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Sucrose non-fermenting related kinase expression in ovarian cancer and correlation with clinical features

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Abstract

Sucrose non-fermenting related kinase (SNRK) is a serine/threonine kinase known to regulate cellular metabolism and adipocyte inflammation. Since alterations in adipocyte metabolism play a role in ovarian cancer metastasis, we investigated the expression of SNRK in benign and malignant human ovarian tissue using immunohistochemistry and qPCR. The number of SNRK positive (+) nuclei is increased in malignant tissue compared to benign tissue (21.03% versus 14.90%, $p<0.0431$). The most strongly stained malignant SNRK+ nuclei were stage 1 compared to stage 2–4 disease. Differential expression of SNRK in early versus late stage disease suggests specific roles for SNRK in ovarian cancer metastasis.

Keywords

Ovarian Cancer; Cancer Biomarkers; Invasion & Metastasis

Introduction

Ovarian cancer is the leading cause of gynecologic mortality in the United States with over 22,000 cases diagnosed annually and 14,000 deaths. This high mortality is attributed to lack of symptoms or screening tests with the majority of disease diagnosed at late stages(1). Ovarian cancer commonly spreads to the omentum, an apron of tissue in the abdomen primarily comprised of adipocytes. The mechanisms that drive the development of ovarian cancer and subsequent metastasis are an area of active research. Metabolic changes in tumor

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cells and omental adipocytes that allow for metastasis and tumor survival have been described (2).

Sucrose non-fermenting related kinase (SNRK) is a serine/threonine kinase with sequence similarity to AMP-activated kinase (AMPK). These protein kinases belong to a family of evolutionarily conserved metabolic sensors found in all eukaryotic organisms(3). Celenza and Carlson found that in the yeast *Saccharomyces cerevisiae,* SNF1 (sucrose nonfermenting) is needed for expression of glucose-repressible genes that allow for the organism to use non-fermentable carbon sources in times of glucose deprivation(4, 5). AMPK family members are activated by starvation as well as energy-depleting stress conditions and enable energy homeostasis and survival by up-regulating energy-conserving/energy-producing catabolic processes, and by limiting energy-consuming anabolic metabolism. In addition, they control normal growth and development as well as metabolic homeostasis at the organismal level(5).

Little is known about the specific function of SNRK. Studies show it is involved in neuronal apoptosis(6), blood vessel development(7), and cardiac development, metabolism and function in mammals(8). SNRK is also reported to directly phosphorylate the mTOR binding partner raptor which results in inhibition of mTOR complex 1 (mTORC1) and causes cell cycle arrest(9). Simultaneous activation of AKT and mTORC1 has been observed in 87% of human ovarian carcinomas, and elevated mTOR signaling is associated with poor prognosis(10). The mTOR inhibitor Temsirolimus has been tested in ovarian cancer clinical trials and was shown to have a modest effect, although results from the trial did not meet criteria to be included in a Phase III study(11). Upstream regulators of mTOR signaling include Liver kinase B1 (LKB1 also known as STK11), phosphatase and tensin homolog (PTEN), tuberous sclerosis 1 (TSC1), and tuberous sclerosis 2 (TSC2), of which LKB1 is known to activate several AMPK family members including SNRK(12).

SNRK expression in cancer is not well described. SNRK overexpression has been shown to reduce colon cancer cell proliferation with stable SNRK knockdown resulting in increased *in vitro* colon cancer cell tumorigenicity(13).

Based on the circumstantial evidence in the literature, what is known about the function of SNRK, and the fact that ovarian cancer metastasis involves alterations in metabolism, we sought to determine the expression of SNRK in ovarian tissue. The objective of this study was to describe the expression pattern of SNRK in both malignant and benign ovarian tissue and correlate expression with clinicopathological variables.

Materials and Methods

Tissue samples

The Medical College of Wisconsin/Froedtert Hospital Institutional Review Board (PRO00022211) approved this study. Benign and malignant ovarian tissue samples were obtained from the Medical College of Wisconsin Tissue Bank. High-grade serous epithelial ovarian cancer tissue samples were obtained from subjects who had not yet undergone chemotherapy. Ovarian tissue samples with benign pathology, such as ovarian fibroma and

simple cyst, were obtained. Clinicopathological information from subjects including demographic data and clinical data such as tumor grade, histology, stage, treatment response, and survival were obtained by a retrospective chart review.

Immunohistochemistry (IHC)

Cryo embedded 10 micron thick tissue sections were rehydrated in PBS and fixed with 4% paraformaldehyde. Sections were blocked for non-specific binding in 1.5% normal goat serum and incubated with the primary SNRK antibody (1:100; Abcam, ab96762) overnight at 4°C. The specificity of this antibody for SNRK has been previously validated by our lab(8). For negative controls, a section from each sample was incubated in PBS without primary antibody. For immunoperoxidase staining, sections were incubated with biotin labeled anti-rabbit secondary antibody (1:250; Vector labs, BA-1000) for one hour at room temperature and were then subjected to DAB peroxidase substrate reaction staining (Vector PK-6100 and SK-4100). Tissue sections were then dehydrated and mounted with Acrytol mounting medium (Leica, 3801720).

Measurement of SNRK Expression

Immunohistochemistry (IHC) stained slides were scanned in a batch on a high resolution, whole slide scanner (NanoZoomer HT 2.0, Hamamatsu, Japan) at 40X magnification and the stored data are accessed through proprietary NDPview software for virtual image exploration. NanoZoomer utilizes line scanner and 3-Chip time delay integration technology (single RGB image) to provide high speed and high sensitivity simultaneously. These high resolution images can be viewed (virtual images) at 0.25x - >100x magnification that enhances quality of slide viewing and analysis. For image data quantification, we utilized software assisted, unbiased quantification method. The scanned images were imported to Visiopharm software (Denmark) and using the micro imager module we extracted 20x ROI images that covered 95% of the tissue slice and these ROI images were utilized to estimate the extent of SNRK (+) nuclei. The staining profile showed two distinct populations, one with lighter (Weak) DAB staining and the other with dark (stronger) DAB staining (Figure 1a). These two different cell populations were identified by preset thresholding and linear Bayesian classification to generate processed image that yields SNRK (+) cells and total tissue region. The non DAB stained tissue regions were captured (highlighted in faint orange color) separately and added to DAB positive region to calculate the total tissue area. The white pixel area in and around the tissue section represented the background area.

Total RNA extraction

RNA was collected from ovarian or tumor tissue from patients who underwent surgery for benign indications (n=3) or ovarian cancer (stage 1 n=2; stage 2 n=5; stage 3 n=7) using TRIzol reagent (LifeTechnologies). NanoDrop ND-2000 (Thermo Scientific) was used to determine RNA quantity and quality.

qPCR analysis

Using 500ng of total RNA from each sample, cDNA was synthesized with SuperScript III (Life Sciences) reverse transcription kit that has genetically engineered MMLV reverse

transcriptase for improved cDNA yield, cDNA length and efficiency on GC-rich target RNAs. *SNRK* mRNA expression in ovarian/tumor samples was analyzed using Life Technologies Taqman Primers: *18S* Hs99999901_s1, *SNRK* Hs00299395_m1. Samples were assembled in TaqMan gene expression master mix (Life Technologies) and run in a Bio-Rad real-time thermal cycler (Cycle 1: 50'C for 2 minutes and 95'C for 10 minutes; Cycle 2–40: 95'C for 15 seconds and 60'C for 1 minutes followed by data acquisition. The gene expression levels in ovarian/tumor tissue were expressed as a ratio, normalized to *18S* rRNA.

Statistical Analysis

For immunohistochemical staining, group comparisons were made using the Kruskal-Wallis/Wilcoxon and Jonckheere-Terpstra tests. SNRK positivity was examined with a generalized linear model with logit link, normal error, and generalized estimating equations (GEE) to control for repeated measures. Results for mean SNRK positivity are the inverse-logit of the least-square mean estimates. Results were considered significant at the 0.05 level. The analysis was performed using SAS version 9.4 (The SAS Institute, Cary, NC). For qPCR, student's unpaired t-test and one-way ANOVA was used for comparison analysis and the results are described as means (± standard error of the mean, SEM).

Results

Thirty-one ovarian tissue samples consisting of twenty-one high-grade serous epithelial ovarian cancer samples and ten benign ovarian tissue samples were analyzed with IHC for SNRK expression. Patient characteristics are shown in Table 1.

Immunohistochemistry

SNRK demonstrated a nuclear staining pattern in both the malignant and benign tissue sections (Figure 1). There was a significant increase in the amount of SNRK (+) cells in malignant ovarian cancer tissue versus benign ovarian tissue as shown via quantification in Figure 2. In serous epithelial ovarian cancer specimens, 21.03% of nuclei showed strong SNRK positivity versus 14.90% benign ovarian nuclei (p=0.0431). When examining patient characteristics, the malignant samples with the highest percentage of strongly stained SNRK + nuclei were Stage I (24.39%) compared to Stage II-IV disease which had a lower percentage of SNRK + nuclei per stage with a high incidence of recurrence and death due to disease based on subject characteristics (Table 1, Table 2). However, due to the small sample size and censoring, reliable correlation with survival could not be made.

qPCR gene expression analysis

SNRK gene expression analysis by qPCR method validates and supports the findings from IHC. Data from 3 benign ovarian samples and 14 ovarian cancer samples (stage 1 n=2; stage 2 n=5; stage 3 n=7) indicate that *SNRK* mRNA is increased in malignant tissue compared to benign ovarian tissue (p=0.16; Figure 3A). When *SNRK* mRNA expression levels in benign ovarian tissue were compared to each stage of ovarian cancer there was increase in mRNA expression between benign and stage 1 disease (p=0.07) and benign and stage 3 disease (p=0.08). There is a significant decrease in mRNA expression between stage 1 and stage 2

disease (p = 0.02) and a significant increase in mRNA expression between stage 2 and stage 3 disease (p=0.03; Figure 3B). This is consistent with the IHC data, where SNRK expression also decreased between stage 1 and 2 disease and then increased in more advanced disease (stage 3) (Table 2).

Discussion

In this study, we describe SNRK expression in benign ovarian tissue and high-grade serous epithelial ovarian cancer tissue. We performed both qPCR for *RNA* gene expression and immunostaining for protein detection in tissues. Utilizing both of these probing methods, we demonstrate that SNRK has a nuclear staining pattern, a finding that has been shown in mouse ovarian tissue in our lab as well (14), and that expression is significantly greater in malignant compared to benign ovarian tissue. We demonstrate a trend indicating lower SNRK expression in those subjects with metastatic disease and poor outcome.

While the sample size in this study is small, it is intriguing that, compared to benign ovarian tissue, SNRK expression is increased in stage 1 ovarian cancer when malignancy is confined to the ovary, and then decreased in stage 2 disease, when the tumor becomes metastatic. Increased SNRK expression in early stage high-grade serous epithelial ovarian cancer tissue may be a result of higher metabolic stress in cells with higher proliferation rates, a topic that needs further investigation. Our results show a change in expression of SNRK between early stage and late stage disease. In late stage ovarian cancer (stages 3 and 4) there is a predilection for metastasis to the omentum, an apron of tissue in the abdomen that is primarily comprised of adipocytes. Alterations in SNRK expression may lead to changes in tumor or adipocyte metabolism and subsequent signaling within this metastatic microenvironment.

Alterations in metabolism within tumor cells and metabolic signaling from adipocytes in the omentum are known to be important in allowing ovarian cancer to metastasize and survive. Studies show that human omental adipocytes promote metastasis of ovarian cancer cells and that interactions between omental adipocytes and ovarian cancer cells lead to metabolic changes that allow adipocytes to act as an energy source for cancer cells(2). SNRK is known to play a role in adipocyte metabolism. Li et al has shown that SNRK is a suppressor of adipocyte inflammation in that, when there is less adipocyte SNRK expression, inflammatory pathways are activated causing compromised insulin signaling, increased lipolysis, and defective autophagy(15). With our findings showing lower SNRK expression in metastatic tumors, combined with the Li et al study that loss of SNRK in adipocytes show pro-inflammatory pathways, we speculate that in the metastatic microenvironment loss of SNRK expression has a profound influence on tumor cells and their interaction with the microenvironment, some of which may be metabolically related. These questions need further investigation, and are currently under study in our laboratory.

SNRK expression has been investigated in other cancers. It is noteworthy that Rines et al demonstrated lower SNRK expression in a colon cancer cell line (HCT116), and that SNRK inhibited colon cancer cell proliferation through reduced beta-catenin signaling(13). Furthermore, AMPK activation has also been shown to be reduced in melanoma cell lines,

which have the oncogenic V600E mutation and reduced in breast cancers where the mechanism of AMPK down-regulation is unknown(16).

In summary, we show that SNRK demonstrates a nuclear staining pattern in human ovarian cancer tissue with a significant increase in SNRK expression in high-grade serous epithelial ovarian cancer tissue compared to benign ovarian tissue. A trend towards decreased SNRK expression in tumors with a higher disease stage and poor outcome is seen. This correlation with disease stage is intriguing and warrants further investigation into the potential role for SNRK in the metastatic process of ovarian cancer.

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Declaration of Interest

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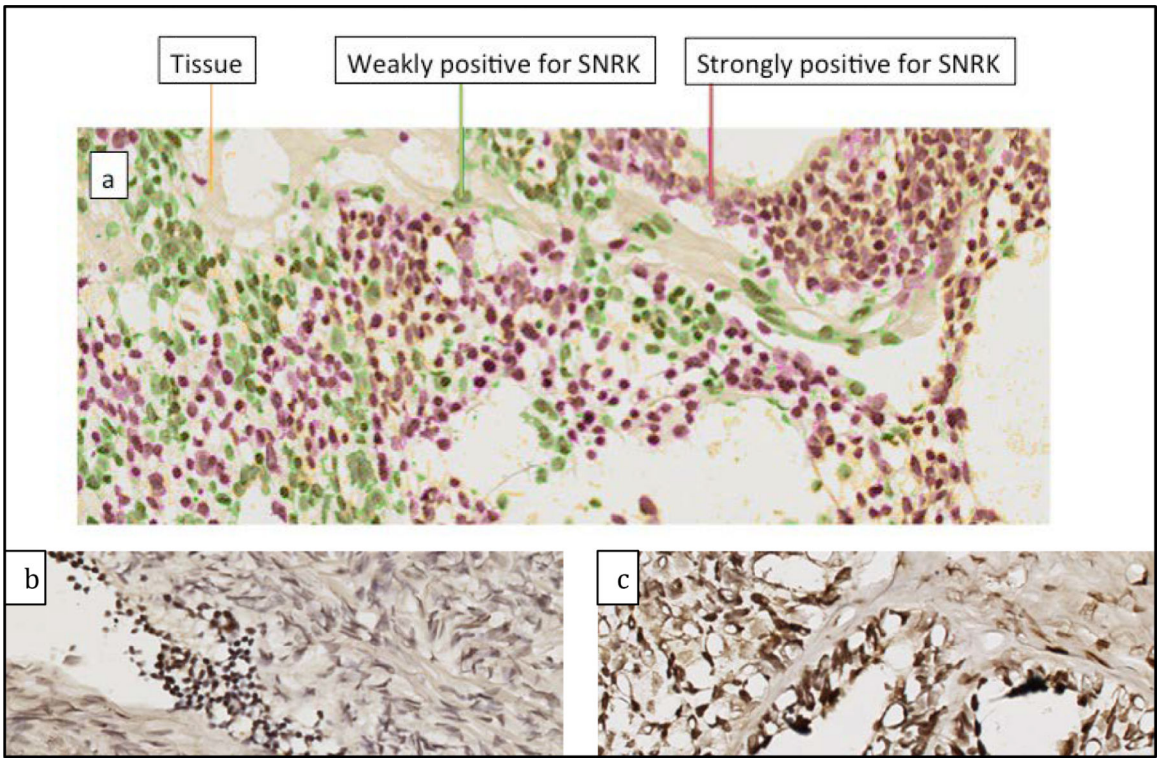

Figure 1.

Tissue sections demonstrating SNRK staining. A) Using Nanozoomer and Visiopharm softwares, nuclei highlighted in green are weakly positive for SNRK and nuclei highlighted in pink are strongly positive for SNRK. B) Tissue section from benign ovarian sample. C) Tissue section from malignant ovarian sample.

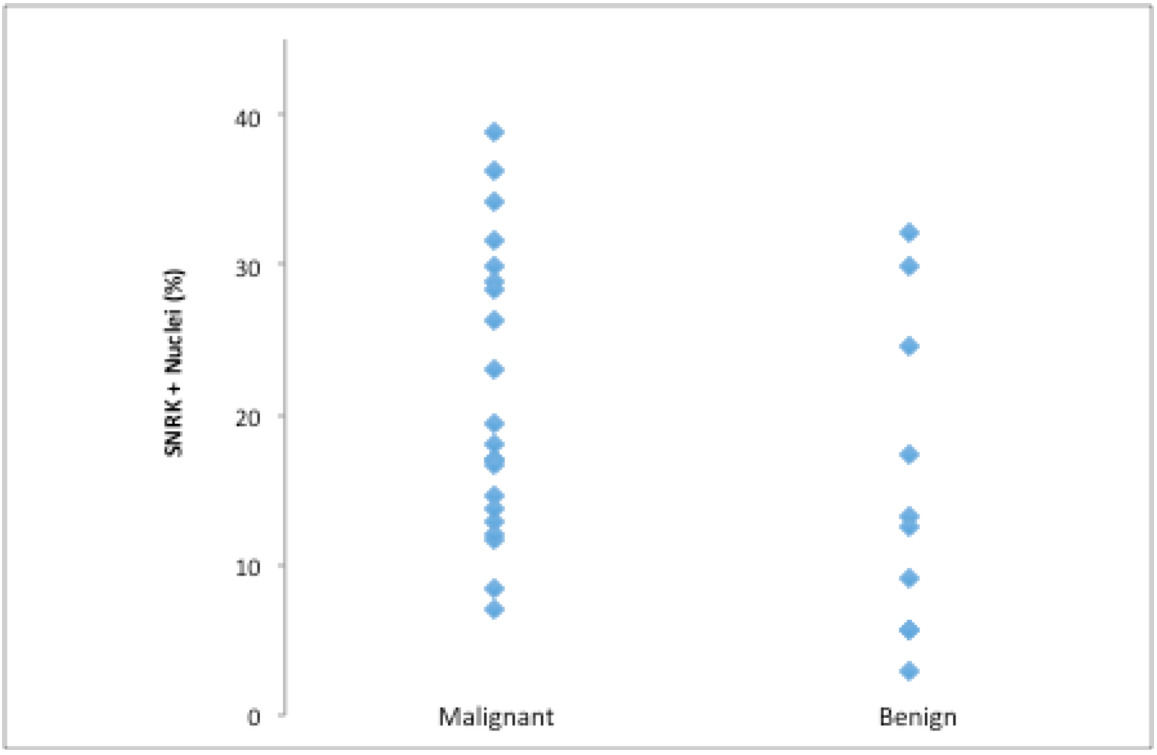

Figure 2.

Scatterplot demonstrates SNRK positivity in serous epithelial ovarian cancer tissue versus benign tissue. SNRK+ malignant nuclei 21.03%, 95% CI (0.1848–0.2384); SNRK+ benign nuclei 14.90%, 95% CI (0.1065–0.2048); p=0.0431.

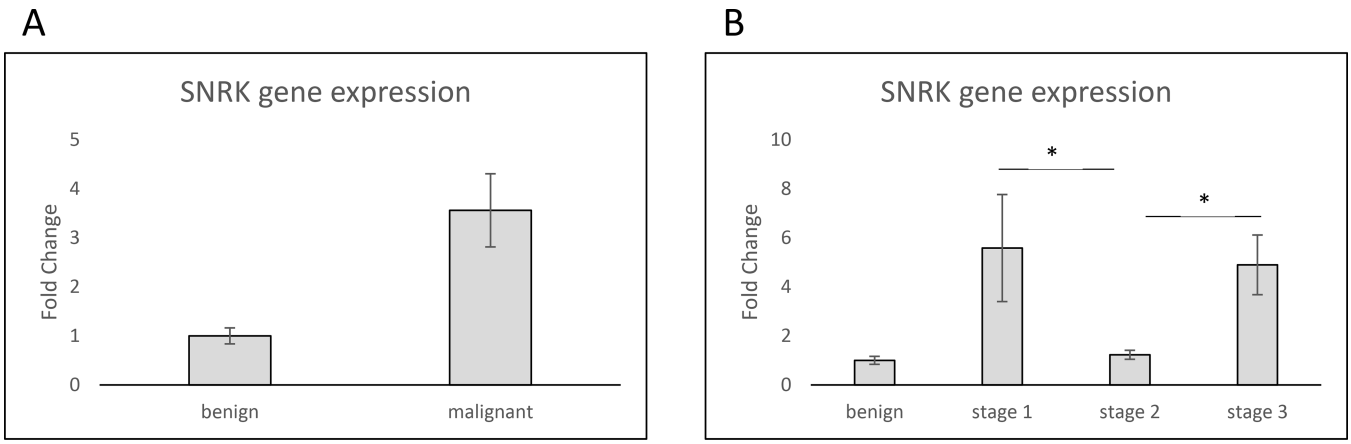

Figure 3.

qPCR analysis. A. *SNRK* mRNA expression is increased in malignant ovarian tissue compared to benign (p=0.16). B. *SNRK* mRNA expression is increased in stage 1 and stage 3 ovarian cancer compared to benign (p=0.07 and p=0.08 respectively). *SNRK* mRNA expression is significantly decreased from stage 1 to stage 2 cancer (p=0.02) and significantly increased between stage 2 and 3 (p=0.03). The results are the mean of the fold change of the Δ^2CT ± SEM from 3 benign and 14 malignant samples. The expression levels were normalized to 18S mRNA. *p-value *<0.05.*

Table 1.

Subject Characteristics.

Subject	Age	BMI	Menopausal Status	Pathology	FIGO Stage	Status	Months to Recurrence or death
Malignant							
1	57	43	Postmenopausal	High grade serous carcinoma	IIIC	Recurrence, NED	44
2	74	34	Postmenopausal	High grade serous carcinoma	IIIC	Deceased	2
3	42	39	Premenopausal	High grade serous carcinoma	IIIC	Recurrence, AWD	12
4	68	27	Postmenopausal	High grade serous carcinoma	IC	NED	NA
5	82	23	Postmenopausal	High grade serous carcinoma	IIIC	Lost to follow up	NA
6	57	30	Postmenopausal	High grade serous carcinoma	IIB	NED	NA
7	60	25	Postmenopausal	High grade serous carcinoma	IIIC	Recurrence, AWD	16
8	56	36	Postmenopausal	High grade serous carcinoma	IIB	Recurrence, AWD	27
9	49	22	Premenopausal	High grade serous carcinoma	IIIC	Recurrence, AWD	23
10	78	32	Postmenopausal	High grade serous carcinoma	IIIC	Recurrence, AWD	21
11	59	34	Postmenopausal	High grade serous carcinoma	IIA	Recurrence, AWD	33
12	64	45	Postmenopausal	High grade serous carcinoma	IA	NED	NA
13	63	21	Postmenopausal	High grade serous carcinoma	IIIC	Deceased	12
14	74	20	Postmenopausal	High grade serous carcinoma	IIIC	Lost to follow up	NA
15	70	36	Postmenopausal	High grade serous carcinoma	IIIC	Recurrence, AWD	16
16	60	53	Postmenopausal	High grade serous carcinoma	IC	Deceased, but not of disease	NA
17	59	33	Postmenopausal	High grade serous carcinoma	IIC	NED	NA
18	72	32	Postmenopausal	High grade serous carcinoma	IIIC	Recurrence, AWD	22
19	43	32	Premenopausal	High grade serous carcinoma	IIC	NED	NA
20	61	25	Postmenopausal	High grade serous carcinoma	IV	NED	NA
21	65	25	Postmenopausal	High grade serous carcinoma	IIIB	Recurrence, AWD	19
Benign							
22	64	35	Postmenopausal	Ovarian fibroma	NA	NA	NA
23	67	21	Postmenopausal	Mucinous cystadenofibroma	NA	NA	NA
24	41	29	Premenopausal	Serous cystadenoma	NA	NA	NA
25	77	32	Postmenopausal	Serous cystadenofibroma	NA	NA	NA
26	49	34	Postmenopausal	Ovarian stromal hyperthecosis	NA	NA	NA
27	67	24	Postmenopausal	Papillary cystadenofibroma	NA	NA	NA
28	60	29	Postmenopausal	Fibrothecoma	NA	NA	NA
29	55	24	Postmenopausal	Epithelial inclusion cysts	NA	NA	NA
30	67	23	Postmenopausal	Mucinous cystadenoma	NA	NA	NA
31	59	37	Postmenopausal	Cystic corpus albicans	NA	NA	NA

(NED: No evidence of disease; AWD: Alive with disease; NA: not applicable; Age in years; BMI: Body Mass Index kg/m^2).

Table 2.

SNRK positivity in serous epithelial ovarian cancer tissue FIGO stages I-IV.

	SNRK+ Nuclei (%)	95% Confidence Interval
Stage I (n=3)	24.39	(0.1643–0.3461)
Stage II (n=5)	19.15	(0.07866–0.3966)
Stage III (n=12)	22.54	(0.1970–0.2567)
Stage IV (n=1)	15.17	(0.1134–0.1998)