic killing mechanism; on the other, that loss of SRSF2 favors the balance toward the anti-apoptotic isoforms, thus likely conferring resistance to cisplatin-induced apoptosis

Overall these *in vitro* data, together with the data from our previous work [12], support the importance of SRSF2-driven splicing mechanism in platinum-induced EOC cell death.

SRSF2 targeted sequencing

Based on our *in vitro* results ([12] and Figure 1), and on the notion that several splicing factors, including SRSF2 are mutated in human malignancies [5, 6], we sequenced SRSF2 in an EOC cohort, hypothesizing that SRSF2 mutations could explain differences in the clinical presentation and/or response to chemotherapy of patients with EOC. Applying a variant call cut of 5%, we found that SRSF2 was somatically mutated in 3.8% of EOC samples analyzed (5 out of 130) (Table 2). Four of the mutated samples were of high-grade serous histology (1 stage IIC, 2 stage IIIC and 1 stage IV) and 1 was of clear-cell histotype (stage IC). Interestingly, 2 HGSOC-mutated samples were found in recurrent samples (n=14) that were of intermediate platinum sensitivity (8 and 10 months for OV39 and OV69, respectively). If we consider the frequency of mutations found in recurrent samples (2/14) *versus* that of the total patient cohort (5/130) or of the primary samples (only 3/116), we can observe an enrichment for mutations in patients that were previously exposed to platinum treatment or that tend to recur (14.3% *vs* 3.8% and 2.6%, respectively). Similarly, if we consider the frequency of mutations in clear-cell (1/7) compared to high-grade serous histotypes (4/108), we can observe that clear-cell histology, which is considered intrinsically chemoresistant,

Table 2. SRSF2 mutations.

EOC ID	Variant	Chr 17 position	% MAF	Type of mutation	Mutation effect	cDNA position	CDS position	Protein position	AA change	Histotype	Stage	Sample type
OV039	G/G>G/A	7473329	6.88	snv	5' UTR variant	165	/	/	/	HGSOC	IIIC	Recurrent
OV069	T/T>T/C	74733112	21.73	snv	Missense*	382	131	44	Y/C	HGSOC	IIC	Recurrent
OV097	C/C>C/G	74733280	76.31	snv	5' UTR variant	214	/	/	/	CCOC	IC	Primary
OV117	T/T> T/TGGGC	74733413	24.20	Insertion	5' UTR variant	80	/	/	/	HGSOC	IIIC	Primary
OV118	G/G>G/T	74733377	83.79	snv	5' UTR variant	117	/	/	/	HGSOC	IV	Primary

*Deleterious variant.
AA: amino acid; CCOC: clear-cell EOC; EOC: endometrial ovarian cancer; HGSOC: high-grade serous EOC; MAF: mutant allele frequency; snv: single nucleotide variant.

has an overrepresentation of SRSF2 mutations (14.3% *vs* 3.2%). Of note, both the clear-cell and the stage IV high-grade serous mutated EOC samples (OV097 and OV118) presented a high variant call (MAF 76.3% and 83.8%), possibly indicating a clonal evolution of the disease.

Conclusions

In this study, we report the first dedicated analyses of SRFS2 mutations in human EOC. Using a large series of consecutively collected samples, we verified a potential increase of SRFS2 mutation in recurrent EOC and in the clear cell subtype. Interestingly, 4 out of the 5 mutations we found inside SRSF2 are located within the 5' untranslated region (5'UTR) of SRSF2 mRNA. 5'UTR mutations can regulate protein expression by interfering with translation efficiency [16]; for example, a mutation inside the 5'UTR of BRCA1 found in aggressive breast cancer reduces BRCA1 expression levels by 30-50% [17]. Thus, it would be interesting to see whether the 5'UTR mutations found in SRSF2 can also regulate its expression. Further experiments are warranted to support this hypothesis, together with additional analyses on a larger collection of samples in order to confirm SRFS2 mutational occurrence in EOC and to verify if SRSF2 mutations and/or expression changes could contribute to the intrinsic or acquired platinum resistance of EOC.

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Conflicts of Interest

The Authors declare there are no conflicts of interest in relation to this article.

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