SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 10: 3722-3726, 2015

A novel splicing mutation in the SLC9A3R1 gene in tumors from ovarian cancer patients

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Received December 2, 2014; Accepted August 25, 2015

DOI: 10.3892/ol.2015.3796

Abstract. The aim of the present study was to investigate novel molecular markers that could improve the diagnosis of ovarian cancer patients or be of predictive value. The sequence of the sodium-hydrogen antiporter 3 regulator 1 (SLC9A3R1) gene that codes for the PDZ2 motif of the Na^+/H^+ exchanger regulatory factor 1 (NHERF1) protein was analyzed. Changes in migration and cell transformation, and alterations of growth factor signaling pathways have been described in cells lacking endogenous NHERF1 or expressing an isoform lacking the function of the PDZ2 domain. Exons 2 and 3, together with flanking intronic sequences of the SLC9A3R1 gene, were amplified and bi-directionally sequenced in 31 primary tumor samples from epithelial ovarian cancer patients. In total, 3 different previously undescribed mutations were detected in 8 out of 31 serous adenocarcinoma tumor samples (25.8%). Bioinformatics analysis predicted a significant effect in the splicing process as a result of the mutations that could disrupt the NHERF1 PDZ2 domain. Point mutations in consensus splicing recognition are a major cause of the splicing defects that are found in several diseases, including cancer. It has previously been shown that a lack of exon 2 and disruption of the PDZ2 domain contribute to cell transformation and leads to modifications in the physiological regulation of the conformational state of NHERF1. Further studies in bigger groups of ovarian cancer patients will determine the importance of this mutation in disease progression and patient survival.

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Key words: ovarian cancer, SLC9A3R1 gene, mutation, splicing, PDZ2

Introduction

Epithelial ovarian cancer (EOC) is the seven most frequent type of cancer in women and the eighth cause of mortality from cancer in women worldwide (1). In contrast to the continuous development in molecular characterization of a number of neoplasms, the progress made in understanding the molecular background of ovarian cancer is limited. This could be due to the complexity of the disease, but also due to certain limitations of study designs and experimental data collection (2).

The sodium-hydrogen antiporter 3 regulator 1 (SLC9A3R1) gene is located on chromosome 17q25.1, consists of six exons and encodes Na^+/H^+ exchanger regulatory factor 1 (NHERF1). The isolated protein has a molecular weight of 50-53 kD, contains 357 amino acids and is structured in three protein domains (3,4). NHERF1 has two PDZ domains (PDZ1 and PDZ2) located in tandem (PSD-95/Dlg/ZO1), mediating protein-protein interaction (5), and a C-terminal ezrin-binding (EB) domain that binds to the ezrin-radixin-moesin (ERM) family of proteins (6).

NHERF1 is expressed primarily at the plasma membrane of polarized epithelia, including that of the kidney, intestine, colon, lungs and uterus. The main function of this adaptor protein is stabilization of protein complexes at the plasma membrane connecting signaling pathways and structural proteins to the cell cytoskeleton (7). NHERF1 binds to β-catenin through PDZ2, and stabilizes the interaction between β-catenin and E-cadherin in the adherent junction of epithelial cells (8,9). In the absence of NHERF1, β-Catenin accumulates in the cytoplasm and E-Cadherin localization at the cell membrane is reduced, resembling the process of epithelial to mesenchymal-like transition (EMT). EMT is observed in normal embryonic development and is recreated during tumor progression (10-12).

NHERF1 has been extensively studied at the protein level, principally in its interactions at the cell membrane, but its gene regulation remains largely unexplored. Thus far, only a few gene mutations associated with human cancer have been characterized. For instance, one previous study (13) in breast cancer showed that the combination of the intragenic mutation rate of 48 breast cancer cell lines and 37 primary breast tumors was 4%. Two missense mutations were described. One of them, a somatic sequence variant of AAG→AAC in the NHERF1 PDZ2

domain that produces a switch in codon 172 (Lys to Asn), was found in primary breast cancer. The other, a missense mutation in codon 180 of exon 2 (CGG→TGG) with a replacement of Arg to Trp, which corresponds to a conserved basic residue in the PDZ2 domain, was found in the MDA-MB-231 breast cancer cell line. Two of the mutations that occur in the PDZ2 domain (codons 172 and 182) decreased the interaction of NHERF1 with SYK (spleen tyrosine kinase), a tumor suppressor gene in the mammary gland. Additionally, the mutation in codon 180 disrupted the interaction with another tumor suppressor gene (Merlin), which shows the importance of the integrity of the PDZ2 motif in NHERF1 tumor suppressor activity in breast cancer (13). A recent study performed by The Cancer Genome Atlas research network analyzed the DNA sequence from coding genes in 316 high-grade serous ovarian adenocarcinomas. No mutations were detected in the coding sequence of the SLCA9AR1 gene despite changes in expression levels and copy number amplification in 7.6% of the cases; splicing sites were not selected for the analysis (14).

The present study reports the results of mutation analysis in the SLC9A3R1 gene that revealed the presence of splice mutations in 8 out of 31 screened EOC samples (25.8%). To the best of our knowledge, this is the first study on SLC9A3R1 point mutations in EOC. Further studies in a larger cohort of ovarian cancer patients will determine the predictive and prognostic value of this mutation.

Materials and methods

Patients and tumor samples. The analysis was performed in 31 EOC tumor adenocarcinoma samples (25 high-grade serous, 4 undifferentiated, 1 clear cell and 1 endometrioid sample) from patients who had undergone primary surgery in the Department of Gynecological Oncology, Medical University of Gdansk (Gdansk, Poland) between 1995 and 1996, and between 2002 and 2004. Informed consent was obtained from all patients, and the study was approved by the Medical Review Board of Gdansk Medical University. The patients treated between 1995 and 1996 received 6 cycles of postoperative chemotherapy combination of cisplatin (75 mg/m^2) and cyclophosphamide (750 mg/m^2) every 3 weeks. The patients treated between 2002 and 2004 received 6 cycles of postoperative chemotherapy combination of cisplatin (75 mg/m^2) and paclitaxal 175 mg/m2 (over 3 h) every 3 weeks. Only 3 cases did not receive any adjuvant treatment due to a poor performance status (PS 3/4). The disease was classified according to the histological grade (G1-G3) and the International Federation of Gynecology and Obstetrics stage (I-IV) (15). Residual disease was defined by the diameter of the largest tumor left in the abdominal cavity after cytoreductive surgery for advanced stages. Patients with and without a family history of the disease were included in the study. The samples of fresh tumor were immediately frozen at -80˚C for molecular analysis; a portion of each tumor was fixed in formalin and embedded in paraffin. Tissue sections (5 μm) were obtained from the blocks and stained with hematoxylin and eosin for histopathological analysis.

Molecular screening. The DNA from the ovarian tumors was extracted from fresh tumor tissues by standard phenol-chloroform procedures. The sequences of exons 2 and 3, and the flanking sequences of the SLC9A3R1 gene were amplified with specific primers (13). The sequences were as follows: Exon 2 forward, 5'-AATTGCTGTGTAGGGATCTAG-3' and reverse, 5'-GGAAGAGAGCGAGAAGCATC-3' (322-bp product); and exon 3 forward, 5'ACTGCAAACTGGCTGAGAAC-3' and reverse, 5'-TGGCTCACATCCCTGACTTG-3' (331-bp product). The PCR reaction was carried with the following conditions: 30 ng of DNA/sample in presence of 1.5 mM $MgCl_2$, (95˚C for 5 sec, followed by 95˚C for 30 sec, gradient 56-63˚C for 30 sec and 72˚C for 30 sec repeated 34 cycles, and a final amplification step at 72˚C for 7 min) using the Taq DNA recombinant polymerase (Fermentas, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Samples harboring the mutation were re-amplified using high fidelity polymerase (Thermo Scientific) to ensure the result was accurate. Following PCR amplification, the PCR products were cleaned using the Axyprep-96 PCR Cleanup kit following the manufacturer's instruction (Axigen, Corning, Tewksbury, MA, USA). The PCR products were prepare for sequencing using the Big Day reaction. Briefly, the PCR products were amplified with the forward or reverse primers separately according to the manufacturer's instruction. Following the PCR amplification, the product was cleaned using the ExTerminator Nucleotide Terminators Removal kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions, and sequenced directly by bi-directional sequencing (ABI Prism 3130; Applied Biosystems, Life Technologies, Foster City, CA, USA). Electropherograms were analyzed by the free BioEdit Sequence Alignment Editor program (16).

Bioinformatics analyses. To predict the splicing signals in wild-type and mutated DNA sequences, the mutations were analyzed using the bioinformatics Human Splicing Finder (HSF) free program (17). The software allows the comparison of a wild-type and mutant sequence in order to predict the impact of in the splicing process.

Results

Mutational analysis. Cases characteristics of the 31 patients with EOC included in the study are presented in Table I. The sequences of exons 2 and 3, together with the flanking intronic sequences of the SLC9A3R1 gene that codes for the PDZ2 domain of the NHERF1 protein, were analyzed. In total, 8 out of the 31 analyzed samples (25.8%) were found to carry a potentially harmful alteration located in the splicing donor site of intron 2 (Fig. 1; Table II). While 3 samples displayed two different substitutions in the +2 position (c.603+2T>A; c.603+2T>C), 5 other samples exhibited co-occurrence of two substitutions (c.603+2T>C; c.603+3G>A) located in the same splicing donor site (in the +2 and +3 positions) (Table I). Moreover, reported alterations were only identified in the tumor tissue of the tested cohort of EOC, no alterations were found in the blood of the patients indicating that they were somatic mutations. All identified alterations were located in the consensus sequence of the splice donor site of intron 2, suggesting a detrimental effect on the splicing process.

Bioinformatics analysis. The mutations were analyzed using the bioinformatics HSF (version 2.4.1), which compares the

Table I. Characteristic of the 31 patients with epithelial ovarian carcinoma.

Case no.	Tumor	FHOC	Age, years	CA-125, U/ml[a]	EOC Histology[b]	Grade	FIGO stage	Residual disease, cm[c]	OS time, months
1	2	Negative	63	>600	Serous	G3	IIIC	>5	12.3
2	21	Negative	58	>600	Serous	G1	IIIC	>5	10.1
3	23	Negative	42	1161	Undifferentiated	G3	IIIC	<1	106.7
4	32	Negative	74	4207	Serous	G3	IIIC	1-5	6.9
5	37	Positive	55	399	Serous	G3	IIIC	1-5	33.2
6	40	Negative	48	No data	Serous	G1	IIIC	<1	7.2
7	49	Negative	41	No data	Serous	G2	IIIC	<1	63.0
8	56	Positive	46	654	Serous	G3	IIIC	<1	104.3
9	58	Negative	77	No data	Serous	G2	IIIC	<1	0.3
10	63	Negative	60	14	Serous	G1	IC	0	32.5
11	74	Positive	45	599	Serous	G2	IV	>5	19.6
12	93	Negative	75	319	Serous	G3	IIIC	1-5	19.9
13	102	Negative	32	326	Clear cell	G2	IIC	0	130.9
14	118	Negative	79	7368	Serous	G2	IIIC	>5	35.0
15	127	Negative	47	198	Undifferentiated	G3	IIIB	<1	100.4
16	135	Negative	55	>600	Serous	G1	IIIC	1-5	16.9
17	137	Negative	63	No data	Serous	G1	IIIC	<1	17.8
18	150	Negative	56	403	Serous	G3	IIIC	<1	0.2
19	153	Positive	48	1089	Serous	G3	IIIC	<1	87.3
20	157	Negative	37	>600	Serous	G2	IIIC	<1	21.5
21	165	Negative	59	10	Serous	G2	IIIC	>5	14.6
22	170	Positive	78	No data	Serous	G1	IIIC	>5	18.0
23	172	Negative	54	1001	Serous	G2	IIIC	1-5	94.3
24	182	Negative	73	563	Undifferentiated	G3	IV	<1	26.6
25	200	Negative	73	4851	Serous	G3	IV	<1	7.0
26	211	Positive	82	802	Serous	G2	IIIA	<1	41.7
27	218	Negative	88	No data	Serous	G1	IV	<1	No FU
28	219	Negative	54	311	Undifferentiated	G3	IIIC	1-5	84.5
29	257	Negative	62	300	Serous	G2	IIIC	>5	25.8
30	766	Positive	46	156	Endometrioid	G3	IIB	0	43.3
31	792	Negative	36	136	Serous	G1	IIIB	0	106.9

[a]CA125 normal range, 4-41 U/ml (29). [b](15). [c]Residual disease, R, is presented as the diameter of the largest tumor left after primary surgery; FHOC, familiar history of ovarian cancer. OS, overall survival; NA, not applicable; CA-125, cancer antigen 125; FIGO, International Federation of Gynecology and Obstetrics; FU, follow-up.

wild-type and mutated sequences to the consensus splice site sequences from the HSF database. The 'consensus splicing site' in the database was determined previously by the analysis of data extracted from Ensembl containing ~22,000 genes and 46,000 transcripts of *Homo sapiens*, which includes introns and exons of all human genes (17). The impact of the mutation is analyzed by using matrices from the study by Shapiro and Senepathy (18) where a consensus value is attributed to each sequence. If the difference between the sequences is >10%, the program predicts a significant effect in the splicing process. The analysis showed that two of the mutations in the +2 splicing site (c.603+2T>A and c.603+2T>C) could exhibit a significant effect in the splicing process. By contrast, the mutation in the +3 position (c.603+3G>A) did not appreciably modify the site (Table III).

Discussion

The present study analyzed the sequence of exons 2 and 3 of the SLC9A3R1 gene, which encodes the PDZ2 domain of the NHERF1 protein. Through this domain, NHERF1 binds to β-Catenin and stabilizes the interaction with E-cadherin at cell-cell junctions (9). The PDZ2 domain also has a significant role in the regulation of the conformational state of NHERF1 by an intramolecular interaction with the C-terminal EB region, which is able to mask other protein domains in order to bind to other partner proteins (19).

The present study found two intronic mutations in the donor splicing site of exon 2 of the SLC9A3R1 gene that, to the best of our knowledge, had not been previously described and could affect the expression of the NHERF1 isoforms. Point

Table II. Mutations in the splicing donor site of the SCLC9A3R1 gene in ovarian cancer.

Case no.	Tumor	FHOC	Age at diagnosis, years	CA-125, U/ml[a]	EOC histology[b]	Grade	FIGO stage	Residual disease, cm[c]	OS time, months	Splice donor site mutation in SLC9A3R1 gene
1	2	Negative	63	>600	Serous	G3	IIIC	>5	12,3	c.603+2T>A
2	21	Negative	58	>600	Serous	G1	IIIC	>5	10,1	c.603+2T>C
7	49	Negative	41	no data	Serous	G2	IIIC	<1	63,0	c.603+2T>C,c.603+3G>A
10	63	Negative	60	14	Serous	G1	IC	NA	32,5	c.603+2T>C,c.603+3G>A
14	118	Negative	79	7368	Serous	G2	IIIC	>5	35,0	c.603+2T>C,c.603+3G>A
15	127	Negative	47	198	Undifferentiated	G3	IIIB	<1	100,4	c.603+2T>C,c.603+3G>A
20	157	Negative	37	>600	Serous	G2	IIIC	<1	21,5	c.603+2T>C
26	211	Positive	82	802	Serous	G2	IIIA	<1	41,7	c.603+2T>C,c.603+3G>A

[a]CA125 normal range, 4-41 U/ml (20). [b](15). [c]Residual disease, R, is presented as the diameter of the largest tumor left after primary surgery. NA, not aplicable; SLC9A3R1, sodium-hydrogen antiporter 3 regulator 1; FHOC, familiar history of ovarian cancer; OS, overall survival; CA-125, cancer antigen 125; FIGO, International Federation of Gynecology and Obstetrics.

Table III. Bioinformatics analyses.

No. of cases	Gene SLC9A3R1 mutations	Human splice finder[a]			Software prediction
		WT CV[b]	Mut CV	ΔCV, %	
7[c]	c.603+2T>C	82.83	56	-32.4	ΔCV reduction >10%, significant effect in 'splicing'
1[d]	c.603+2T>A	82.83	56	-32.4	ΔCV reduction >10%, significant effect in 'splicing'
5[e]	c.603+3G>A	82.83	83.99	1.4	ΔCV reduction <10%, splicing site not affected

[a](17). [b]CV to assess the strength of the splicing (16). CV, consensus value; Mut, mutant; WT, wild-type; SLC9A3R1, sodium-hydrogen antiporter 3 regulator 1. [c]Cases 2, 7, 10, 14, 15, 20 and 26. [d]Case 1. [e]Cases 7, 10, 14, 15 and 26.

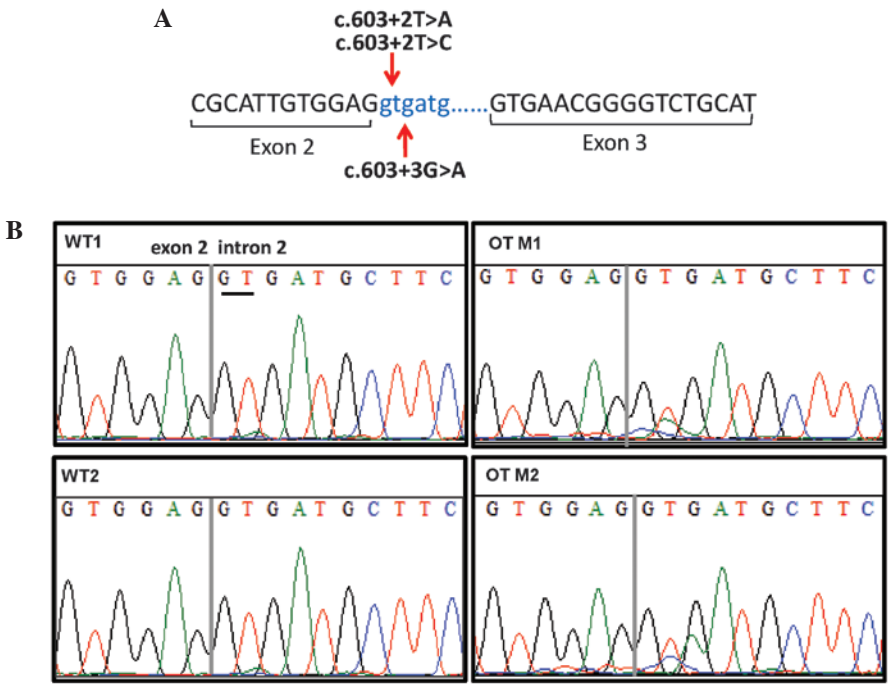

Figure 1. Donor splices site mutations of the sodium-hydrogen antiporter 3 regulator 1 gene in ovarian cancer tumors. (A) Schematic representation of the mutations in the splicing donor consensus sequence (second base of the dinucleotide GT) and the third base of intron 2 found in ovarian tumors. (B) Electropherograms showing the wild type (left), and mutated alleles (c.603+2T>C, c.603+3G>A) of ovarian tumors (right).

mutations in splicing recognition sites are a major cause of splicing defects, such as exon skipping of one or more adjacent exons, or inclusion of the intronic sequence, and are frequently found in different diseases (21-25).

It has been reported that the alteration of splicing could have a huge impact during tumorigenesis, as several genes express cancer-specific splicing isoforms (26-28). It was previously shown that one of the tumor suppressor mechanisms displayed by the PDZ2 domain of NHERF1 was the selective stabilization of the interaction between β-catenin and E-cadherin, which contributes to the maintenance of the structure of polarized epithelial cells. In the absence of NHERF1 expression, the β-catenin/E-cadherin association is disrupted and leads to decreased β-catenin at the plasma-membrane localization, reduced expression of E-cadherin at the cell-cell junction and cell transformation (9,29). The potential disruption of the PDZ2 domain as a result of the mutation could modify the interaction of NHERF1 with proteins that interact directly with the PDZ2 domain, such as β-catenin, as well it possibly affecting the regulation of the conformational state of the protein, and its binding to phosphatase and tensin homolog and ERM proteins (19).

In summary, mutations of splicing recognition sites of the SLC9A3R1 gene in malignant ovarian tumors may potentially affect the behavior of cancer cells. The present study found mutations in early low-grade and advanced (G1-G3) EOC tumors, however, future studies are required in order to understand the clinical implications of these mutations in the prognosis of ovarian cancer patients.

Acknowledgements

The authors are especially grateful to Dr Alberto R. Kornblihtt for his contribution to the analysis of the results and to Dr Guillermo Juvenal for useful discussions. This study was supported by a grant from the Medical University of Gdansk: ST-2, Polish National Science Centre projects: 2011/02/A/NZ2/00017, the National Research Council of Argentina and the National Agency for the Promotion of Science and Technology of Argentina (PICT 0087, 2008).

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