Type-Specific Cell Line Models for Type-Specific Ovarian Cancer Research

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Abstract

Background: Ovarian carcinomas consist of at least five distinct diseases: high-grade serous, low-grade serous, clear cell, endometrioid, and mucinous. Biomarker and molecular characterization may represent a more biologically relevant basis for grouping and treating this family of tumors, rather than site of origin. Molecular characteristics have become the new standard for clinical pathology, however development of tailored type-specific therapies is hampered by a failure of basic research to recognize that model systems used to study these diseases must also be stratified. Unrelated model systems do offer value for study of biochemical processes but specific cellular context needs to be applied to assess relevant therapeutic strategies.

Methods: We have focused on the identification of clear cell carcinoma cell line models. A panel of 32 “ovarian cancer” cell lines has been classified into histotypes using a combination of mutation profiles, IHC mutation-surrogates, and a validated immunohistochemical model. All cell lines were identity verified using STR analysis.

Results: Many described ovarian clear cell lines have characteristic mutations (including ARID1A and PIK3CA) and an overall molecular/immuno-profile typical of primary tumors. Mutations in TP53 were present in the majority of high-grade serous cell lines. Advanced genomic analysis of bona-fide clear cell carcinoma cell lines also support copy number changes in typical biomarkers such at MET and HNF1B and a lack of any recurrent expressed re-arrangements. Conclusions: As with primary ovarian tumors, mutation status of cancer genes like ARID1A and TP53 and a general immuno-profile serve well for establishing histotype of ovarian cancer cell We describe specific biomarkers and molecular features to re-classify generic “ovarian carcinoma” cell lines into type specific categories. Our data supports the use of prototype clear cell lines, such as TOV21G and JHOC-5, and questions the use of SKOV3 and A2780 as models of high-grade serous carcinoma.

Introduction

Ovarian cancer is a diverse set of diseases and amongst the most clinically significant, epithelial ovarian cancers (EOC), at least five distinct entities exist [1–9]. At a broad level, the terms type I and type II EOCs are often applied, wherein high-grade serous carcinomas (HGS Cs) are type II and all other histologies are type I cancers [8]. However, even within type I, distinct entities exist, namely low-grade serous carcinoma (LGSC), endometroid carcinoma (ENOCa), clear cell carcinoma (CCC) and mucinous carcinoma (MUC). There is significant data suggesting that a majority of HGSC originate from fallopian tube epithelium [1,10–13], while low-grade serous tumors are generally still thought to arise from the ovarian surface epithelium – though this relationship is being questioned [7,14]. ENOCa and CCC tumors occur in a background of endometriosis and could represent a spectrum of displaced, malignant endometrium [15–20]. Finally, mucinous tumors are exceedingly rare and their true origin is difficult to ascertain with subgroups of distinct histology. Their resemblance to other mucinous epithelial malignancies, most notably gastric cancers, has added to the confusion of their origin [3,21–23]. Clinical responses and epidemiological differences are also apparent between histotypes. High-grade serous cancers show the best initial response rates to the current standard chemotherapy regime of platinum and taxanes [24,25]. Familial BRCA1/BRCA2 mutations also appear largely restricted to this histology [26–28]. Conversely, the minor histotypes tend to occur in younger patient populations and more frequently present at lower stage [29–31]. A
list of some of the more distinguishing features between histotypes types is given in Table 1.

Regardless of origin or histological similarities and differences, biomarker and genomic studies have been successfully used to distinguish each histotype and may represent a far more biologically relevant basis for classifying and subsequently treating EOCs. Although this concept is well-accepted, and gaining traction on becoming a new clinical standard, ambiguous cell line model systems perpetuated through molecular biology bench research hamper the development of tailored type-specific therapies. Those using bench experiment model systems must recognize that, like primary cancers, the models used to study these diseases must also be stratified. Although biochemical studies can generate useful information from using a variety of unrelated model systems, disease specific studies need to apply cellular context. The vast majority of research employing functional studies on “ovarian” cell lines does not properly ascertain the background of their model systems. Resulting conclusions may be difficult to interpret and the value of potential therapeutic targets may be questionable as is the true relevance to a particular disease.

Cell line studies of ovarian cancer have been severely hampered due to the lack of proper annotation of “ovarian” carcinoma cell lines. Once in culture, cells no longer have easily identifiable morphological traits to aid in histological classification. Additionally, human error, mislabeling and the generic feature of “epithelial-like” cell lines have also led to mix ups of cell lines and contamination which has resulted in un-interpretable data [32,33]. In the post-genome era, biomarkers and genomic features for ovarian carcinoma subtypes are very well established. Screening techniques to assay biomarkers and verify genomic features for ovarian carcinoma subtypes are very well established.

Table 1. Discriminating Features Of The Five Major Histotypes Of Ovarian Carcinoma.

| Preparative | Clear Cell Carcinoma | Endometrioid Carcinoma | Mucinous Carcinomas & Mucinous Borderline Tumors | Low-Grade Serous Carcinomas & Serous Borderline Tumors | High-grade serous carcinoma |
|------------|----------------------|------------------------|-----------------------------------------------|------------------------------------------------------|---------------------------|
| Presentation | Presents at younger age and low stage (pelvic mass) [4,29–31] | Presents at younger age (than HGSC) [4,29–31] | Presents at younger age (than HGSC) [4,29–31] | Presents at younger age (than HGSC) [4,29–31] | Presents at older age (than other histotypes) and high stage (ascites common) [4,8,29–31] |
| Precursors | Associated with Endometriosis [1,8,16,82] | Associated with Endometriosis [1,8,16,82] | Potential link to Walthard cell nests [83] | Association between ovarian surface and fallopian tube epithelium is unclear [14] | Significant subset associated with serous tubal intraepithelial carcinoma (STIC) [1,8,11,84] |
| Genetics, Genomics & Biomarkers | TPS3 wild-type [4,15] | TPS3 mutations rare [4] | TPS3 wild-type (borderline) | TPS3 mutant (~1/2 of carcinomas) [4,8] | TPS3 wild-type (4,8) |
| | Negligible occurrence of (germline) BRCA1/2 mutations [26–28,86] | Negligible occurrence of (germline) BRCA1/2 mutations [26–28,86] | Negligible occurrence of (germline) BRCA1/2 mutations [26–28,86] | Frequency of BRCA1/2 mutations presumed low | Germline and somatic BRCA dysfunction/ high proportion of hereditary (germline) BRCA1/2 mutation carriers [9,26–28,86] |
| High frequency of ARID1A and PIK3CA mutations; frequent loss of PTEN expression; near ubiquitous expression of HNF1β [15,16,45] | High frequency of ARID1A mutations; Moderate frequency of PIK3CA, CTNNB1, and PTEN (loss/LOH) mutations [16,45] | High frequency (55–75%, carcinoma-borderline) of KRAS mutations; KRAS mutations (ras-pathway mutation almost exclusively KRAS); Frequent (19%) of high-level ERBB2 amplification [22] | High frequency mutually exclusive RAS-pathway mutations (KRAS, BRAF, NRAS, or ERBB2) typical of borderline serous tumors [5,8,10,66] | Complex karyotypes suggestive of a period of massive genomic instability [9,87] |
| Treatment Response and Outcomes | Higher frequency of thromboembolic complications [15,88] | Low stage outcome better than (stage matched) HGSC; poor initial response to therapy and worse high stage outcomes (vs. HGSC) [15,89] | Typically longer interval to progression or death than HGSC (confounded by stage). Stage matched analysis (Stage III) suggests little difference in outcome to HGSC [90] | Overall favorable (due to prevalence of low-stage disease), however very poor outcome on recurrence [31,43] | Poor response to current treatment standards (Platinum/taxane) [91,92] |
| | Good initial response rates to current treatment standards (Platinum/taxane); relapse and eventual treatment failure is common [4,24] |

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Methods

Cell culture

Cells were maintained in a humidified incubator at 37°C with 5% CO₂. See Table S1 for a list of cell lines, culture conditions and contributing labs and repositories. Some cell lines were derived in-house (labeled with “VOA#”) through continuous in vivo culture of primary patient material obtained through the OVCARE Tumor bank. All patients with tissue deposited in the OVCARE tumor bank provided written consent for experimental studies including sequencing. HIC characterization, and derivation of long-term cell lines from tissue samples. The OVCARE tumor bank study was approved under University of British Columbia and British Columbia Cancer Agency Research Ethics Board H05-60119 protocol.

All cell lines were subjected to identity testing using STR genotyping (AmpFISTR Identifier, Applied Biosystems) at the College of American Pathologist’s (CAP) accredited Centre For Translational and Applied Genomics (CTAG) as per manufacturer directives. Only lines with profiles matching public repository records, reported STR [32], and/or original patient tumors (in the case of in-house derived cell lines) were retained for further study.

Immunohistochemistry and Calculator of Subtype Prediction (COSP)

Cell lines were scraped from culture plates, washed 2× with PBS and pelleted. Cell pellets were re-suspended in ~500 µl 10% Neutral Buffered Formalin (NBF) and allowed to fix overnight. Cells were pelleted again and re-suspended in a Histo-gel (Thermo-Fisher) plug prior to embedding in paraffin. A tissue microarray (TMA) was constructed as previously described [4] taking 3×2 mm cores from the cell line plugs. Immunohistochemistry (IHC) was performed on 4 µm sections on a Ventana Discovery XT system as previously described [2,34], refer to table S2 for details of antibodies used. Histotype prediction was done using the Calculator of Subtype Prediction (COSP) [2] in tumor bank mode. Tumour bank mode was chosen due to the nature of the fixed cell lines and the controlled fixation period similar to the tumor bank process on which this predictor was trained. Scoring criteria for IHC was done visually and followed similar to the tumor bank process on which this predictor was nature of the fixed cell lines and the controlled fixation period.

mRNA transcripts

RNA was extracted from cell lines using Qiazol-miRNeasy kit (Qiagen) protocol and from primary tumors, 12 randomly selected from each histotype, using the miRNeasy FFPE kit (Qiagen). All RNA transcript levels were measured using the NanoString nCounter system [36] and data normalized with nSolver software v1.1 (NanoString Inc.) using endogenous control genes (ACTB, SDHA, RPL19, POLR1B, PGK1) as per manufacturers directives. In the case of TFF3 mRNA levels we considered any sample with detectable transcripts to be positive and substituted a score of “1” in place of TFF3 IHC when using COSP. The detection threshold (DT) for mRNA was considered to be the maximum count from spike-in negative control probes (across all cell line samples) plus 2 standard deviations. Statistical tests were calculated using GraphPad Prism v6.0c software.

Mutation Testing and Genomic Analysis

Genomic DNA was extracted using standard methods (Genta Puregene kit; Qiagen). Regions encompassing mutations of known significance (Cancer hotspots) were Sanger sequenced using M13-tagged primers. Sequencing of ARID1A was done through a combination of custom hybrid capture and transcriptome sequencing on an Illumina GAII next generation sequencing (NGS) system as described previously [16,37]. Associated raw data is deposited in the NCBI Sequence Read Archive under BioProjects PRJNA209481, PRJNA209482, and PRJNA209484. All noted variants were either verified by Sanger sequencing or considered valid if recorded in the Cancer Cell Line Encyclopedia (CCLE) [38] and/or the COSMIC database [39]. Expressed re-arrangements were predicted from transcriptome sequencing data for CCC cell lines TOV21G, JHOC5-JHOC7, JHOC-9, and RMG-2 using deFuse [40] (Table S3).

Copy Number Analysis

DNA copy number was inferred from Affymetrix SNP 6.0 genome-wide microarrays. Arrays were run as per manufacturers directives and copy number ratio generated from an unpaired reference. Detection of copy number changed regions was done using a segmentation algorithm. All analysis and visualization was executed with Partek Genomics Suite 6.6, raw data is available from NCBI GEO [Accession GSE48351].

Results

Histotype by COSP in ovarian cancer cell lines

Ovarian cancer cell lines grown in culture do not exhibit the histological phenotypes that are useful for classification into the major disease types. Our group has described a large number of immunohistochemical biomarkers that show specific profiles across these histotypes [4,41–43]. A core panel of 9 IHC markers combined with a predictive algorithm, the Calculator for Ovarian Subtype Prediction (COSP), can be used to reliably distinguish between types [2]. We have previously demonstrated a high level of concordance between our predictive immune-classifier and consensus expert gynecopathological review [2,34]. Initially, we applied this panel (Fig 1A–B), and the COSP predictive algorithm, to 32 ovarian cancer cell lines of ambiguous histotype to establish if cell lines retained representative characteristics sufficient to classify cell lines to their true disease origins and allow for type-specific ovarian cancer model development. The TFF3 IHC marker, which is normally strongly associated with the mucinous type and seen at moderate frequency in ENOCa and LGSC [2], was negative across all samples (Table S4), suggesting this secreted factor, if expressed at all, may be expelled quickly from the cells and washed away in media. Consequently, TFF3 IHC may not be a reliable biomarker measurement for use with cultured cells. However, the prevalence of TFF3 mRNA in primary samples appeared similar to that reported by IHC [2,44], with consistently higher expression in mucinous carcinomas (p<0.01; Fig. 1C). We therefore substituted detectable TFF3 mRNA for IHC and scored any cell line with detectable mRNA as “1” in our COSP algorithm (Fig. 1D and Table 2).

Many previously described CCC lines showed features characteristic of their expected origins. In addition to the COSP 9-marker panel, we added IHC for ARID1A (BAF250a). Given the strong negative association of mutation status and detectable protein expression [16] we considered this assay as a surrogate mutation test useful in segregating endometriosis associated ovarian cancer from other subtypes, most notably high-grade
Table 2. Validation of the histotype of commonly used ovarian carcinoma cell lines using immunohistochemistry based prediction via COSP and mutational profiling.

| Cell Line | Reported Histotype | COSP Markers | COSP Prediction (Clinical) | Non-COSP Markers | DNA Mutational Profile | Validated Cell Line Histotype |
|-----------|--------------------|--------------|-----------------------------|------------------|------------------------|-----------------------------|
| JHOC-5    | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| JHOC-7    | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| JHOC-9    | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| RMG-2     | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| TOV21G    | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| OVTOKO    | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| A2780     | Adenocarcinoma     | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| IGROV1    | Mixed              | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| TOV112D   | ENOCa              | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| 2008      | ENOCa              | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| OVIDE     | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| ES-2      | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| SKOV3     | adenocarcinoma     | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| OVSAYO    | CCOC               | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| CAO3V     | Adenocarcinoma     | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| Kuramochi | Undifferentiated   | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| OVCAR-3   | Adenocarcinoma     | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| OVCAR-4   | Serous Adenocarc. | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| OVCAR-5   | Adenocarcinoma     | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| OVCAR-8   | Adenocarcinoma     | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| COLO-7020E| carcinoma          | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| COLO-704  | carcinoma          | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| Hey       | carcinoma          | p16 (CDKN2A) MDM2 TFF2 (mRNA) p53 VIMENTIN WT1 HNF1B PR DKK1 CCC ENOCa HGSC MUC ARID1A (BAF250A) TPS3 Other* |
| Cell Line      | Reported Histotype | COSP Markers | COSP Prediction (Clinical) | Non-COSP Markers | DNA Mutational Profile | Validated Cell Line Histotype |
|---------------|-------------------|--------------|----------------------------|------------------|------------------------|-----------------------------|
|               |                   | p16 (CDKN2A) | MDM2 | TFF3 [mRNA] | p53 | VIMENTIN | WT1 | HNF1B | PR | DKK1 | CCC | ENOCa | HGSC | MUC | ARID1A (BAF250A) | TP53 | Other* |
| VOA1400_CL    | HGSC primary tumour | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 100 | 0 | 1 | E198* (het) | none detected |
| VOA1416_CL    | HGSC ascites      | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 100 | 0 | 1 | nc | none detected |
| VOA1072_CL    | HGSC primary tumour | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 1 | R248Q (Hm) | none detected |
| VOA1312_CL    | LGSC ascites      | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 100 | 0 | 1 | nc | XKRAS |
| VOA1056_CL    | LGSC primary tumour | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 39 | 61 | 0 | 1 | nc | NRAS |
| MCAS          | mucinous carcinoma | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 100 | 1b | 127bp del (Hm, Ex XKRAS 4) | mucinous carcinoma |
| RMG-1         | CCOC              | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 22 | 0 | 76 | 3 | 1 | nc | none detected |
| OV90          | Adenocarcinoma    | 1 | 1 | 1 | 2 | 0 | 0 | 1 | 1 | 0 | 0 | 53 | 47 | 0 | 1b | p.S215R (Hm) | none detected |

COSP and ARID1A markers were scored as positive (1) or negative (0), except for p53: null mutation (0), wildtype (1), mutated (2). ARID1A IHC: a – corresponding ARID1A nonsense or frameshift mutation detected, b – no ARID1A mutation detected in sequencing data (if no letter code, sequence information was unavailable) COSP algorithm can be found at [http://www.gpec.ubc.ca/index.php?content=papers/ovcasubtype.php](http://www.gpec.ubc.ca/index.php?content=papers/ovcasubtype.php) TP53 mutations are noted as heterozygous (het) or Homozygous/Hemizygous (Hm) *Sequencing of BRAF, KRAS, ERBB2, NRAS, CTNNB1, EGFR, PTEN, PIK3CA, PPP2R1A, DICER1 and ARID1A doi:10.1371/journal.pone.0072162.t002
Figure 1. Prediction of histotype was in part based on the COSP algorithm using 9 IHC markers [2]. (A–B) Representative IHC from a typical high-grade serous ovarian carcinoma cell line, Kuramochi, and a clear cell carcinoma cell line, TOV21G. In addition to the 9-marker COSP panel, IHC for ARID1A (BAF250a) is also shown as a mutation surrogate. (C) TFF3 mRNA expression from 60 ovarian cancer samples (12 of each histotype). As noted previously high expression is most prevalent in MUC, followed by ENOCa and LGSC [2,4]. Expression in our pilot cohort suggests the highest levels of TFF3 in MUC, which was significantly higher than all other groups (Tukey’s adjusted p<0.01); no other pairwise comparisons had p<0.05. (D) TFF3 mRNA detected in ovarian cancer cell lines was used in place of an IHC score as the secreted TFF3 was considered a poor biomarker for cell culture conditions. Any cell line with measurable TFF3 mRNA above the NanoString detection threshold (see methods) was considered positive (score of 1 for use in the COSP algorithm).

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Table 3. Copy number changes across putative CCC oncogenes, tumor suppressors, and biomarkers.

| Segment Copy Number | JHOC-5 | JHOC-7 | JHOC-9 | OVMANA | OVTKO | RMG-2 | TOV21G | References |
|---------------------|--------|--------|--------|--------|-------|-------|--------|------------|
| ARID1A              | 3.422  | 2.397  | NC     | 2.314  | NC    | NC    | NC     | [16,45]    |
| ERBB2               | 3.061  | NC     | NC     | 2.514  | 2.382 | NC    | NC     | [52]       |
| HNF1B               | 3.061  | 3.109  | 3.533  | NC     | 2.514 | 2.382 | NC     | [53]       |
| MAP1LC3A            | NC     | NC     | NC     | NC     | NC    | NC    | NC     | [54]       |
| MET                 | 8.465  | NC     | NC     | 3.451  | 2.346 | 2.667 | NC     | [53,56,57] |
| PIK3CA              | 0.971  | 1.222  | NC     | NC     | 6.482 | NC    | NC     | [48,49]    |
| PPP1ID              | 3.157  | 2.329  | NC     | 3.004  | 3.009 | 2.382 | NC     | [50,51]    |
| STAT3               | 3.314  | 3.142  | NC     | NC     | 2.522 | 2.382 | NC     | [53]       |
| TP53                | NC     | NC     | NC     | 1.320  | 2.410 | NC    | NC     | [4]        |
| YAP1                | NC     | NC     | NC     | NC     | 1.264 | NC    | NC     | [59]       |
| ZNF217              | 2.893  | 5.897  | 4.717  | 3.412  | 3.648 | 2.589 | NC     | [55,58]    |
| CDKN2A              | 0.163  | NC     | NC     | NC     | 0.246 | 1.244 | NC     | [58]       |
| CDKN2B              | 0.454  | NC     | NC     | 0.602  | 1.244 | NC    | NC     | [58]       |

NC = no change in copy number was detected.

Mutational Profiles: Clear cell specific molecular features

We next tested cell lines for mutations in common ovarian cancer associated genes (Table 2 and Table S5). As some of the cell lines we tested are also part of a larger Cancer Cell Line Encyclopedia (CCLE) repository data set [36], we cross-validated our mutation testing with this database as well as the COSMIC database [39]. We focused on regions of known significance in common cancer genes including hotspots in BRAF, KRAS, NRAS, ERBB2, EGFR, CTNNB1, PIK3CA, PPP2R1A and Dicer1. All coding exons of TP53 were verified in all cell lines. ARID1A mutations were tested using a custom NGS gene hybrid capture strategy [37] in RMG-1, RMG-2, JHOC-5, JHOC-7, JHOC-9, TOV21G, and ES-2; for all other cell lines we used ARID1A data from COSMIC and CCLE in addition to IHC as an ARID1A mutation-testing surrogate (Table 2).

As with our IHC data most CCC lines maintained a profile consistent with the CCC histotype including mutations in PIK3CA and ARID1A. Further, loss of ARID1A expression, demonstrated by IHC, showed good concordance with presence of known truncating mutations, as noted for primary tumor specimens [16]. As expected IHC for p53 correlated well with occurrence of truncating mutations, as noted for primary tumor specimens [16].

ARID1A mutations provided compelling evidence of a non-HGSC origin. The incidence of TP53 and ARID1A mutation was near mutually exclusive with the exceptions of IGROV1 and 2008. IGROV1 carries two frame shift mutations in ARID1A (p.M274fs/p.G184fs) and a mutation of unknown significance in TP53 (p.Y126C [het]), though p53 expression by IHC appeared normal.

The 2008 cell line had undetectable ARID1A, suggesting loss of function, and also carried two TP53 mutations (c.572_574 delCTC [het]/c.673-1 G>T [het, splice site]) and corresponding p53 IHC overexpression. These atypical combinations of mutation could plausibly be explained by a propensity to accumulate mutations in cell lines with DNA mismatch repair (MMR) deficiencies, as has been reported for IGROV1, SKOV3, and A2780 [46,47]. We validated MMR pathway protein expression with IHC for MLH-1, PMS-2, MSH-2, and MSH-6 (Table S5) and observed loss of two or more MMR proteins in IGROV1, SKOV3, A2780 TOV21G, COLO-704 and COLO-720E; no MMR protein deficiency was noted in the 2008 cell line.

Copy Number profiles of clear cell lines

As our primary objective was to describe CCC cell lines we generated copy number profiles of bona-fide CCC cell lines using Affymetrix SNP 6.0 microarrays. Consistent with previous reports using primary tumor samples, CCC lines showed a moderate degree of copy number abnormalities, suggesting a genome that has undergone some degree of genomic instability.

A limited number of literature reports have highlighted genes with mutations, overexpression and/or amplification amongst primary CCC, some with a relationship to survival or advanced disease [4,16,43,48–59]. As we observed in mutation profiles, our bona-fide CCC cell line panel was representative of clear-cell associated copy number changes (Figure 2, Table 3). Most showed modest copy number gains for HNF1B (5/7) and MET (4/7), including one with high-level amplification (JHOC-5), similar to previous reports for CCC tumors [53,56,57]. Although 3/7 CCC lines showed copy number gain of ERBB2, in all cases the amplicon segment also encompassed the nearby CCC biomarker HNF1B, and none were positive for HER2 protein expression by IHC (not shown). Copy number loss around TP53 was observed.

Linear SVM analysis of gene expression profiles of時候 ovarian carcinoma modeled as a binary class (HGSC or CCC) identified genes with altered expression in CCC relative to HGSC (Figure 3). Gene set enrichment analysis of genes with altered expression in CCC relative to HGSC identified a set of genes involved in the cell cycle and DNA damage response pathways (Figure 3), suggesting a mechanism for the observed genomic instability in CCC.

Table 4. Gene expression profiles of times ovarian carcinoma modeled as a binary class (HGSC or CCC) identified genes with altered expression in CCC relative to HGSC (Figure 3). Gene set enrichment analysis of genes with altered expression in CCC relative to HGSC identified a set of genes involved in the cell cycle and DNA damage response pathways (Figure 3), suggesting a mechanism for the observed genomic instability in CCC.
only in a single CCC cell line (OVMANA; heterozygous loss) and, as noted above, all CCC lines appeared to have a normal-like expression pattern for p53 (IHC score 1).

**Transcriptome profile of clear cell lines**

As with other ovarian carcinoma types, recurrent translocations amongst CCC have not been described, though only a minimal number of studies have been undertaken [16].

![Figure 2. Genome-wide copy number profiles of bona-fide ovarian CCC cell lines. A large range of copy number changes are seen including typical Chr8 gains and Chr17 gains surrounding the CCC biomarker HNF1B gene, see also Table 3. doi:10.1371/journal.pone.0072162.g002](image-url)
Type-Specific Ovarian Cancer Cell Line Models

A

Karyotype: 92<4n>, XXXX, -3, der(3)t(3;4)(?;q21;?), der(4)t(3;4)
(?;q?)x2,-5,-6, del(7)(?), del(8)(p?)x4, der(8)t(8;19)
(p?q?)-9,-9, der(10)t(10;22)(p?q?)t(10;20)(q?),
der(10)t(10;20)(q?), -10, der(11)t(7;11)(?;q22.2), -11, -12,
der(14)t(14;15)(?;q)x2,-16,-16,-18,-18, der(19)t(19)?(,)
der(20)t(16;20)(?;?)

B

Karyotype: 46<2n>, XX, der(3)t(3;4)(?;q21;?), -5, del(8)(p?),
+der(8)t(8;19)(p?q?), der(10)t(10;20)(q?),
der(14)t(14;15)(?;q)

Figure A shows the karyotype of JHOC-9 (clone 1) with various chromosomal abnormalities. Figure B displays the karyotype of JHOC-9 (clone 2) with different structural changes.
preference for observed in primary CCC [16,45] and may indicate some PIK3CA detected mutations (chance occurrence and does not appear to be significant. Other PLOS ONE | www.plosone.org 10 September 2013 | Volume 8 | Issue 9 | e72162 doi:10.1371/journal.pone.0072162.g003 notations). (B) Circos plot of RNAseq data and deFuse analysis depicting expressed genomic rearrangements in the JHOC-9 cell line. Translocations seen in the 24-color FISH profile are also visible as expressed transcripts including (t8;19) observed in both 2N and 4N dominant clones. No recurrent translocations were seen across our series (see also Table S3).

Figure 3. Genomic structure of CCC cell line JHOC-9. (A) 24 color FISH analysis suggested the presence of two dominant clones; one near-diploid and one near-tetraploid in the JHOC-9 CCC cell line. A number of translocations and rearrangements can be seen in each representative clone. The complex karyotype of each dominant clone is noted below the corresponding 24-colour FISH results. Not all derivative chromosomes were identifiable resulting in a large number of ambiguous translocations and fragments (denoted by question marks in the karyotype notations). (B) Circos plot of RNAseq data and deFuse analysis expressing genomic rearrangements in the JHOC-9 cell line. Translocations in 2N and 4N cell lines are also visible as expressed transcripts including (t8;19) observed in both 2N and 4N dominant clones. No recurrent translocations were seen across our series (see also Table S3).

Discussion and Conclusions

As our initial goal was identification of bona fide CCC cell lines, we are pleased to report that the majority of reported CCC lines were representative of the primary tumors’ molecular and pathological phenotype. Our immuno-classification scheme, COSP, predicted most to be CCC and our own mutation data, as well as that from COSMIC and CCLE, suggested loss of function ARID1A mutations were prevalent in these cell lines. Although three CCC lines did not have identifiable ARID1A mutations, only JHOC-5 cells appeared to have both wild-type sequence and detectable protein expression. The number of ARID1A “normal” CCC lines is lower than might be expected given the frequency of ARID1A mutations (and negative IHC) observed in primary CCC [16,45] and may indicate some preferential selection for ARID1A null CCC lines to adapt to in vitro culture. However, given the small sample size it may well be a chance occurrence and does not appear to be significant. Other detected mutations (PIK3CA, Pten, KRAS, PPP2R1A) are all consistent with varying frequencies in CCC. TP53 mutations are notably absent in all of our validated CCC cell lines, as a de-facto defining characteristic, and only a single CCC line had heterozygous copy number loss though still retained normal-like p53 IHC.

Both CCC and EOCa appear to arise in a background of endometriosis. Atypical endometriosis adjacent to, or contiguous with, either histotype is not unusual for either CCC or EOCa [16,60,61]. Co-occurrence (sometimes contiguous) of both CCC and EOCa histologies in a mixed-cell type tumor has been reported [62] (and Dr. Blake Gilks, personal communication). Mutualized profiles including ARID1A and PIK3CA, are common to both types, overall supporting a related origin and similar route to transformation [16,45,48,49]. We found that both ES2 and OVIS cell lines, reportedly derived from CCC, largely resembled the immuno-profiles of EOCa. Conversely the 2008 cell line, reportedly derived from serous carcinoma [63], though often referred to as EOCa [64], appeared more EOCa-like from COSP alone. The 2008 line did show mutant p53 staining and has two confirmed TP53 mutations, atypical for true CCC. IHC was negative for ARID1A, supporting a non-serous origin. We favored an assignment of EOCa base largely on the TP53 mutation though note that this cell line is quite atypical as it may carry loss of function changes for ARID1A, mutation of TP53, and is positive for the CCC biomarker HNF1B. Arguably errors in cell line histotype reports may be explained simply by historically poor reproducibility in cell type assignment, though it is not unforeseeable that the biological relationship between CCC and EOCa could be influencing these phenotypes. Given the high degree of overlap between the mutational characteristics of CCC and EOCa, our panel was not able to further segregate or clarify this apparent confusion. SKOV3 is another unique case as it’s immuno-phenotype most closely resembles HGSC, yet it carries a truncating mutation for ARID1A, a mutation that has not been observed in HGSC despite widespread testing [16,45]. Previous studies with SKOV3 have pointed to a clear cell-like histology when grown as xenograft [64] and this may also favor an endometriosis-associated ovarian cancer diagnosis as does the presence of a PIK3CA mutation. Finally, the TOV112D cell line also presents as an exception with a moderately strong prediction of HGSC immuno-phenotype. In spite of this finding we suggest this line is representative of TP53 mutant EOCa, based on the presence of an EOCa characteristic CTNNB1 mutation, pathological review of the primary tumor material in the originating laboratory and expression profiling experiments supporting this conclusion [65]. We propose that these atypical CCC/EOCa may be useful in exploration of some common endometriosis-associated ovarian cancer biology though care should be undertaken to allow proper interpretation of the results.

Unfortunately our COSP tool is unable to differentiate LGSC. Based on expert re-review of primary material we are aware of two cell lines derived from LGSC primary tumors. We therefore confidently favor this classification for VOA1056_CL and VOA1312_CL despite predictions of HGSC or EOCa obtained from COSP. The VOA1056_CL line carries a Ras-pathway mutation as might be expected of an LGSC tumor, however this is an NRAS Q61R activating mutation. Activating NRAS mutations were recently described in LGSC at the 2012 AACR annual meeting [66] however, this represent the first validated report of an NRAS mutant LGSC tumor and the first validated LGSC derived cell line carrying this mutation. The COSMIC database suggests the cell lines LG-1 (G12D; defined as ovarian carcinoma, type not specified) and TYK-nu (G12D and Q61K; defined as ovarian “serous carcinoma”) also carry activating NRAS mutations, however we were unable to source these cell lines to confirm/reject their histological identity. In the cases of LGSC cell lines derived in-house, mutations of TP53 were not observed, consistent with IHC based literature reports suggesting this is a major molecular discriminator between HGSC and LGSC [67].

Finally only a single cell line in our collection was reported to be of mucinous carcinoma origin. The mutation profile of this cell line is consistent with this diagnosis, including a 127-bp TP53 homozygous deletion, overexpression by IHC, and KRAS G12V mutation.

Cell line records for epithelial ovarian carcinoma have recently come into question with a number contaminated and redundant cell lines acknowledged in a recent study [32]. Most notably 2008 (aka. ov2008) was reported to be frequently contaminated with, or a mislabeled version of, the HPV-positive ME-180 cell line (ATCC.
HTB-33), the “true” HPV-negative 2008 line defined in the report from Korch et al. [32], is the one used in our study. Maintaining appropriate records, testing and, most importantly, re-testing identity of cell lines in each individual lab’s stocks should be paramount even if cell lines are obtained directly from repositories. Here we report only on cell lines that matched the originating repository STR DNA profile or the STR profile of their originating primary tumors (in the case of in-house derived lines). Despite our own best efforts our exercise did yield the discovery of 3 cell lines in our own stocks that were either mislabeled or contaminated, including our original stock of the 2008 cell line noted above. All contaminated lines have since been discarded/replaced. It should be noted that none of our assays were designed or tested to discriminate ovarian from non-ovarian malignancies, and although STR analysis would have eliminated any obviously male cancers (through detection of Chr Y markers), some level of accuracy in repository reported origin of “ovarian” must be assumed. In the case of the more atypical cell lines it is possible these may be of non-ovarian origin, e.g. endometrial carcinomas or other peritoneal cancers of unknown primary, we are currently unable to assess this idea. Further, our analysis may be confounded by dominant expansion of rare tumor sub-clones [68], acquisition of spontaneous mutations during culturing, and MMR deficiency (whether acquired or present in the originating primary tumor). MMR deficiencies have been reported to be prevalent in endometriosis-associated ovarian cancers (CCC and ENOCa) [69–72] and the potential acquisition of mutations as a result of MMR deficiency may influence some of the more ambiguous biomarker phenotypes within this group, as well as observed atypical mutation patterns. We noted MMR deficiencies in the non-serous lines TOV121G, SKOV3, A2780, and IGROV1 as well as the HGSC cell lines COLO-704 and COLO-720E (Table S5). MMR-protein deficiencies were not observed in our in-house derived LGSC cell lines (or their corresponding primary tumors) or in the mucinous carcinoma line MCAS.

In the spectrum of ovarian carcinomas, recent evidence strongly supports diagnosis and treatment of the five major histotypes of carcinomas as distinct diseases. Cancer cell lines provide an important intermediate tool for clinically relevant translational science, allowing genomic manipulation and cell biology studies beyond what can be reasonably achieved in clinical trials or animal models of cancer. In order to develop appropriate treatments, translational researchers need to use model systems appropriate to each ovarian carcinoma type. Unfortunately, historical records of ovarian cancer cell lines have rarely included information on histological origin [32,64]; this is further hampered by a historical lack of reproducibility in histological diagnosis [73–75]. Histopathological reproducibility is steadily improving as grading criteria become unified [78–81], and objective biomarker based tools to delineate histotypes are developed [2,4,41,44]. However, cell lines lack morphological features that are recognizable in culture and development of new, well-defined, cell lines is laborious with poor long-term success rates. Assigning histotype to readily available and well-used cell lines will undoubtedly lead to better interpretation of new data and re-interpretation of already published findings.

Supporting Information

Figure S1 127bp homozygous (or hemizygous) deletion affecting TP53 exon 4 in the MCAS mucinous carcinoma cell line. This mutation was apparent by Sanger sequencing though not annotated in the CCLE database. Coding bases are annotated in upper case. (PDF)

Table S1 Cell Lines & Sources.

(PDF)

Table S2 Antibodies and Dilutions.

(PDF)

Table S3 deFuse predicted expressed re-arrangements from transcriptome sequencing data.

(XLS)

Table S4 Mutations Found In Ovarian Carcinoma Cell Lines.

(PDF)

Table S5 Mismatch Repair IHC.

(PDF)

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Author Contributions

Conceived and designed the experiments: MSA SEK DGH.Performed the experiments: MSA KGW CS CC JS WY NM LMP. Analyzed the data: MSA KS SEK. Contributed reagents/materials/analysis tools: MSA KGW DGH. Wrote the paper: MSA SEK DGH LMP KCW.

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