

## Ectopic Expression of Sortilin 1 (NTR-3) in Patients with Ovarian Carcinoma

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Received: 31 Aug 2009

Accepted: 27 Sep 2009

## Abstract

Gene expression profiling of ovarian carcinoma tissues has shown an increase of four-fold expression of *SORT1* gene. Sortilin 1 (NTR-3) is a 95-100 kDa protein normally expressed in heart, brain, placenta, skeletal muscle, spinal cord, thyroid, and testis. However, its expression has never been reported in normal ovary. Here, we report expression of sortilin 1 in ovarian carcinoma tissues both at gene and protein levels. Sortilin 1 was expressed in all ovarian carcinoma patients (n=15) as well as ovarian carcinoma cell lines (n=5) regardless of their phenotypic characteristics. Non-malignant ovaries (n=6) did not express sortilin 1. The molecular basis for this ectopic expression is not yet clear. Our results showed a major cell surface expression of sortilin 1 rather than ER-Golgi compartment where it is mainly expressed. This finding may introduce sortilin 1 as a novel tumor marker for diagnosis of ovarian carcinoma and may signify its therapeutic value in targeted therapy.

*Avicenna J Med Biotech* 2009; 1(2): 125-131

**Keywords:** Ectopic, Expression, NTR, Ovarian carcinoma, Sortilin

## Introduction

Ovarian cancer is one of the leading causes of cancer deaths in women. Due to the lack of any specific tumor marker, the diagnosis of this disease usually happens in advanced stages leading to high mortality rate. Although, the increased level of CA125 is considered to be a diagnostic marker (in about 80% of cases), but it is still not reliable and usually increases at very late stages. Since no suitable diagnostic and curative therapy is available, there is a high demand for finding

novel tumor markers in this malignancy both for diagnostic and therapeutic purposes.

Gene expression profiling of ovarian carcinoma patients in early and late stages or with low malignant potential has shown a nearly four-fold increase of *SORT1* gene expression<sup>(1)</sup>.

The human *SORT1* gene consists of 22 exons located on the short arm of chromosome 1 (1p21.3-p13.1) encoding a type I receptor of 831 amino acids with a molecular

weight of 95-100 *kDa*. Seven non-coding transcript variants for this gene have also been reported.

*SORT1* is expressed at gene level in heart, brain, placenta, skeletal muscle, spinal cord, thyroid, and testis <sup>(2)</sup>. Its expression, however, has never been reported in normal ovary. Sortilin 1 is a member of family of Vps10p-domain receptors. It is known as a multi-ligand molecule and such proteins as lipoprotein lipase (LPL), Neurotensin 3 (NT3), Receptor associated protein (RAP), proNGF, and thyroglobulin are among its potential ligands <sup>(2, 3-5)</sup>.

The function of sortilin 1 varies depending on its location and the presence of its ligands. Sortilin 1 acts as a sorting receptor when it is expressed in trans-golgi network and also as a clearance receptor when it is expressed on cell surface <sup>(2, 3-5)</sup>.

The role of sortilin 1 and NT3 as its ligand in cancer cell growth has previously been reported <sup>(6)</sup>. Although, sortilin 1 as a type I receptor lacks the necessary domains involved in signaling, but is an essential part of a receptor complex for pro-NGF induced neural cell death <sup>(5, 7)</sup>.

The four-fold overexpression of *SORT1* in ovarian carcinoma together with its driving role in cancer cell growth brings forward the potential involvement of this protein in the pathobiology of this disorder. To test this hypothesis, we studied the expression of sortilin 1 in 15 ovarian carcinoma patients both at gene and protein levels. Sortilin 1 was expressed in all 15 ovarian carcinoma tumor tissues as well as related cell lines, while no expression of this molecule in non-malignant ovarian tissue was found. The expression of sortilin 1 in ovarian carcinoma patients may represent a crucial role of this protein in tumorigenesis driving machinery.

## Materials and Methods

### *Patients and tissue samples*

Ovarian tissue samples were obtained from patients at Imam Khomeini Hospital. After a consent letter was signed by each patient, all

the aspects of this study were approved by the Avicenna Local Ethics Committee. After surgical resection, each fresh tumor specimen was immediately divided into two portions: one portion was instantly frozen in liquid nitrogen and the other was OCT (Optimal Cutting Compound) embedded using Jung freezing medium (Leica Instruments, Nussloch, Germany) for immunohistochemical investigations.

Fifteen malignant ovarian tissue samples including papillary serous carcinomas (n=9; mean age 48.3±16.08 yr), endometrioid carcinoma (n=1; age 39), mucinous carcinomas (n=2; age 59 and 70 yrs), clear cell carcinoma (n=1; age 45 yr), germ cell tumor (n=1; age 28 yr), and sex cord-stroma tumor (n=1; age 22 yr) were investigated. A total of six non-malignant ovarian tissues (mean age 45.3±4.2 yr) served as control group (Table 1). Tissue sections made from each sample, stained with Hematoxylin and Eosin (H&E) and examined by two independent pathologists to confirm the pathological state of each sample.

### *Cell lines*

The human ovarian carcinoma cell lines (Table 2) were cultured in RPMI 1640 (Gibco, Invitrogen, USA), containing 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 100 *units/ml* penicillin (ICN Biomedicals, Ohio, USA), and 100  $\mu\text{g/ml}$  streptomycin (Sigma, St Louis, MO). All cell lines were cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub> atmosphere.

### *RNA extraction and cDNA synthesis*

Total RNA was extracted from cell lines and tissue samples using RNA-Bee reagent (BioSite, Täby, Sweden) according to the manufacturer's instruction. The quality of the RNA samples was determined by agarose gel electrophoresis after staining with ethidium bromide, and visualization under UV light. Total RNA was unfolded at 65 °C for 10 *min*. cDNA was then synthesized using 5  $\mu\text{g}$  of total RNA in 20  $\mu\text{L}$  reaction mixture consisting of 4  $\mu\text{L}$  of 5x reaction buffer, 1  $\mu\text{L}$

Table 1. Expression of sortilin 1 and phenotypic characterization of 15 ovarian carcinoma patients compared to the normal ovary tissues from 6 healthy individuals

| Patients | Morphology            | Type of differentiation    | Age (yr) | Stage | CA125 level (U/ml) | Level of differentiation | RT-PCR (nested) and Western blot |
|----------|-----------------------|----------------------------|----------|-------|--------------------|--------------------------|----------------------------------|
| T4       | Epithelial            | Serous cyst adenocarcinoma | 40       | NA    | NA                 | NA                       | +                                |
| T5       | Epithelial            | Serous cyst adenocarcinoma | 48       | IV    | 38.6               | NA                       | +                                |
| T7       | Sex cord-stroma       | Granulosa cell tumor       | 22       | NA    | NA                 | NA                       | +                                |
| T9       | Epithelial            | Serous cyst adenocarcinoma | 23       | III   | 66.4               | NA                       | +                                |
| T10      | Epithelial            | Clear cell                 | 45       | III   | 183                | NA                       | +                                |
| T14      | Epithelial            | Serous cyst adenocarcinoma | 66       | IV    | 1890               | Moderate                 | +                                |
| T15      | Epithelial            | Serous adenocarcinoma      | 33       | I     | >1000              | Well                     | +                                |
| T18      | Epithelial            | Serous adenocarcinoma      | 66       | NA    | >1000              | Well /moderate           | +                                |
| T19      | Epithelial            | Endometrioid               | 39       | III   | 48                 | Moderate                 | +                                |
| T22      | Epithelial            | Serous adenocarcinoma      | 69       | II    | 2490               | Moderate                 | +                                |
| T25      | Epithelial            | Mucinous                   | 59       | NA    | 124                | NA                       | +                                |
| T32      | Epithelial            | Serous cyst adenocarcinoma | 50       | NA    | 448                | NA                       | +                                |
| T34      | Germ cell             | Yolk sac tumor             | 28       | NA    | 31.9               | NA                       | +                                |
| T37      | Epithelial            | Mucinous                   | 70       | NA    | NA                 | NA                       | +                                |
| T39      | Epithelial            | Serous adenocarcinoma      | 40       | III   | NA                 | Well                     | +                                |
| Normal   | Non-malignant tissues | -                          | 40-49    | -     | NA                 | -                        | Negative (6 out of 6)            |

NA: not assigned

of 10 mM dNTPs, 1  $\mu$ L of 10 pmol/mL random hexamers (N6), and 200 U M-MuLV reverse transcriptase (Fermentas GmbH, St. Leon-Rot, Germany). The reaction mixture was incubated at 42 °C for 45 min.

#### RT-PCR amplification of SORT1

RT-PCR amplification was performed using SORT1 gene (g.b. NM\_002959) specific primers of “CAGTCCAAGCTATATCGAA GTGAGG” as sense and “AAGATGGTGTT GTCTGATCCCCATTT” as antisense (Operon, Köln, Germany). Briefly, 25  $\mu$ L reaction mixture of PCR was prepared using 2.5  $\mu$ L 10x buffer, 3  $\mu$ L 25 mM MgCl<sub>2</sub>, 1  $\mu$ L dNTPs (10 mM), 5 pmol of each primer, 1 Unit Taq DNA polymerase (Roche, Germany) and 1  $\mu$ L template cDNA. PCR was then followed by 35 cycles (for SORT1) or 30 cycles (for  $\beta$ -actin) at 92 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec leading to a 383 bp SORT1 or 155-bp  $\beta$ -actin amplicons. To ensure the specificity of primers, some of the SORT1 PCR products were cloned into

pGEM-T easy vector (Promega, Madison, WI) and were subjected to sequencing.

Due to the poor quality of RNA prepared from some tissue samples, nested-PCR was carried out for detection of SORT1 gene in ovary tissues using “GTAAACAGCAGAG GTGTCTGGAG” as sense primer and “AA ACATACTGCTTTGTGGATTTC” as anti-sense primer. Briefly, 1  $\mu$ L of RT-PCR product was added to a new 24  $\mu$ L reaction mixture containing 2.5  $\mu$ L 10x PCR buffer, 3  $\mu$ L 25 mM MgCl<sub>2</sub>, 1  $\mu$ L 10 mM dNTPs, 5 pmol of each primer, and 1 Unit Taq DNA polymerase. Nested PCR was performed for 20 cycles generating an amplicon of 225 bp. PCR products were finally visualized by running agarose gel electrophoresis containing ethidium bromide.

#### Western blotting

Tissues and cell lines were lysed in lysis buffer containing 2% Triton X-100, 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 20 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1% glycerol, 0.1%

Table 2. Expression of sortilin 1 in ovarian cancer cell lines

| Cell line  | Morphology | Western blot | RT-PCR | Reference      |
|------------|------------|--------------|--------|----------------|
| CAOV-4     | Epithelial | +            | +      | ATCC (HTB-76)  |
| A2780S     | Epithelial | +            | +      | NCBI (C461)    |
| A2780CP    | Epithelial | +            | +      | NCBI (C454)    |
| 2008/C13.R | Epithelial | +            | +      | NCBI (C446)    |
| OVCAR-3    | Epithelial | +            | +      | ATCC (HTB-161) |
| SKOV-3     | Epithelial | +            | +      | ATCC (HTB-77)  |

NCBI; National Cell Bank of Iran, ATCC; American Type Culture Collection

sodium dodecyl sulfate (SDS), and 1% protease inhibitor cocktail (Sigma) on crashed ice for 1 hr. The lysates were then centrifuged for 10 min (9300 g, 4 °C). The protein concentration of the supernatant was estimated by the Bradford method. Equal amounts of protein (30 µg) were run on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 2 hr under non-reducing condition.

After electrophoresis, resolved proteins were transferred onto PVDF (polyvinylidene difluoride) membranes (Millipore, Bedford, USA). The membranes were then blocked overnight at 4 °C with 5% nonfat milk in phosphate-buffered saline (PBS)-Tween 20 (0.15 M PBS pH 7.4, 0.1% Tween 20). All additional incubations were performed in PBS-Tween 20 supplemented with 5% nonfat milk (Merck, Darmstadt, Germany). Membranes were incubated with a rabbit polyclonal antibody against human sortilin 1 (Abcam, Cambridge, UK) at a concentration of 1 µg/mL for 1 hr. After 4 times of 15 min washings with PBS-Tween 20, membranes were incubated with a horseradish peroxidase (HRP)-conjugated sheep anti-rabbit immunoglobulin (Avicenna Research Institute, Tehran, Iran) as secondary antibody for 1 hr and washed as above.

To verify the relative amounts of protein in each lane, the level of β-actin as an internal control was measured with anti-β-actin monoclonal antibody (Sigma). Membranes were developed using the ECL detection system (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instruction.

#### Immunohistochemistry (IHC)

Five µm sections of OCT-embedded ovary tissues were cut on a cryostat (Thermo-Shandon, UK), dried overnight, fixed in acetone for 2 min at -20 °C and kept at -70 °C until use. Slides were then thawed and washed three times (3×3 min) with Tris-Buffered Saline, pH 7.4 containing 5% bovine serum albumin (TBS-BSA). At the next step, the slides were blocked with 5% goat serum diluted in TBS-BSA for 30 min and incubated

with 1:500 diluted primary anti-sortilin 1 polyclonal antibody (Abcam, Cambridge, UK) overnight at 4 °C. To confirm the specificity of the IHC staining, negative reagent control slides were incubated with TBS-BSA without any primary antibody (no-antibody control) or with pre-immune rabbit serum with the same IgG concentration. Following three washes, the slides were then incubated with 1:75 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Ray Biotech, Inc. GA, USA) for 45 min.

After washing with TBS-BSA, the nuclei were counterstained by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Calbiochem, USA) at 1µg/ml for 5 min, then the slides were washed, mounted in PBS-glycerol 90% and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

## Results

#### *SORT1* gene expression

Representative RT-PCR gel of ovarian carcinoma patients is shown in Figure 1. All ovarian carcinoma patients (n=15) expressed *SORT1* at the mRNA level (Table 1). *SORT1* was also expressed in all six ovarian carcinoma cell lines (Table 2). Non-malignant ovary, however, failed to express this gene (data not shown).

#### *Sortilin 1* protein expression

Western blot analyses of six ovarian carcinoma cell lines showed a band of 95-100 kDa as well as an extra band of 50 kDa (Figure 2A). Cell lysates from primary ovarian carcinoma tissues of different subtypes showed only a band of 95-100 kDa

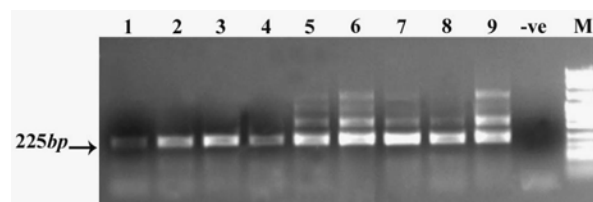


Figure 1. *SORT1*-specific mRNA expression (nested RT-PCR) in ovarian cancer tissue samples. Lanes 1-9 tissue samples, -ve: Negative control PCR reaction without template, M: DNA size marker. The figure represents 9 experiments out of 15. The extra bands over 225 bp are due to the overload of primary PCR reactions

of sortilin 1 (Figure 2B). None of the non-malignant ovary samples expressed sortilin 1 at protein level (Figure 2C).

To confirm the expression and localization of sortilin 1 in tumor samples, we performed indirect immunofluorescence staining on 7 malignant ovarian tissue samples and 5 normal ovaries. The positive staining was only observed in the cell membrane of malignant epithelial cells. Consistent with the results obtained by Western blotting, the ovarian carcinomas (four of seven specimens) expressed sortilin 1 mainly localized to cell surface rather than ER-Golgi compartment in immunohistochemistry & tainins. Three out of seven ovarian carcinoma samples showed lower levels of sortilin 1 in immunofluorescence experiments. Similar to Western blot results, no sortilin 1 expression was observed in normal ovary tissue sections. One representative out of seven immunofluorescence experiments is shown in Figure 3.

### Discussion

The functional roles of neuropeptides (NT) and their receptors in cancer cell growth are now well established. The growth effect of

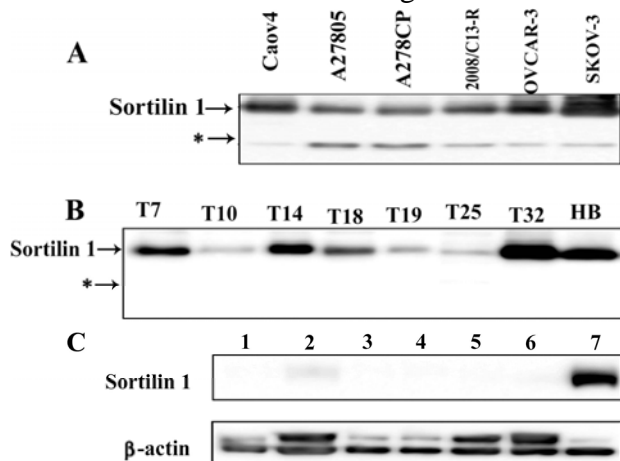


Figure 2. Western blot analysis of sortilin 1 using rabbit polyclonal anti-sortilin antibody under non-reducing conditions. A) All ovarian cancer cell lines exhibited expression of about 100 *kDa* sortilin 1 protein. \*A band of about 50 *kDa* was also expressed by these cell lines. B) Ovarian cancer tissue samples T7, T10, T14, T18, T19, T25, and T32 corresponding to patients in Table I selected based on their differentiation. Human brain (HB) cell lysate served as positive control. C) Lanes 1-6 representing non-malignant ovarian tissue samples. Lane 7 represents human brain cell lysate.  $\beta$ -actin expression was used as an internal loading control

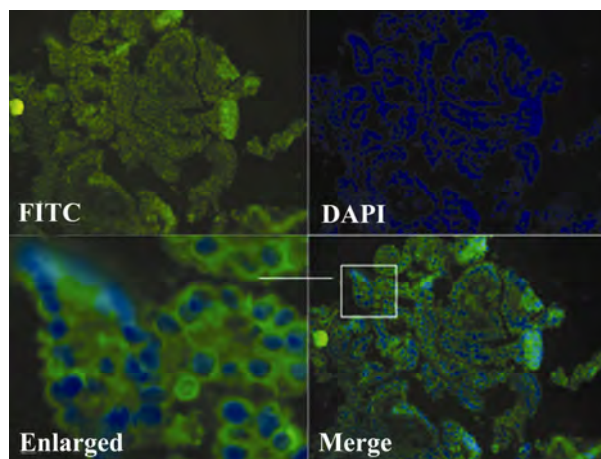


Figure 3. Immunofluorescence staining of sortilin 1 in ovarian carcinoma tissue (T15) using rabbit anti-sortilin 1 polyclonal antibody. The enlarged part clearly shows the cell surface expression of sortilin 1. The figure represents one experiment out of seven

sortilin 1 as neurotensin-3 receptor (NTR-3) in prostate, colon, and pancreatic cancer cell lines strongly suggests the involvement of this protein in cell growth<sup>(8)</sup>. Sortilin 1 was also expressed in a panel of breast cancer cell lines (our unpublished data). It seems that the expression of sortilin 1 is not restricted to ovarian carcinoma and it might be also expressed in other solid tumors. This may signify more important function of this receptor in cancer development.

In our study, the cell lysates from all ovarian cancer tissues and cell lines but not from non-malignant ovary tissues, showed expression of 95-100 *kDa* sortilin 1 protein indicating an ectopic expression. Interestingly, a 50 *kDa* band was only observed in ovarian carcinoma cell lines whereas it was absent in breast cancer cell lines (data not shown). This 50 *kDa* band may represent an unidentified isoform of sortilin 1 which requires further characterization.

Although, sortilin 1 is mainly localized to ER-Golgi apparatus, it is also expressed as a mature cell surface receptor<sup>(9)</sup>. Our immunofluorescence experiments showed that sortilin 1 is mainly expressed on tumor cell surface rather than ER-Golgi compartment. The surface expression of this protein may provide a more significant role for this protein in ERK signaling pathway<sup>(10)</sup> leading to proliferation

of cancer cells. Apparently, expression of sortilin 1 in ovarian cancer cells as a cell surface receptor does not induce apoptosis excluding its proapoptotic activity. This might be due to the lack of other co-receptors required for triggering apoptosis in ovary cancer cells and the absence of their signaling mediators (proNGF, proBDNF). It could be also due to structural differences in sortilin 1 expressed by cancer cells leading to impaired heterodimerization with other co-receptors (p75NTR) which is necessary for signal transduction. In general, the dual functional proapoptotic and anti-apoptotic roles of sortilin 1 must be considered in normal and pathogenic conditions.

Among patients enrolled in this study two patients had tumors with morphology of non-epithelial origin indicating that expression of sortilin 1 is not restricted to epithelial cells. Due to the lack of patient samples from early stages and also limited number of patients, it was not possible to correlate the expression level of sortilin 1 to the stage of the disease as well as to CA125 level or age of the patients. In this regard the expansion of the study in more patients is warranted.

Recent report shows that sortilin 1 is also expressed in thyroid epithelial cells capable of binding with high affinity to thyroglobulin, another evidence for sortilin 1 as a multi-ligand receptor<sup>(4)</sup>. This finding together with the expression of sortilin 1 in ovarian carcinoma may explain the 4% incidence of metastasis of ovarian carcinoma to thyroid glands, rich in thyroglobulin<sup>(11)</sup> as well as the same incidence in brain metastases<sup>(12)</sup>.

Altogether, ectopic expression of sortilin 1 in ovarian carcinoma tissues, its absence in normal human ovary, its involvement in ERK signaling pathway<sup>(10)</sup> as well as its restricted tissue expression in women makes it a potential candidate for developing more selective targeted therapy approach in patients with ovarian carcinoma. In this regard, generation of monoclonal antibodies capable of inducing apoptosis and also competing with sortilin 1

ligand binding site (Vps10p domain) is recommended.

Women with cancer may suffer from infertility after medical treatment. The current approach to protect reproductive capability in women with cancer is ovarian cortex cryopreservation prior to any medical treatment and re-implanting the frozen ovary tissues after cancer therapy. Detection of sortilin 1, as a unique marker in the cryopreserved tissue for distinguishing malignant ovaries from non malignant ones before reimplantation may decrease the risk of relapse in these patients leading to regaining their reproductive capability.

To the best of our knowledge, this study is the first report describing ectopic expression of sortilin 1 in patients with ovarian carcinoma. Further functional impacts and structural characterization are needed to explore its potential role in the pathogenesis of ovarian carcinoma.

### Acknowledgment

This work was supported by grants from Presidential Science and Technology Deputy of Islamic Republic of Iran. We thank Mrs. Zohre Sadeghian (Imam Khomeini Hospital, Tehran, Iran) for her assistance in collecting tissue samples.

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