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ALDH1A1-related stemness in high-grade serous ovarian cancer is a negative prognostic indicator but potentially targetable by EGFR/mTOR-PI3K/aurora kinase inhibitors

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Running title. Features and drug sensitivity of ovarian cancer stem-like cells

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Abstract

Poor chemotherapy response remains a major treatment challenge for high-grade serous ovarian cancer. Cancer stem cells are the major contributors to relapse and treatment failure as they can survive conventional therapy. Our objectives were to characterise stemness features in primary patient derived cell lines, correlate stemness markers with clinical outcome, and test the response of our cells to both conventional and exploratory drugs. Tissue and ascites samples, treatment-naïve and/or after neoadjuvant chemotherapy, were prospectively collected. Primary cancer cells, cultured under conditions favouring either adherent or spheroid growth, were tested for stemness markers; the same markers were analysed in tissue and correlated with chemotherapy response and survival. Drug sensitivity and resistance testing was performed with 306 oncology compounds.

Spheroid growth-condition HGSC cells showed increased stemness marker expression (including ALDH1A1) as compared to adherent growth-condition cells, and increased resistance to platinum and taxane. A set of eight stemness markers separated treatment-naïve tumours into two clusters and identified a distinct subgroup of HGSC with enriched stemness features. Expression of ALDH1A1, but not most other stemness markers, was increased after neoadjuvant chemotherapy and its expression in treatment-naïve tumours correlated with chemoresistance and reduced survival. In DSRT, five compounds, including two PI3K-mTOR inhibitors, demonstrated significant activity in both cell culture conditions. Thirteen compounds, including EGFR, PI3K-mTOR and aurora kinase inhibitors, were more toxic to spheroid cells than adherent cells.

Our results identify stemness markers in HGSC that are associated with a decreased response to conventional chemotherapy and reduced survival if expressed by treatment-naïve tumours. EGFR, mTOR-PI3K and aurora kinase inhibitors are candidates for targeting this cell population.

Keywords. Ovarian cancer, stem-like cell, patient derived cell line, survival, drug sensitivity

Introduction

High grade serous ovarian carcinoma (HGSC) is an aggressive gynaecological cancer with a 30% survival rate at five years. While most tumours initially respond to treatment, relapse followed by chemoresistance is common [1,2]. The standard therapy for advanced HGSC is surgery combined with platinum-taxane chemotherapy.

A subpopulation of treatment-refractory cancer cells with stem-cell properties is a likely cause of relapse and treatment failure in ovarian cancer [3]. Cancer stem cells (CSCs) represent an important target for novel therapeutic strategies aimed at eradicating ovarian cancer [4]. CSCs are characterised by their capacity to self-renew, resistance to apoptosis, and their ability to generate daughter cells which differentiate into a variety of cancer cell types [5]. Similarly, CSCs can be defined by these functional traits [6]. CSCs were originally characterized in acute myeloid leukaemia [7] and later in many different cancers including breast cancer [8], brain cancer [9], colon cancer [5,10] and ovarian cancer [11]. Several stemness marker combinations have been suggested for HGSC CSCs, but a definite marker set remains to be established [12]. One of the best described stemness markers is aldehyde dehydrogenase isoform I (ALDH1A1), an enzyme that has been used to define CSCs in many cancer types [13], including ovarian cancer [14,15]. In ovarian cancer, ALDH1A1 is correlated with stemness and poor prognosis [15–17]. The surface marker PROM1 (Prominin-1, CD133) has been associated with ovarian cancer, though more recently it has been considered controversial [18]. Transcription factors such as POU5F1, SOX2, LIN28A, NANOG and MYC have been associated with the reprogramming of human somatic cells to pluripotent stem cells [19], as well as cancer cells with stemness properties. In ovarian cancer, these transcription factors have been associated with poor prognosis [20–22], but only SOX2 has been associated with a cancer stem cell phenotype [23].

BMI1 overexpression has shown a significant association with low overall survival in ovarian cancer [24]. In other tumour types, BMI1 has been linked to cancer cell survival and senescence, self-renewing cell divisions of somatic stem cells, and has been found to be overexpressed in, for example, Ewing sarcoma family tumours [25,26]. While the markers described above have been associated with many cancers, including ovarian cancer, they have typically been studied as individual markers and not as a marker panel.

Little is known about the drug response of primary HGSC cells with stemness features. Drug sensitivity testing has typically been carried out using cells of unknown origin or those lacking genomic features of HGSC [27–29] and using adherent cell culture conditions, which do not support stemness features [29]. Drug sensitivity testing with conditions favouring stemness features is technically challenging and only few studies have been reported so far [27,30,31].

The aims of this work were to (1) identify stemness phenotype markers in primary HGSC cells, (2) determine if the identified stemness markers are enriched after platinum-taxane therapy, (3) determine whether the same markers are prognostic in treatment-naïve HGSC, and (4) carry out large scale oncology drug testing to study whether HGSC cells cultured in conventional adherent methods and stemness promoting conditions exhibit different drug response profiles.

Materials and methods

Patient-derived materials

Tissue and ascites specimens were collected from consented patients at the Department of Obstetrics and Gynaecology, Turku University Central Hospital, as part of a prospective ovarian cancer study

(MUPET/HERCULES) (ClinicalTrials.gov Id: NCT01276574). The study protocol and use of all material has been approved by (1) The Ethics Committee of the Hospital District of Southwest Finland (ETMK): ETMK 53/180/2009 § 238 and ETMK 69/180/2010, and (2) The Finnish National Supervisory Authority for Welfare and Health in Finland (Valvira): DNRO 6550/05.01.00.06/2010 and STH507A.

Tumour samples, ascites samples, and longitudinal clinical information were collected from 40 patients with stage III or IV HGSC (Figure S1 and Table S1). Treatment-naïve samples were collected during primary debulking surgery (PDS) or diagnostic laparoscopy. Patients considered primarily inoperable received three cycles of neoadjuvant chemotherapy (NACT) and samples were obtained during interval debulking surgery (IDS). Altogether, 36 treatment-naïve and 18 NACT samples, including ten paired samples, were available. Primary cell cultures were established from 25 patients, from which 19 treatment-naïve and 6 NACT cell lines were created. Of these, 3 were paired sets (treatment-naïve and NACT) established from the same patients. For RNA sequencing, tumour tissue was collected at the treatment-naïve stage and at interval surgery following 3-4 cycles of NACT; paired material was available from 21 patients.

Longitudinal clinical information was collected as described previously [32], and included stage, intraoperative description of tumour dissemination, treatment, and survival data.

To study the co-expression of stemness markers and their association with survival in FIGO stage III-IV HGSC patients, we used two existing datasets: (1) the microarray expression data of 144 treatment-naïve primary tumour samples from patients with clinical data available [33] and (2) the RNA sequencing data of 66 primary HGSC patients [34]. To make the two datasets comparable, we used eight stemness markers that were included in both platforms.

Cell lines and primary cell culture

Primary cell cultures were established from ascites and tumour tissue. Ascites samples were centrifuged at 1500 rpm for 15 min (Centrifuge 5810R, Eppendorf, Hamburg, Germany), followed by gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) to enrich the cancer cell component (Cell lines M019i, M022i and M068i). Cells from primary tumours were isolated by plating approximately 1 mm^3 pieces on 6-well plates. Detached cells were collected weekly for 4 weeks and cultured as adherent or spheroids (see below). Attached cells were also collected when the wells were grown to confluency. The contaminating stromal and immune cells were grown out by passaging the cells approximately 5 times. During passaging the contaminating cells were either discarded or died. A cell smear was stained with toluidine blue to confirm uniformity of the cell population morphologically. Cell block samples of cultured cells were stained with PAX8 and WT1 antibodies to confirm the ovarian cancer origin of the cells. The uniform cell cultures were designated patient derived cell lines.

Conventional HGSC cell lines included CAOV3 (American Type Culture Collection (ATCC) http://www.lgcstandards-atcc.org), CAOV4 (ATCC), OVCAR4 (National Cancer Institute, NCI, USA), TYK-nu (Health Science Research Resources Bank, (JCRB) Japan), TYK-nuCP-r (JCRB), NIHOVCAR3 (ATCC), and OVCAR8 (NCI).

The primary and conventional cell lines were investigated in two culture conditions; adherent (2D) and spheroidal (non-adherent, 3D). Adherent cells were cultured in DMEM (Euroclone, Milano, Italy) or RPMI medium containing 5–10 % FBS (Lonza, Basel, Switzerland), 100 µg/ml penicillin-streptomycin (Gibco Life Technologies, Grand Island, NY, USA) and 2 mM Ultraglutamine (Lonza).

Spheroidal cell culture medium DMEM-F12 (Lonza) was supplemented with 20 ng/ml EGF (Gibco Life Technologies), 10 ng/ml bFGF (Gibco Life Technologies) and 1x B27 (Gibco Life Technologies). CCD-18Co myofibroblasts (ATCC) were used as feeder cells in the analysis of flow cytometry sorted ALDH-positive and -negative cells. Cell morphology was analysed using a Primo Vert light microscope and Zen lite software (Carl Zeiss Microscopy Ltd, Goettingen, Germany). Immunofluorescence was detected with a LSM780 laser scanning confocal microscope (Carl Zeiss Microscopy Ltd).

Statistical analysis

Differences between the study groups in RT-qPCR and ADLH flow cytometric data were analysed using a two-sided *t*-test. The Kaplan–Meier method with the log-rank test were used for survival analyses. Immunohistochemical as well as RNA sequencing data were analysed with Spearman's correlation analyses. DSRT data was analysed using a web-based pipeline BREEZE (breeze.fimm.fi).

Detailed information about the ATP cell proliferation assay, RT-qPCR, aldehyde dehydrogenase enzyme activity and ALDH1A1 analysis, RNA sequencing and expression analysis for HGSC tumours, and drug sensitivity and resistance testing (DSRT) can be found in supplementary material, Supplementary materials and methods.

Results

Cultured primary spheroid HGSC cells express stemness markers and are more resistant to

conventional chemotherapy than adherent cells

We identified ten stem cell markers that have been extensively documented in a variety of cancers and analysed their expression in primary HGSC cells grown under spheroid and adherent conditions (Figure 1A). Of the ten markers, five *(ALDH1A1*, p=0.042; *CIP2A*, p=0.005; *POU5F1*, p=0.021; *SOX2*, p=0.019; *BMI1*, p=0.003) were differentially expressed in the spheroid cells as compared with the adherent cells (Figure 1B).

We next compared the response of spheroidal and adherent patient derived cell lines to cisplatin and paclitaxel, derivatives of platinum and taxane that form the standard of care in HGSC. A paired set of cell lines from the same patient, M022 (sample from treatment-naïve patient) and M022i (i=sample from an interval surgery), and three conventional cell lines (CAOV4, OVCAR4 and OVCAR8) were tested. In all cell lines, cells grown under spheroid conditions were clearly more resistant to both cisplatin (p=0.0141) and paclitaxel (p=0.0188) than adherently grown cells. The results are shown in Figures 1C,D.

Taken together, these results indicated that primary patient derived cells, and especially cells cultured in spheroid conditions, serve as a relevant model to study stemness features in HGSC. Further, of the ten stemness markers previously reported in a variety of cancers, five (*ALDH1A1, CIP2A, POU5F1, SOX2* and *BMI1*) were relevant to the HGSC model.

Stemness markers in treatment-naïve HGSC define a tumour subset with potential prognostic significance

The ability of the ten stemness markers to classify tumour subsets and predict survival was first studied in the microarray expression dataset, which consists of 144 treatment-naïve samples from

stage III-IV HGSC patients. The mRNA expression of eight markers (*ALDH1A1*, *CIP2A*, *MYC*, *LIN28A*, *NANOG*, *SOX2*, *PROM1* and *BMI1*) stratified patient samples into two clusters. Of these, Cluster 2] (enriched stemness cluster, 32% of all cases) showed significantly higher stemness marker expression than Cluster 1 (baseline cluster, 68% of all cases) (Figure 2A). Analysis of each of these markers showed significant enrichment in Cluster 2 as compared to Cluster 1 (Figure 2B). A similar division into two clusters was identified in an independent RNA sequencing dataset of 66 treatment-naïve samples, with an identical ratio between the two clusters (supplementary material, Figure S2). In a Kaplan–Meier survival analysis of the larger dataset, patients belonging to Cluster 2 (enriched stemness) had shorter overall survival than Cluster 1 (baseline) patients (median OS 20 months versus 33 months, log-rank test p=0.047) (Figure 2C). In the smaller validation set, significant OS differences were not detected.

ALDH-positive cells give rise to stem cell-like colonies

As ALDH1A1 is one of the strongest markers of stemness we wanted to further study the association between ALDH and stemness features. We detected the enzyme activity of cultured primary HGSC cells with Aldehyde Dehydrogenase Based Cell Detection Kit, which labels live cells possessing ALDH activity. With live cell ALDH staining, cells were separated by flow cytometry to study ALDH high ($ALDH^{bright}$) and low ($ALDH^{dim}$) populations. We consistently observed that only the cells with high ALDHactivity formed stem-like colonies (Figure 3A). Further analysis of ALDH1A1 expression by western blotting demonstrated that colony-derived cells in spheroid conditions expressed more ALDH1A1 than adherently cultured cells (Figure 3B). Quantitation of the results showed

significantly more ALDH1A1 in spheroid cells in three out of four cell lines ($p<0.001$) (Figure 3C).

ALDH1A1 expression is increased after platinum-taxane chemotherapy and is associated with poor outcome indicators

We next explored whether stemness features, and especially expression of ALDH1A1, were induced by chemotherapy. We compared the ALDH activity of nine treatment-naïve HGSC cell cultures with four NACT HGSC cell cultures. The amount of ALDH positivity, as defined by live cell ALDH labelling, was significantly higher in cells obtained after NACT than in cells collected from treatment-naïve patients ($p<0.001$) (Figure 4A). This difference was also identified by immunofluorescence staining of the cells for ALDH1A1 (Figure 4B). To study this finding further, we compared *ALDH1A1* RT-qPCR-based mRNA expression in tumour tissue obtained from treatment-naïve and chemotherapy-treated interval samples. The results indicated that *ALDH1A1* expression was increased after platinum-taxane combination therapy ($p<0.05$) (Figure 4C). The increased *ALDH1A1* expression could also be visualized by IHC (Figure 4D). When RNA-seq results from a separate set of 21 naïve - interval tissue pairs were compared, we were able to conclude that *ALDH1A1* levels in interval tissue were significantly higher than *ALDH1A1* levels in naïve tissue ($p<0.0001$) (Figure 4E). In pairwise analysis, 71.4 % (15/21) of the pairs showed increased ALDH1A1 expression in the interval sample; in 12.8% (3/21) expression remained stable, and in the remaining 12.8% (3/21) expression was decreased in the interval sample (supplementary material, Table S2). Interestingly, in this paired analysis, the ALDH1A1 increase in post-treatment samples correlated with an increase in MYC and BMI1 expression, but showed an inverse correlation with expression of POU5F1, NANOG, SOX2 and LIN28A. Of the nineteen ALDH protein isoforms, the mRNA for six (*ALDH1A1*,

ALDH1A3, ALDH1L1, ALDH2, ALDH6A1, ALDH9A1) showed significantly increased levels in interval samples as compared to treatment-naïve samples (supplementary material, Table S3). Two mRNA transcripts (*ALDH18A1, ALDH3B2*) were reduced in the interval samples. Of all mRNA transcripts, the expression of *ALDH1A1* was highest in the interval samples. Furthermore, the absolute change between interval and naïve samples was most pronounced for the *ALDH1A1* transcript.

We correlated the RNA-seq-based *ALDH1A1* expression in naïve samples with disease dissemination at IDS, primary therapy outcome, and residual tumour volume. The Spearman's correlation analyses demonstrated that higher levels of *ALDH1A1* in naïve samples correlated with more disseminated disease at IDS, suggesting that tumours with higher *ALDH1A1* expression do not respond to NACT as well as tumours with lower levels of expression (p=0.009). Furthermore, the results showed that high expression of *ALDH1A1* is indicative of the primary therapy outcome (p=0.058) or residual tumour volume (p=0.082), although these changes did not reach statistical significance.

We performed gene enrichment ontology analysis of the paired treatment-naïve and interval sample RNA-seq data to study potential differences in stemness-related pathways. The results demonstrated a significant enrichment in cell number maintenance and stemness features after neoadjuvant chemotherapy. The most pronounced changes were seen in the maintenance of cell number (GO:0098727, FDR-corrected p-value 1.7×10^{-66}), stem cell population maintenance (GO:0019827, FDR-corrected p-value 2.1×10^{-66}) and somatic stem cell population maintenance (GO:0035019, FDR-corrected p-value 1.2×10^{-44}), all of which showed >100-fold enrichment in the interval samples.

The results demonstrate that HGSC cells surviving standard chemotherapy are enriched for stemness features. Additionally, the results indicate that ALDH1A1 may be one of the most prominent stemness markers that can be visualized in live cells and in tumour tissue with western blotting, immunofluorescence, RNA expression and IHC.

High-throughput drug sensitivity testing reveals heterogeneity in the drug response of cells cultured in adherent and spheroid conditions

Cancer cells with stemness features are thought to be resistant to chemotherapy, but no comparative high throughput drug tests have been performed with primary HGSC cultures. Therefore, we carried out drug testing with a panel of 306 oncology compounds using three ascites- derived primary HGSC cell lines after NACT (M019i, M068i and M022i), and both adherent and spheroid conditions (see Figures 3B,C). The results were calibrated against fresh normal bone marrow cells to compare the general toxicity of the drug with a sensitive healthy tissue (Drug Sensitivity Score, DSS).

The comparison of selective DSS (sDSS) values between cell lines revealed significant differences between individual cell lines and between cell culture conditions. Generally, the M022i cells were more resistant to the compounds tested than the other two primary cell lines M019i and M068i, which showed rather similar response profiles. This is in line with the exceptionally short patient survival of M022i (Progression free survival (PFS) 3.1, overall survival (OS) 4.0) when compared to the survival of M019i (PFS 2.4, OS 34.3) and M068i (PFS 9.3, OS 11.2) patients. Of the 306 compounds, 31.8% and 31.5% were effective (sDSS >5) against M019i grown in adherent and spheroid conditions, respectively (Figure 5A). For M068i cells the corresponding values were 27.3% and 32.8% (Figure 5B). The proportion of highly effective compounds (sDSS > 15) for M019i was 6.7%

and 9.9%, and for M068i 5.7% and 8.6%, again tested separately for adherent and spheroid conditions. M022i cells responded to 22.2% of the compounds when grown in adherent conditions and 14.0% when grown as spheroids (Figure 5C); the proportion of highly effective drugs was 3.8% and 1.9%. Interestingly, many classical chemotherapy agents, including cytarabine, decitabine, vinblastine, vincristine, and vinorelbine were not cytotoxic against M022i, although they were effective or highly effective on M019i and M068i cell lines, independent of growth conditions. The efficacy of the drug compounds in each cell line is presented in supplementary material, Table S4.

We identified five compounds that were highly effective against all three cell lines, independent of growth conditions. Of these, omipalisib and PF-04691502 are inhibitors of the PI3K-mTOR-pathway, refametinib is a MEK inhibitor, BIIB021 is an HSP90 inhibitor, and BMS-754807 is an IGF-1R/InsR inhibitor.

Thirteen compounds appeared to be more effective against cells grown in spheroid conditions than in adherent conditions. These included three EGFR inhibitors (afatinib, erlotinib and gefitinib), three aurora kinase inhibitors (AZD1152-HQPA, alisertib, and AT9283), two PI3K-mTOR pathway inhibitors (apitolisib and AZD2014/vistusertib), two topoisomerase inhibitors (camptothecin and topotecan), a MEK inhibitor (trametinib), a polo-like kinase 1 inhibitor (volasertib) and a nucleoside analogue (gemcitabine). Additionally, the growth inhibitory effect of three EGFR inhibitors (canertinib, dacominitib and neratinib) was more prominent in spheroid conditions in the M019i and M068i cell lines.

Considered together, these results suggest that ovarian cancer cells with stemness features can be targeted with compounds already in trials or clinical use. Of special interest are compounds inhibiting EGFR, PI3K-mTOR and aurora kinase activity.

Discussion

Inter- and intra-tumour heterogeneity of HGSC creates a great challenge for effective treatment and for selecting an optimal study design for translational research. One facet of the heterogeneity is the presence of a rare population of cells with stemness features, which may play a pivotal role in tumour initiation, growth, chemoresistance, and recurrence [35,36]. In this study, we have correlated clinical samples with *in vitro* analyses to identify stemness-feature promoting conditions, and to define a stemness marker panel for HGSC. Using these markers, we showed that expression of stemness markers in treatment-naïve tumours indicates reduced survival and that stemness markers are upregulated in cancer cells surviving chemotherapy. We further show that cells grown under conditions favouring stemness demonstrate sensitivity to a limited number of oncology compounds.

In spite of numerous studies, a definitive HGSC stemness marker panel is still to be defined. Due to the heterogeneity of proposed cell surface markers, we focused primarily on intracellular markers with a key role in controlling the pathways leading to a spheroidal, stem cell-like, phenotype. Each of the included markers (*ALDH1A1, PROM1, MYC, LIN28A, NANOG, POU5F1B, POU5F1, SOX2, CIP2A,* and *BMI1*) has been previously associated with stemness, but they have not been studied to this extent as a marker set. Of the analysed markers, five (*ALDH1A1, CIP2A, SOX2, POU5F1,* and *BMI1*) were significantly increased in primary HGSC cells cultured under conditions favouring stemness. However, when expression was analysed in tumour tissue, *MYC, LIN28A* and *NANOG* also clustered in these same tumours, with the other upregulated markers. These results show that several transcription factors and pathway regulators are concomitantly altered in HGSC cells with stemness features.

There is evidence that stemness features in treatment-naïve tumours indicate poor response to chemotherapy and aggressive behaviour. The most consistent results have been achieved using immunohistochemistry for ALDH1A1, where abundant ALDH1A1 immunoreactivity has been associated with poor PFS and OS (reviewed in [37]). Correlation has also been achieved with other putative stemness markers, including CD44 and PROM1, although these results have been more ambiguous. Only a few studies have tested the correlation between HGSC outcome and mRNA expression of stem cell marker panels. In the current study we demonstrated that in treatment-naïve tumours, *ALDH1A1* expression coincided with several other stemness markers, and that high expression of the marker panel correlated inversely with overall survival. The expression of ALDH1A1, the most prominent ALDH isoform in HGSC, was increased not only in HGSC tumours after chemotherapy, in line with earlier studies [38], but also in primary cell cultures from these tumours. Interestingly, pairwise comparison of samples collected at the treatment-naïve stage and after NACT demonstrated an increased *ALDH1A1* expression but stable or reduced expression of several other stemness markers. Further studies, including single cell analyses, are needed to explain this finding, which may be indicative of phenotypic plasticity of CSCs challenged by chemotherapy.

HGSC stem cells are thought to convey chemoresistance, which results in treatment failure with conventional chemotherapy. Therefore, one of our primary aims was to identify compounds that are effective against HGSC cells with stemness features. We performed comparative high throughput drug screening with 306 compounds under conventional culture conditions and with conditions favouring the stemness features, using primary cell cultures. We are not aware of any previous studies in which a similar approach has been used. A general finding in these experiments was that there is individual variation between patients, but also between culture conditions; the most sensitive donor

responded to twice as many compounds as the most resistant donor. Surprisingly, we did not find significant overall differences in response rates between cells grown in adherent and spheroid growth conditions. The results pinpoint candidate compounds with general efficacy independent of donor, and compounds that demonstrate some selectivity towards stemness conditions. Altogether, five drugs were highly effective independent of the donor or culture condition, and thirteen compounds showed preference towards cells grown in conditions favouring stemness features. A potential weakness of the drug screen results is the fact that adherent and spheroid cells were grown in different culture media, the effect of which cannot be ruled out.

The compounds that demonstrated efficacy independent of culture conditions or donor included kinase inhibitors targeting PI3K and mTOR. These results are consistent with findings that indicate a central role for the PI3K-mTOR pathway both in ovarian cancer biology [39] and in adverse outcome of the disease [40]. Interestingly, the IGF-1R receptor, a transmembrane tyrosine kinase receptor that transmits signals via the AKT-PI3K or MAPK-ERK pathways, has been associated with cancer stem cell functionality in ovarian cancer, as well as tumorigenicity and maintenance of a cancer stem cell phenotype in breast cancer [41,42]. Our discovery of a tyrosine kinase inhibitor (BMS-754807) showing effectivity towards stem-like cells is in agreement with these findings. Furthermore, there are recent promising results from a study of combinatorial therapy of mTOR- PI3K and MEK inhibitors in ovarian cancer [43]. Combined inhibition of MEK and Src was recently shown to deplete ALDH1A1-positive ovarian cancer cells with stem cell characteristics [44].

Altogether six EGFR inhibitors demonstrated preference towards cells grown in stemness conditions in at least two of the three donors. While clinical trials with afatinib, erlotinib and gefitinib have failed to demonstrate efficacy in HGSC, at least as single agents, no studies using the irreversible pan-ERBB

inhibitors canertinib, dacominitib and neratinimib in HGSC have been published. There is limited information on the association of chemotherapy resistance and EGFR expression or activation in HGSC cells. The above-referred MEK/Src inhibition study [44] showed a critical role for EGFR signalling as an alternative pathway to MEK/MAPK in supporting cancer stem cell viability. Another recent study showed that dacomitinib, one of the effective EGFR inhibitors in our panel, prevents growth of chemoresistant cells by inhibiting aurora kinase B activity [45]. This is in line with our results demonstrating that several aurora kinase inhibitors effectively killed cells grown in stemness conditions. Aurora kinases have been reported to play a role in ovarian cancer stem cell biology [46,47] and aurora kinase inhibitors have shown efficacy towards conventional ovarian cancer cell lines. The current study is the first to demonstrate efficacy in primary cell cultures. Recently, clinical trials with aurora kinase inhibitors alisertib and AMG-900 [48,49] have demonstrated clinical benefit. Similarly, the first phase I study combining mTOR inhibitor AZD2014 and weekly paclitaxel showed promising results in HGSC, warranting further clinical trials [50].

In conclusion, we have established an experimental model to study the HGSC cancer stem cell phenotype using primary cell cultures. We demonstrate that stemness markers are enriched after neoadjuvant chemotherapy, both in tumours and in cell cultures derived from them. We also show that in treatment-naïve tumours, expression of stemness markers predicts poor survival. Most notably, compounds targeting EGFR, PI3K and aurora kinase demonstrate activity towards CSCs. In the future, treatments targeting the signalling pathways vital for CSCs could help to eradicate the cell population that remains resistant to conventional chemotherapy.

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Author contributions statement

JH and AA contributed to material acquisition. KK, PR, KH, LL and PM carried out experiments. KK, PR, PC, KH, TK, NM and SP analysed the data. KK, PR, PC and KH generated figures. KK and OC contributed to study design, data interpretation and literature search. PÖ, SG, KW, SH, KK and OC were involved in writing the paper. All authors had final approval of the submitted version.

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Figure legends

Figure 1. HGSC cell phenotype depends on culture conditions.

Eleven patient-derived and seven commonly used HGSC cell lines were grown as adherent cells or under conditions supporting spheroidal growth. (A) Morphology of two patient-derived cell lines, M022 and M022i (i = interval) grown in spheroid (sph) or adherent (adh) conditions. Scale bar = 100 µm. (B) Expression of selected stemness-related markers in spheroidal and adherent cells. Out of ten stemness related markers, five (*ALDH1A1, CIP2A, POU5F1, SOX2 and BMI1*) were significantly upregulated in primary spheroidal cell cultures. Three replicates. * $p < 0.05$, ** $p < 0.01$ (n=16, two-sided t-test). (C) Platinum (cisplatin) and taxane (paclitaxel) sensitivity of HGSC cells grown under spheroidal or adherent conditions. Higher activity area (AA) indicates reduced sensitivity. * $p < 0.05$. (paired t-test) 3 replicates. (D) Platinum (Cisp; blue) and taxane (PTX; red) drug response curves for each cell line. Spheroids = triangles, adherent cells = boxes. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

Figure 2. Expression of stemness markers in treatment-naïve HGSC tumours is associated with survival.

(A) Expression heatmap of eight stemness markers in 144 treatment-naïve HGSC tumours in a microarray dataset [33]. Tumour samples were clustered by k-means clustering (k=2). Cluster 1 = baseline cluster. Cluster 2 = enriched stemness cluster. (B) Violin plots of stemness marker expression in Cluster 1 and Cluster 2 samples. Results are deduced from the same dataset. Significant expression differences for each gene between Cluster 1 and Cluster 2 patients are marked by asterisk (* for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, **** for $p < 0.0001$ and ***** for $p < 0.00001$). (C)) Kaplan-

Meier survival curves of the different clusters. Cluster 1 patients are associated with shorter overall survival than Cluster 2 patients (Median OS 20 mo versus 33 mo, log-rank test p=0.047).

Figure 3. ALDH as a marker for stemness-like phenotype

(A) Primary HGSC cells were sorted by flow cytometry for $ALDH^{bright}$ and $ALDH^{dim}$ cells and single cells were plated on myofibroblast feeder cells. Only $ALDH^{bright}$ cells gave rise to colonies (marked with a circle). Scale bar 100 µm. (B) Primary HGSC cells grown under spheroid or adherent conditions were analysed for ALDH1A1 protein expression. (C) Quantitation of western blotting results (n = 4). (*, p< 0.05, **, p< 0.01, ***, p< 0.001, ****, p< 0.0001)

Figure 4. ALDH is increased in HGSC tumors after platinum-taxane chemotherapy.

(A) Flow cytometry analysis of Aldefluor-stained treatment-naïve (n=21) and chemotherapy-treated (n=6) adherent (2-5 replicates) and spheroidal (2–20 replicates) cells after three platinum-taxane chemotherapy cycles. The percentage of Aldefluor-positive cells is significantly higher in the interval HGSC cells (*** p< 0.001). (B) Immunofluorescence staining for ALDH1A1 shows increased number of ALDH1A1-positive cells (red) in the interval sample. Blue = DAPI. Scale bar 100 µm. (C) *ALDH1A1* mRNA expression in tumour tissues (n=25) measured by RT-qPCR from treatment-naïve and chemotherapy-treated interval samples. *ALDH1A1* expression is significantly increased in chemotherapy-treated samples (* p<0.05). (D) ALDH1A1 IHC of representative treatment-naïve and chemotherapy-treated tissue specimens. Note strong ALDH1A1 immunoreactivity in cancer cells of chemotherapy treatment. Scale bar 100 µm. (E) RNA-seq-based *ALDH1A1* expression in tumour tissues (n=21) from treatment-naïve and chemotherapy-treated samples. *ALDH1A1* expression is

significantly higher in interval tissues (*** $p<0.001$).

Figure 5. Response of spheroid and adherent primary HGSC cells to oncology compounds.

Comprehensive DSRT was performed with 306 compounds. Three correlation plots are presented on primary ovarian cancer cell lines: (A) M019i, (B) M068i and (C) M022i. Correlation plots represent drug sensitivity scores (sDSS) of spheroidal (y-axis) and adherent (x-axis) cells. Drugs with sDSS score over 5 are considered effective and sDSS scores over 15 represent highly effective drugs. Dots on the correlation plots represent drugs.

BM = BMS-754807, Om = omipalisib, R e= refametinib, BI = BIIB021, PF = PF-04691502, Cn = canertinib, Da = dacomitininb, Ne = neratinib, Er = erlotinib, Af = afatinib, Gf = gefitinib, Tr = trametinib, 2014 = AZD2014, Ap = apitolisib, Ca = camptothecin, To = topotecan, Ge = gemcitabine, 928 3= AT9283, Al = alisertib, 1152 = AZD1152-HQPA, De = decitabine, Vn = vinorelbine, Vb = vinblastine, Vc = vincristine, Cy = cytarabine, Vo = volasertib

Red = EGFR inhibitor (6), Pink = Aurora-kinase inhibitor (3), Orange = mTOR/PI3K inhibitor/MEK inhibitor (6), Green = IGFR -/HSP90 -/topoisomerase -/polo-like kinase inhibitor (5), Blue = Classic/Nucleoside analogue (6).