Expression of Wnt pathway molecules is associated with disease outcome in metastatic high-grade serous carcinoma

Running title: Wnt in high-grade serous carcinoma

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Abstract

The objective of this study was to analyze the expression and clinical role of Wnt pathway molecules in metastatic high-grade serous carcinoma (HGSC). mRNA expression by qPCR of 20 molecules related to Wnt signaling (*WNT1*, *WNT2*, *WNT3*, *WNT4*, *WNT5A*, *WNT6*, *WNT7*, *WNT11*, *FZD1*, *FZD4*, *FZD5*, *FZD6*, *FZD7*, *FZD8*, *FZD10*, *LRP5*, *LRP6*, *DKK*, *CCND*, *RUNX2*) was analyzed in 87 HGSC effusions. Thirty-nine surgical specimens (19 ovarian, 20 intra-abdominal) were analyzed for comparative purposes. Protein expression of YAP and LRP and their phosphorylated forms by Western blotting was analyzed in 52 tumors. Significant differences in mRNA expression as function of anatomic site were observed for *WNT3* ($p=0.005$), *WNT5A* ($p=0.008$), *WNT7* ($p<0.001$), *FRZ5* ($p=0.04$) and *FRZ6* ($p<0.001$). YAP and LRP and their phosphorylated forms were detected in HGSC specimens. *FZD10* was overexpressed in effusions from patients who had complete response to chemotherapy compared to those with less favorable response ($p=0.037$). *WNT4* ($p=0.005$), *WNT7* ($p=0.047$), *RUNX2* ($p=0.038$), *LRP5* ($p=0.022$), *LRP6* ($p=0.011$), *FZD6* ($p=0.036$), *FZD7* ($p=0.004$) and *FZD10* ($p=0.015$) levels were inversely related to primary chemoresistance. High *FZD5* levels in pre-chemotherapy effusions tapped at diagnosis and high *WNT2* levels in post-chemotherapy disease recurrence effusions were related to shorter overall survival ($p=0.018$ and $p=0.011$, respectively), whereas high *RUNX2* ($p=0.031$) and *FZD1* ($p=0.029$) in post-chemotherapy effusions were associated with longer overall survival. In multivariate analysis of post-chemotherapy cases, *WNT2* ($p=0.002$), *RUNX2* ($p=0.017$), *FZD1* ($p=0.036$) and *FZD4* ($p=0.013$) were independent prognosticators. In conclusion, expression of Wnt pathway molecules is anatomic site-dependent. In HGSC effusions, it is informative of chemoresponse and survival.

Keywords: Wnt; Quantitative PCR; Effusion; High-grade serous carcinoma; Survival

Introduction

Ovarian cancer, the 2nd most common gynecological cancer, was diagnosed in 239,000 women and was the cause of 152,000 deaths globally in 2018, making it the 7th most common cancer and the 8th most common cause of cancer death in women [1]. Recent years have seen longer overall survival (OS) in this disease, with approximately 45% of patients alive at 5 years, due to improved surgery and chemotherapy protocols, as well as targeted therapy. However, recurrence occurs in the majority of patients, particularly those diagnosed with advanced-stage (FIGO stage III-IV) disease, with a death-of-disease observed in the majority of patients in this group [1]. One of the difficulties in treating this cancer is metastasis to effusions within the serosal cavities, i.e. the pleural, pericardial and peritoneal spaces, in which tumor cells possess cancer stem cell characteristics and are chemoresistant [2]. Furthermore, others and we have repeatedly observed in previous research that cancer cells in effusions are unique, presumably due to difference in the microenvironment and the ability to survive and proliferate in anchorage-free conditions, thus avoiding anoikis. This suggests that anatomic localization is responsible to some of the observed tumor heterogeneity in HGSC. Better understanding of the molecular profile of cancer cells in effusions is therefore an important challenge.

The Wingless-type (Wnt) signaling pathway has been implicated in development and progression of numerous cancers. The pathway is initiated by binding of various Wnt ligands to members of the Frizzled (Fz) family of receptors. Upon receptor activation, a signal is forwarded to the phosphoprotein Disheveled. Wnt signaling can occur via two pathways - the canonical and the non-canonical pathway. In the canonical Wnt pathway, phosphorylation of Disheveled leads to translocation of β -catenin to the nucleus that induces a cellular response through gene transcription together with the TCF/LEF transcription factors [3]. In the absence of Wnt signaling, β -catenin is degraded [4]. The non-canonical signal transduction is β -catenin-independent and involves

Wnt/ Ca^{2+} and planar cell polarity pathways regulating cell adhesion and migration and activation of the Rho system [5,6].

The expression, biological role and clinical relevance of Wnt signaling in ovarian carcinoma have been previously investigated in experimental models and clinical specimens [7-15; reviewed in 16-17]. The association between Wnt signaling and stemness, epithelial-to-mesenchymal transition (EMT) and resistance to therapy was recently reviewed [18]. However, data regarding the clinical relevance of this pathway in high-grade serous carcinoma (HGSC) effusions are to date limited to a single study of 28 specimens [14]. The objective of the present study was to analyze the anatomic site-related expression of the Wnt pathway in HGSC and assess its clinical relevance in a large series of patients with HGSC effusions with full clinical data. Our data suggest that Wnt pathway molecule expression may be informative of chemotherapy response and survival in metastatic HGSC.

Material and Methods

Patients and specimens

HGSC specimens (n=126; 87 effusions, 39 solid specimens) and clinical data were obtained from patients treated at the Department of Gynecologic Oncology, Norwegian Radium Hospital, during the period of 1998 to 2008. As the fallopian tubes have not been adequately assessed in this cohort, tumors in the ovary are specified as such without reference to primary site. All tumors were reviewed by a surgical pathologist with experience in gynecologic pathology and cytopathology (BD) and diagnosed based on the combination of morphology and immunohistochemistry (IHC) according to the WHO 2014 guidelines [19].

Effusions consisted of 70 peritoneal and 17 pleural specimens. Effusions were centrifuged immediately after tapping, and cell pellets were frozen at -70°C in equal amounts of RPMI 1640 medium (GIBCO-Invitrogen, Carlsbad, CA) containing 50% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 20% dimethylsulfoxide (Merck KGaA, Darmstadt, Germany).

Tumor cell content in all effusions was >50%, based on assessment of H&E sections from cell blocks prepared using the Thrombin clot method. Clinicopathologic data for the effusions cohort are detailed in **Table 1**.

Surgical specimens (19 ovarian, 20 intra-abdominal, predominantly omental) were frozen at -70°C without any treatment. Frozen sections from all solid tumors were reviewed by one of the authors (BD), and only specimens with tumor cell population >50% and minimal or no necrosis were included in this study. The majority of tumors were not patient-matched.

Informed consent was obtained according to national and institutional guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway.

RNA extraction and qRT-PCR

Solid samples were thawed and homogenized using mixed 1 mm and 2 mm zirconium oxide beads in the Bullet Blender (Next Advance, Inc., Troy NY). Effusions were centrifuged after thawing and

cell pellets were transferred to a new tube before homogenization. Total RNA was extracted using the TRI Reagent® protocol (Sigma-Aldrich, St Louis MI). RNA quantity and quality were measured by NanoDrop 2000 (Thermo Scientific, Waltham MA).

One microgram of total RNA was transformed to cDNA using qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg MD) according to the manufacturer's protocol. qRT-PCR was performed on the cDNA using the KAPA SYBERFAST Universal qPCR kit (Kapa Biosystems, Wilmington MA) with the CFX Connect Real-Time system (Bio-Rad Laboratories, Hercules CA). The final concentrations of template and primers were determined individually for each assay, calibrated based on a standard curve. Primer sequences are listed in **Table 2** [20,21]. Analysis was performed with BioRad CFX manager software. The fold change of genes of interest was calculated relative to RPLP0 and expressed as $2^{-\Delta CT}$.

Western blotting (WB)

Fifty-two HGSC specimens, consisting of 34 effusions and 18 surgical specimens (8 ovarian, 10 intra-abdominal) were analyzed for protein expression of LRP6 and YAP, as well as their phosphorylated forms, by WB. Samples were lysed with 1% NP-40, 20mmol/L Tris-HCl (pH 7.5), 137mmol/L NaCl, 0.5mmol/L EDTA, 10% glycerol, 1% protease inhibitor cocktail (Sigma-Aldrich) and 0.1% SDS. After centrifugation, protein content was quantified using the Bradford assay, and 25µg of protein were loaded on 10% SDS polyacrylamide gels, separated by electrophoresis and transferred to PVDF membranes (Millipore, Billerica, MA). Following blocking of nonspecific binding with 5% nonfat milk in TBST, membranes were incubated with primary antibodies (**Table 3**), followed by incubation with goat-anti-rabbit secondary-antibody, attached to horseradish peroxidase (HRP), diluted 1:5000. The Hela cervical carcinoma cell line was used as control and expression >5% of control value was interpreted as positive. The films were scanned and analyzed with the ImageJ 1.46r program (NIH, Bethesda MD). Data was normalized to GAPDH.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections from 81/87 HGSC effusions analyzed using qRT-PCR were analyzed for β -catenin protein expression using the Dako EnVision Flex + System (K8012; Dako, Glostrup, Denmark). The β -catenin antibody was a mouse monoclonal antibody purchased from BD Transduction Laboratories (currently BD Biosciences; cat # 610153, clone 14; Franklin Lakes, NJ), applied at a 1:6000 dilution.

Following deparaffinization, sections were treated with EnVisionTM Flex + mouse linker (15 min) and EnVisionTM Flex/HRP enzyme (30 min) and stained for 10 min with 3'3-diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, dehydrated and mounted in Richard-Allan Scientific Cyto seal XYL (Thermo Fisher Scientific, Waltham, MA). Positive and negative controls consisted of normal skin.

IHC scoring: Staining extent at the cell membrane, cytoplasm and nucleus was scored by an experienced cytopathologist (BD), using a 0-4 scale as follows: 0=no staining, 1=1-5%, 2=6-25%, 3=26-75%, 4=76-100% of tumor cells.

Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 25). Probability of <0.05 was considered statistically significant. The association between mRNA expression and anatomic site (3-tier analysis) was studied using the Kruskal Wallis H test. The Mann-Whitney U test or the Kruskal Wallis H test was applied to analysis of the association between mRNA expression in HGSC effusions and clinicopathologic parameters (for 2-tier or 3-tier analyses, respectively). For this analysis, clinicopathologic parameters were grouped as follows: age: ≤ 60 vs. >60 years; effusion site: peritoneal vs. pleural; FIGO stage: III vs. IV; chemotherapy status: pre- vs. post-chemotherapy specimens; residual disease (RD) volume: 0 cm vs. ≤ 1 cm vs. >1 cm; response to chemotherapy: complete response vs. partial response/stable disease/progressive disease. The

association between Wnt pathway mRNA expression and β -catenin membrane expression (score =4 vs. all others), was analyzed using the Mann-Whitney U test.

Progression-free survival (PFS) and OS were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier method and log-rank test. Multivariate survival analysis was executed using the Cox Regression Model. Platinum resistance was defined as PFS $\leq$ 6 months according to guidelines published by the Gynecologic Oncology Group (GOG) and progressive disease or recurrence was evaluated by the Response Evaluation Criteria In Solid Tumors (RECIST) criteria.

Results

Wnt pathway molecules are differentially expressed in HGSC at different anatomic sites

Eighteen of the 20 Wnt pathway mRNAs were detected in HGSC specimen, whereas *WNT1* and *WNT11* mRNA were absent in both effusions and surgical specimens.

Comparative analysis of HGSC effusions, the ovarian tumors and solid peritoneal/omental metastases showed significantly lower *WNT3* (mean rank = 45.35 vs. 59.68 and 68.92, respectively; $p=0.005$), *WNT7* (mean rank = 42.58 vs. 70.76 and 67.38, respectively; $p<0.001$), *FZD5* (mean rank = 58.56 vs. 67.66 and 81.05, respectively; $p=0.04$) and *FZD6* (mean rank = 55.01 vs. 79.55 and 85.17, respectively; $p<0.001$) levels in effusions compared to the ovarian and peritoneal tumors, whereas the opposite was true for *WNT5A* (mean rank = 70.14 vs. 52.00 and 45.52, respectively; $p=0.008$). Expression of the remaining mRNAs was comparable at these anatomic sites ($p>0.05$; data not shown).

LRP protein was detected in 13/34 (38%) HGSC effusions and 9/18 (50%) surgical specimens. p-LRP protein was detected in 7/34 (21%) HGSC effusions and in 9/18 (50%) surgical specimens. YAP protein was detected in 29/34 (85%) HGSC effusions and in all 18 surgical specimens. p-YAP protein was detected in 27/34 (79%) HGSC effusions and in 17/18 (94%) surgical specimens (**Figure 1**). p-YAP protein expression was significantly lower in effusions compared to the ovarian and peritoneal tumors surgical specimens (mean rank = 22.12 vs. 39.25 and 31.20, respectively; $p=0.009$). Expression of the remaining proteins was comparable at these anatomic sites ($p>0.05$; data not shown).

β -catenin protein expression by IHC was observed in HGSC cells in all 81 effusions, with the following staining extent: score=1: 2; score =2: 3; score =3: 10; score=4: 66 specimens (**Figures 2-A to 2-D**). Combined cytoplasmic and nuclear expression was seen in only 2 specimens, both with only focal membrane expression (**Figures 2-E, 2-F**). Statistical analysis showed that any loss of β -catenin membrane expression (score <4) was significantly associated with higher *WNT4* ($p=0.005$),

WNT6 (p=0.048), *WNT7* (p=0.003), *LRP5* (p=0.014), *LRP6* (p=0.001), *FRZ1* (p=0.004), *FRZ4* (p<0.001), *FRZ5* (p=0.005), *FRZ6* (p=0.033), *FRZ7* (p<0.001) and *FRZ8* (p=0.017).

Wnt pathway molecule expression in HGSC effusions is associated with clinicopathologic parameters

FZD10 was overexpressed in effusions from patients who had complete response to chemotherapy compared to those with less favorable response (mean rank = 46.18 vs. 35.36, respectively; p=0.037). *WNT4* (mean rank = 36.71 vs. 51.81, respectively; p=0.005), *WNT7* (mean rank = 28.69 vs. 38.03 respectively; p=0.047), *RUNX2* (37.64 vs. 48.76; p=0.038), *LRP5* (38.02 vs. 50.40; p=0.022), *LRP6* (37.34 vs. 51.13; p=0.011), *FZD6* (38.50 vs. 49.89; p=0.036), *FZD7* (36.46 vs. 52.08; p=0.004) and *FZD10* (37.67 vs. 50.79; p=0.015) levels were inversely related to primary chemoresistance.

Wnt pathway mRNA expression was unrelated to effusion site, previous exposure to chemotherapy, patient age, FIGO stage and RD volume (p>0.05; data not shown).

Wnt pathway molecule expression in HGSC effusions is significantly associated with survival

The follow-up period for patients with HGSC effusions ranged from 1 to 179 months (mean = 38 months, median = 28 months). PFS ranged from 0 to 81 months (mean = 10 months, median = 6 months). At the last follow-up, 81 patients were dead of disease, 3 were alive with disease and 1 was with no evidence of disease. One patient died of complications and 1 was lost to follow-up. In univariate survival analysis of all cases, mRNA expression of Wnt pathway members was unrelated to OS (**Table 4-A**). However, levels of several of the studied molecules were significantly related to disease outcome when specimens were separated in to chemo-naïve effusions tapped at diagnosis and post-chemotherapy specimens, the majority from patients with disease recurrence.

In patients with pre-chemotherapy effusions, high *FZD5* levels were associated with shorter OS ($p=0.018$; **Figure 3-A**), with no other significant finding with respect to OS (**Table 4-A**) or PFS ($p>0.05$; data not shown).

In survival analysis for patients with post-chemotherapy effusions, high *WNT2* levels were related to shorter OS ($p=0.011$; **Figure 3-B**), whereas high *RUNX2* ($p=0.031$; **Figure 3-C**) and *FZD1* ($p=0.029$; **Figure 3-D**) in post-chemotherapy effusions were associated with longer OS (**Table 4-A**). Higher *RUNX2* levels were additionally associated with longer PFS ($p=0.014$; **Figure 3-E**). Among clinical parameters in the post-chemotherapy group, FIGO stage IV disease was significantly related to shorter OS ($p=0.025$), whereas the association for age was not significant ($p=0.160$). RD volume was not analyzed in this group due to the fact that only 1 patient was debulked to 0 cm. In multivariate OS analysis of this group, in which all parameters with $p<0.2$ except for *WNT7* were entered, *WNT2* ($p=0.002$), *RUNX2* ($p=0.017$), *FZD1* ($p=0.036$), *FZD4* ($p=0.013$) and FIGO stage ($p=0.005$) were independent prognosticators (**Table 4-B**).

Discussion

Ovarian carcinoma is a molecularly heterogeneous disease, particularly across different histotypes [22], and mutations in the β -catenin gene *CTNNB1*, associated with canonical Wnt signaling, are largely confined to endometrioid adenocarcinoma [reviewed in 17]. However, in view of the role of the non-canonical pathway in cancer biology and progression, we investigated the clinical role of Wnt signaling in HGSC, with focus on metastatic cells in effusion specimens.

Experimental data support a role for Wnt signaling in promoting ovarian carcinoma. Niclosamide, an anti-helminthic drug of the salicyclamide family, was found to inhibit the Wnt pathway. Arend et al. observed inhibition of proliferation in chemoresistant A2780cp20 and SKOV3Trip2 ovarian cancer cells and in tumor spheres isolated ovarian cancer ascites using Niclosamide, with inhibition of the mTOR/STAT3 signaling pathway [13]. In another study from the same institution, 60 serous carcinoma ascites specimens, including 59 tapped in the primary setting and 1 at disease recurrence, were treated with the Wnt/ β -catenin inhibitor WNT974. Combination of this drug with carboplatin proved more effective in inducing cytotoxicity and cell cycle arrest than use of single drug [14]. Yoshioka et al. observed overexpression of Wnt7A in serous carcinoma compared to normal ovaries, benign and borderline tumors and endometrioid carcinoma, and this molecule promoted tumor progression in an intra-peritoneal model of ovarian carcinoma, particularly when co-transfected with FZD5 [11]. The Wnt/ β -catenin pathway was further reported to mediate platinum resistance in an experimental model of HGSC [9] and was recently implicated in resistance to Poly (ADP-ribose) polymerase (PARP) inhibitors [7].

Data from the present study suggest that Wnt pathway members, with the exception of *WNT1* and *WNT11*, are frequently expressed in HGSC. Our data with respect to *WNT1* and *WNT11* differ from those of earlier reports, in which these molecules were reported to be expressed in ovarian carcinoma [10,15]. It is difficult to ascertain whether this owes to methodological differences, case

selection or other parameters. Of note, despite the apparent lack of central role for canonical Wnt signaling in HGSC, levels of multiple mRNAs of the Wnt pathway were inversely related to membrane expression of β -catenin, suggesting that activation of this pathway is more pronounced in HGSC effusions with reduced adhesion.

Whether Wnt pathway members are differentially expressed as function of anatomic site in HGSC is unknown to date. In our series, the majority of Wnt pathway molecules were expressed at comparable levels in solid specimens and effusions. With the exception of *WNT5A*, differences in expression as function of anatomic site were observed as overexpression in the solid specimens, and the same was true was p-YAP, member of the HIPPO pathway that regulates the WNT pathway [23].

Potential limitations to this analysis may be related to the relatively small number of solid specimens, the fact that the majority were not patient-matched, and the possibility that changes in host cell components (mesothelial cells vs. stromal myofibroblasts; different leukocyte classes). These differences are nevertheless supported by our repeated observations in previous studies that HGSC cells in effusions are molecularly distinct from their counterparts in solid lesions [2].

Data is limited regarding the potential role of the Wnt pathway in predicting chemoresponse or its prognostic relevance in clinical ovarian carcinoma. Analysis of the link between Wnt pathway expression levels and chemotherapy response in the present study showed significant association between expression of *WNT4*, *WNT7*, *RUNX2*, *LRP5*, *LRP6*, *FZD6*, *FZD7* and *FZD10*, and favorable response at diagnosis and/or absence of primary (intrinsic) resistance. *RUNX2* was further associated with longer PFS, a clinical parameter strongly linked to chemoresponse. These data are in agreement with the findings of Dai et al., who observed association between methylation of 7 genes, including *FZD4* and *LRP5*, and significantly reduced PFS in this cancer, in analysis of a large series with screening and validation sets [12], as well as data by Seagle and co-workers, in which higher *FZD3* levels in HGSC were related to longer PFS [8]. It needs to be stated that the number of analyses performed with respect to association with clinicopathologic parameters in

general, and chemoresponse in particular, raises the possibility of false discovery and requires a Bonferroni correction, implying that values of $p=0.005$ or less may be the more robust observations. Data with respect to OS were less uniform. *FZD5* levels in pre-chemotherapy effusions and *WNT2* levels in post-chemotherapy effusions were significantly associated with shorter OS, whereas the opposite was true for *RUNX2*, *FZD1* and *FZD4* in post-chemotherapy effusions. In the above study by Seagle, higher *FZD3* levels were significantly associated with longer OS [8]. In the only study of effusion specimens with clinical end-points published to date, Reinartz et al. studied the genomic profile of ascites specimens, the majority (28/29) from patients with HGSC, using RNA sequencing. Seven genes which constitute part of the Wnt pathway were shown to be overexpressed in tumor cells compared to tumor-associated macrophages (TAM), supporting activation of the non-canonical pathway in these specimens, and high *WNT11* expression was associated with shorter relapse-free survival [15]. In the study by Bodnar and co-workers, WNT1 protein expression was unrelated to OS or PFS [10]. However, the series analyzed consisted of tumors of various histotype, a fact which limits the ability to compare data to our findings.

Advanced-stage HGSC is almost uniformly lethal, as exemplified in our effusion cohort, in which only 1 of 87 patients was alive with no evidence of disease at the last follow-up. This underscores the need to identify novel therapeutic targets in this cancer. In the present study, Wnt pathway molecules were frequently expressed in HGSC, with anatomic site-related variation that provides further evidence of tumor heterogeneity in this cancer. This likely derives also from the microenvironment, as in other gynecological cancers, e.g. endometrial cancer [24]. While higher Wnt pathway molecule levels in HGSC effusions are generally associated with better chemotherapy response, some molecules in this pathway may be related to clinically aggressive disease. The data in the present study should nevertheless be considered as hypothesis-generating and need to be validated in an independent series. The possibility that the entire non-canonical Wnt pathway is

perturbed in metastatic HGSC, based on the large number of clinically-relevant observations in the present study, supports the rationale of further investigating this pathway in this cancer.

Compliance with Ethical Standards: The study was approved by the Regional Committee for Medical Research Ethics in Norway.

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Conflict of interest: None declared

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Michal Chehovev, Reuven Reich and Ben Davidson. The first draft of the manuscript was written by Michal Chehovev and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Table 1: Clinicopathologic parameters of the HGSC effusion cohort (87 patients)

Parameter	Distribution
Age (mean)	38-83 years (62)
FIGO stage	
II	2
III	47
IV	38
Residual disease ^a	
0 cm	6
≤1 cm	25
>1 cm	23
CA 125 at diagnosis (range; median)	11-25390 (990) ^b
Chemoresponse after primary treatment	
CR	38
PR	26
SD	7
PD	9
NA ^c	7

Abbreviations: NA = not available; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease

^a For 54 patients who received surgery as upfront treatment.

^b Available for 64 patients

^c Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor).

Table 2: qPCR primers

<i>WNT1</i>	forward	5'-CCGTACGACCGTATTCTCCG-3'
	reverse	5'-CGCTGTACGTGCAGAAGTTG-3'
<i>WNT2</i> [24]	forward	5'-CCATCTCCTCAGCTGGAGTTG-3'
	reverse	5'-TGGATCACAGGAACAGGATTTTAC-3'
<i>WNT3</i> ^a	forward	5'- TCCAACTATTGGGGGCGTC-3'
	reverse	5'- CGGGTGGCTTTGTCTGAGGA-3'
<i>WNT4</i>	forward	5'- GAGCAACTGGCTGTACCTGG-3'
	reverse	5'- GGAAGTGGTACTGGCACTCC-3'
<i>WNT5a</i> [24]	forward	5'- AACTCGCCCACCACACAAG-3'
	reverse	5'- TCATTGCGCACGCAGTAGTC-3'
<i>WNT6</i> [24]	forward	5'- CAGCCCCTTGGTTATGGACC-3'
	reverse	5'- CGTCTCCCGAATGTCCTGTT-3'
<i>WNT7</i> ^a	forward	5'- GTCTCGCACACTTGCACCG-3'
	reverse	5'-GAGGAGAAGCCACCGATCC-3'
<i>WNT11</i>	forward	5'- CGGCGTGCAGGACCAG-3'
	reverse	5'- CAGCGCCAGCCACTTGAT-3'
<i>FZD1</i>	forward	5'- AGAGGTCTCCCTGGCCG-3'
	reverse	5'- GACTTCTTAGGCGCCTCCTC-3'
<i>FZD4</i>	forward	5'- CTCGGGCTACAACGTGACCAAGAT-3'
	reverse	5'- AATAGATAAAACACTCAGGGTA-3'
<i>FZD5</i>	forward	5'- ACCAGAACCTGAACTCGCTG-3'
	reverse	5'- GTACTTGAGCACGAGCACCC-3'
<i>FZD6</i>	forward	5'-GTTAGACGGGGACGGGAAG-3'
	reverse	5'- GCGAGAGGAAGAAAATGCTCC-3'

<i>FZD7</i>	forward	5'- CTCCGCTTTCGTCCCTGG-3'
	reverse	5'- GAGATGCCCTTCTCTCCGTG-3'
<i>FZD8</i>	forward	5'- TGGAGTGGGGTTACCTGTTG-3'
	reverse	5'-AGCGGCTTCTTGTAGTCCTC-3'
<i>FZD10</i>	forward	5'- TATCGGGCTCTTCTCTGTGC-3'
	reverse	5'- GACTGGGCAGGGATCTCATA-3'
<i>LRP5</i>	forward	5'- TCATTGTGGACTCGGACATTTAC-3'
	reverse	5'- GTAGAAAGGCTCGCTTGGGGACA-3'
<i>LRP6</i>	forward	5'- CCCATGCACCTGGTTCTACT-3'
	reverse	5'- CCAAGCCACAGGGATACAGT-3'
<i>DKK [25]</i>	forward	5'- AGCGTTGTTACTGTGGAGAAG-3'
	reverse	5'- GTGTGAAGCCTAGAAGAATTGCTG-3'
<i>CCND</i>	forward	5'- TGTCCTACTACCGCCTCACA-3'
	reverse	5'- AGGAGGGACTGTCATGTGGA-3'
<i>RUNX</i>	forward	5'- GAACCCAGAAGGCACAGACA-3'
	reverse	5'- GGCTCAGGTAGGAGGGGTAA-3'
<i>RPLP0</i>	forward	5'-CCAAC TACTTCCTTAAGATCATCCA ACTA-3'
	reverse	5'- ACATGCGGATCTGCTGCA-3'

^a *WNT3* and *WNT7* were analyzed in only 65 of the 87 HGSC effusions.

Table 3: WB antibodies

Antibody	Isotype	Dilution	mW (kDa)	Manufacturer	Cat. #
α -phospho-YAP (ser397) (D1E7Y)	Rabbit monoclonal	1:1000	75	Cell Signaling Biotechnology (Danvers MA)	13619
α -YAP/TAZ (D24E4)	Rabbit monoclonal	1:1000	50 (TAZ) 70 (YAP)	Cell Signaling Biotechnology	8414
α -phospho-LRP (Ser1490)	Rabbit	1:1000	180, 210	Cell Signaling Biotechnology	2568
LRP6 [EPR2423(2)]	Rabbit monoclonal	1:1000	180	Abcam (Cambridge, UK)	ab134146
α -GAPDH [14C10]	Rabbit monoclonal	1:1000	37	Cell Signaling Biotechnology	2118
β -catenin (C- terminal antigen)	Rabbit	1:1000	92	Cell Signaling Biotechnology	9587

Table 4-A: Univariate survival analysis of overall survival for the HGSC effusion cohort (87 patients)^a

Gene	All cases	Pre-chemo	Post-chemo
<i>WNT2</i>	p=0.541	p=0.933	p=0.011^b
<i>WNT3</i>	p=0.650	p=0.520	p=0.969
<i>WNT4</i>	p=0.078	p=0.290	p=0.227
<i>WNT5a</i>	p=0.442	p=0.811	p=0.268
<i>WNT6</i>	p=0.685	p=0.889	p=0.219
<i>WNT7</i>	p=0.246	p=0.514	p=0.065
<i>FZD1</i>	p=0.768	p=0.235	p=0.029^c
<i>FZD4</i>	p=0.870	p=0.295	p=0.138
<i>FZD5</i>	p=0.065	p=0.018^b	p=0.865
<i>FZD6</i>	p=0.944	p=0.466	p=0.840
<i>FZD7</i>	p=0.356	p=0.927	p=0.052
<i>FZD8</i>	p=0.521	p=0.962	p=0.274
<i>FZD10</i>	p=0.706	p=0.603	p=0.550
<i>LRP5</i>	p=0.916	p=0.421	p=0.118
<i>LRP6</i>	p=0.893	p=0.562	p=0.471
<i>DKK</i>	p=0.092	p=0.160	p=0.602
<i>CCND</i>	p=0.691	p=0.324	p=0.491
<i>RUNX</i>	p=0.289	p=0.744	p=0.031^c

^a *WNT3* and *WNT7* were analyzed in only 65 of the 87 HGSC effusions.

^b Shorter survival; ^c longer survival

Table 4-B: Multivariate survival analysis of overall survival for patients with post-chemotherapy effusions (n=42) ^a

Gene	p-value
<i>WNT2</i>	p=0.002 ^b
<i>LRP5</i>	p=0.099
<i>FZD1</i>	p=0.036 ^c
<i>FZD4</i>	p=0.013 ^c
<i>RUNX</i>	p=0.017 ^c
FIGO stage	p=0.005 ^d
Age	p=0.609

^a *WNT7* excluded since it was not analyzed in all cases.

^b Shorter survival; ^c longer survival; ^d FIGO stage III vs. IV

Figure legends

Figure 1: Western blot

LRP, p-LRP, Yap and p-Yap western blot in 8 HGSC effusions.

Figure 2: IHC

(A-C) Diffuse (>75%) membrane staining for β -catenin in 3 HGSC effusions; (D) Focal membrane expression; (E-F) Two effusions with combined cytoplasmic and nuclear β -catenin expression.

Figure 3: Wnt pathway genes are informative of survival in metastatic HGSC

A: Kaplan-Meier survival curve showing the association between *FZD5* mRNA levels in pre-chemotherapy effusions (n=44) and overall survival (OS). Patients with effusions with high (above median) *FZD5* mRNA level (n=22; red line) had mean OS of 33 months compared to 65 months for patients with effusions having low *FZD5* mRNA level (n=22, blue line; p=0.018).

B: Kaplan-Meier survival curve showing the association between *WNT2* mRNA levels in post-chemotherapy effusions (n=42) and OS. Patients with effusions with high (above median) *WNT2* mRNA level (n=16; red line) had mean OS of 24 months compared to 37 months for patients with effusions having low *WNT2* mRNA level (n=26, blue line; p=0.011). Median value is generated from analysis of the entire cohort (n=87).

C: Kaplan-Meier survival curve showing the association between *RUNX* mRNA levels in post-chemotherapy effusions (n=42) and OS. Patients with effusions with high (above median) *RUNX* mRNA level (n=26; blue line) had mean OS of 38 months compared to 23 months for patients with effusions having low *RUNX* mRNA level (n=16, red line; p=0.031). Median value is generated from analysis of the entire cohort (n=87).

D: Kaplan-Meier survival curve showing the association between *FZD1* mRNA levels in post-chemotherapy effusions (n=42) and OS. Patients with effusions with high (above median) *FZD1*

mRNA level (n=20; blue line) had mean OS of 40 months compared to 25 months for patients with effusions having low *FZDI* mRNA level (n=22, red line; p=0.029).

E: Kaplan-Meier survival curve showing the association between *RUNX* mRNA levels in post-chemotherapy effusions (n=42) and progression-free survival (PFS). Patients with effusions with high (above median) *RUNX* mRNA level (n=26; blue line) had mean PFS of 9 months compared to 4 months for patients with effusions having low *RUNX* mRNA level (n=16, red line; p=0.014).

Median value is generated from analysis of the entire cohort (n=87).