# A multiethnic analysis of immunerelated gene expression signatures in patients with ovarian clear cell carcinoma

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# A multi-ethnic analysis of immune-related gene expression signatures in patients with ovarian clear cell carcinoma

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**Abstract**

Little is known about the immune environment of ovarian clear cell carcinoma (OCCC) and its impact on various ethnic backgrounds. The aim of this OCCC immune-related gene expression signatures (irGES) study was to address the interaction between tumour and immune environment of ethnically-diverse Asian and Caucasian populations and to identify relevant molecular subsets of biological and clinical importance. Our study included 264 women from three different countries (Singapore, Japan, and UK) and identified four novel immune subtypes (*PD1-high, CTLA4-high, antigen-presentation* and *pro-angiogenic* subtype) with differentially expressed pathways, and gene ontologies using the NanoString nCounter PanCancer Immune Profiling Panel. The PD1-high and CTLA4-high subtypes demonstrated significantly higher *PD1*, *PDL1* and *CTLA4* expression and were associated with poorer clinical outcomes. Mismatch repair (MMR) protein expression, assessed by immunohistochemistry, revealed that about 5% of OCCC had deficient MMR expression. The prevalence was similar across the three countries and appeared to cluster in the CTLA4-high subtype. Our results suggest OCCC from women of Asian and Caucasian descent share significant clinical and molecular similarities. To our knowledge, our study is the first study to include both Asian and Caucasian women with OCCC and helps shine light on the impact of ethnic differences on the immune microenvironment of OCCC.

Keywords: Ovarian cancer, clear cell cancer, gene expression signatures, immune microenvironment, microsatellite instability, mismatch repair protein, immune subtypes, RNA expression and ethnicity

## Introduction

The prevalence of ovarian clear cell carcinoma (OCCC) varies across ethnicity. In Europe and North America OCCC accounts for 3-12% of epithelial ovarian cancer (EOC) [1–3] while in East Asia, the rates are significantly higher, approaching 25% of EOC [4,5]. However, OCCC is still considered "rare" by ESGO-GCIG and RARECAREnet defined as an annual incidence of < 6 per 100,000 (www.rarecarenet.eu; last accessed Dec 19, 2020). This subset of EOC typically has a poorer prognosis and much lower rates of response to platinum-based chemotherapy compared to patients with high grade serous ovarian carcinoma (HGSOC) [6].

In recurrent OCCC, responses to chemotherapy are uniformly low. A study in the Japanese population observed response rates to second-line chemotherapy of 8% in "platinum sensitive" patients and only 6% in the "platinum resistant" cohort [7]. In another retrospective study of 39 OCCC patients from Australia, patients who had partial response or no response to initial chemotherapy failed to respond to second-line chemotherapy [8]. A retrospective study of response rates in recurrent OCCC patients from Canada and UK revealed an overall response rate of 9% confirming the limited activity of chemotherapy in recurrent disease [9]. Typically, response rates of recurrent OCCC are less than 10% [7] with median progression free survival (PFS) of around 11 weeks and median overall survival (OS) of around 40 weeks [9].

As a consequence of the rare nature of this disease, large scale international trials are required in order to change the standard of care. To date, these have only rarely been completed. One of the first large trials conducted specifically for OCCC was JGOG3017 [10], a randomized phase III trial that compared the efficacy and safety of paclitaxel plus carboplatin (TC) versus irinotecan plus cisplatin (CPT-P) in patients with OCCC. This JGOG/GCIG trial was an international collaboration that accrued 667 patients, mainly from Japan and the remaining from Korea, France, and UK. The main objective was to evaluate if the CPT-P regimen was superior to TC. The trial observed no differences in PFS or OS between the two study regimens and suggested TC therapy remain the standard chemotherapy for patients with OCCC. There is a clear need for new therapeutic strategies for OCCC patients. A recent phase II trial of pembrolizumab for recurrent ovarian cancer (KEYNOTE-100) showed a low overall response (~8%), although the response rate in patients with clear cell histology was 16% [11], suggesting that certain OCCCs may elicit anti-tumour immune responses following PD-1 checkpoint inhibition.

Several reports have demonstrated subsets of OCCC which have immune-active phenotypes. Using exome-sequencing and microarray data, Matsushita *et al* demonstrated OCCC with high immunoediting, based on the number of neoantigens per somatic mutation, was associated with a T-cell inflamed phenotype, and a more favourable prognosis [12]. Willis *et al* reported PD-L1 expression was found in 43% of OCCC, particularly in 67% of OCCC with mismatch repair (MMR) defects [13]. Howitt *et al.* showed that OCCC with MSI exhibited a high number of CD8+ tumour-infiltrating lymphocytes (TILs) and higher PD-1 expressing TILs compared with microsatellite stable (MSS) OCCC. PD-L1 expression in tumour cells or immune cells was also noted in all cases of OCCCs with MSI [14]. In addition, Tan *et al* [15] demonstrated a subset of OCCC enriched for genes associated with extracellular matrix organization, adhesion and collagen binding similar to renal cell cancers that revealed preferential response to anti-angiogenic agents emphasizing the complex pathology of this rare subset of EOC and the importance of understanding the underlying biology of this disease.

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)-mediated and programmed cell death protein 1 (PD-1) are both immune inhibitory checkpoints commonly seen on activated T-cells that make up the tumour-immune environment and regulate immune responses. In our study, we analysed the tumour-immune environment of OCCC from women of different ethnic backgrounds who were treated in Singapore, Japan, or the United Kingdom (UK), based on a panel of immune-related genes using NanoString nCounter® technology and correlated their histopathological characteristics with clinical outcomes.

## Materials and methods

### Patients and collection of specimens

A cohort of 264 women with ovarian clear cell carcinoma of the ovary, primary peritoneal, or Fallopian tube, diagnosed between 1984 and 2016 (Singapore: between 2000 – 2015, Japan: between 2010 – 2016, UK: between 1984 – 2016) were identified through National University Cancer Institute (NCIS) ($n$ = 96), Singapore, Saitama Medical University International Medical Center, Hidaka, Saitama, Japan ($n$ = 84) and Edinburgh Cancer Research UK Centre, UK ($n$ = 84). A summary of clinical details is provided in Table 1. All procedures were conducted in accordance with the approved protocols. Eligible patients were at least 18 years of age (21 years of age for patients from Singapore), newly diagnosed invasive epithelial ovarian, peritoneal, or Fallopian tube cancer and had a histological diagnosis of clear cell carcinoma verified by two

independent pathologists (Singapore: DL, JW; Japan: MY, M Yano; UK: CSH, DL) based on established diagnostic criteria [16,17]. This study was approved by the Institutional Domain Specific Review Board from all participating sites.

**Clinical and pathological data**

Patients' medical records were comprehensively reviewed. Median follow-up for the whole was 41 months (Table 1). Outcome analyses were restricted to binary comparisons for several reasons: (a) OCCC is considered a rare cancer as defined by an annual incidence of < 6 per 100,000 based on classification from ESGO-GCIG and RARECAREnet [18]; (b) OCCC tends to present as early-stage disease resulting in low event rates which precluded the ability to observe significant differences between the subtypes analysed. Disease-free survival (DFS) was defined as the interval between histologic diagnosis and first progression, death as a result of disease, or last follow-up. Death as a result of non-disease-related causes was not considered in the calculation of DFS. Overall survival (OS) was defined as the interval between histologic diagnosis and the date of death as a result of disease, or last follow-up. Histopathology data were assessed by expert pathologists based on classical morphology and IHC analysis for positive HNF1β and napsin A, as well as negative WT1.

**Sample processing and gene expression profiling**

RNA was extracted from 10 µm FFPE sections using QIAGEN RNeasy FFPE Kits (Qiagen N.V, Hilden, Germany) following the manufacturer's protocol (Cat No./ID: 73504). Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to check the quality and quantity of RNA extracted from Singapore samples. Only RNA with length > 300 nt were considered for quantity calculation for downstream application. Unamplified total FFPE RNA (100 ng ) was hybridized to NanoString® Pan-cancer immune panel Reporter CodeSets and Capture ProbeSets at 65 °C for at least 16 h but not more than 48 h in a thermal cycler. The hybridized RNA samples were then loaded onto a NanoString nCounter system (NanoString Technologies Inc, Seattle, WA, USA) for gene expression analysis.

**Gene expression data processing**

Gene expression profiling of the OCCC samples on Pan-cancer immune panel were retrieved. The NanoString Pan-cancer immune panel analysed expression levels of 730 genes

related to immune cell types, CT antigens, responses, and functions. Gene expression normalization of NanoString data was performed using nSolver analysis software version 3.0 (NanoString Technologies Inc, Seattle, WA, USA). Only samples that passed all quality metrics were retained for analysis (supplementary material, Table S1). The raw NanoString counts were subjected to background subtraction, positive control normalization and 40-reference gene normalization. The normalized counts were then log2-transformed prior to down-stream analysis.

**Immune molecular subtype identification**

ConsensusClusterPlus v1.44.0 (with default parameters settings except Euclidean distance, max $K$ = 20, and 1000 permutations) in R v3.5.1 bioconductor v3.8 was employed to identify subtypes in the Singapore cohort using all 730 immune-related genes [19]. Silhouette analysis was used to select core samples (silhouette width > 0.065, first quantile of silhouette width). A total of 519 differentially expressed immune-related genes were identified from the 72 core samples from the Singapore cohort (ANOVA $p < 0.05$ and expressed in more than 10% of the sample) and were used as the gene expression signature (supplementary material, Table S2).

Consensus clustering was subsequently applied to the Japan and UK cohorts individually using the differentially expressed immune-related genes identified. Distinct clusters identified in the Japan and UK cohorts were assigned to the immune subtype that shared the highest similarity to that of Singapore cohort.

**Immunohistochemistry (IHC)**

FFPE sections (4 μm) from all three cohorts were immunostained for mismatch repair (MMR) proteins using antibodies from Ventana (Roche Tissue Diagnostics, Oro Valley, Arizona, USA): MLH1 (mouse clone M1); MSH2 (mouse clone G219-1129); PMS2 (mouse clone A16-4) and MSH6 (rabbit clone SP93). Deparaffinisation was performed using EZ Prep and antigen retrieval was performed using CC1 retrieval solution at 100 °C for 40 to 92 min. Endogenous peroxidase was blocked using 3% Hydrogen Peroxide. All steps were performed using a Roche Ventana Ultra Automated IHC machine (Roche Tissue Diagnostics, Oro Valley, AZ, USA). Tissue sections were counterstained with haematoxylin, dehydrated through a graded ethanol series and coverslipped.

### Statistical analyses

Statistical analyses were conducted using Matlab® R2012a 7.14.0.739, (MathWorks; Natick, MA, USA). Statistical significance of differential expression was evaluated using unpaired *t*-tests. Associations were evaluated using Fisher's exact test. A Spearman correlation coefficient test was applied to assess significance of correlation. Kaplan–Meier analyses were conducted using GraphPad Prism® 5.04 (GraphPad Software, San Diego, CA, USA). Statistical significance of Kaplan–Meier analyses was calculated using a log-rank test. Pathway enrichment scoring was based on Kolmogorov–Smirnov testing as described previously [20].

## Results

### Patient demographics

A total of 264 women with clear cell carcinoma of the ovary, primary peritoneum, or Fallopian tube from three international sites (Singapore, Japan and UK) were included in this study. Nine samples did not pass the quality check of NanoString gene expression profiling and were removed, resulting in a combined dataset of 255 (Figure 1). All samples from UK, Singapore and Japan were reviewed by two independent expert gynaecology pathologists and deemed to be histologically consistent with a diagnosis of OCCC based on classical morphology and/or IHC analysis of HNF1β, napsin A and WT1 [16,17] (Table 1). Subsequently, eligible histologically confirmed OCCC archival samples from all three countries underwent immune related gene expression profiling using the NanoString platform and their associated clinicopathological outcomes were correlated accordingly.

Median age at diagnosis across the three centres was 56 years of age (Singapore: 52 years; Japan: 57 years and UK: 61 years) (Table 1). As expected, early-stage disease predominated in each cohort with the proportions relatively similar across the three countries. Altogether, 139 (54.6%) women had Stage I disease while 113 (44.3%) had Stage II – IV disease. Japan with 65.8% ($n$ = 50), had the highest proportion of patients with Stage I disease followed by UK with 51.8% ($n$ = 43) and Singapore with 48% ($n$ = 46). Information about the initial disease stage was unavailable for three patients (Table 1). Amongst the 139 Stage I patients, 28.7% (40/139) had Stage IA/ IB disease and 71.2% (99/139) had Stage IC disease. Within the patients with Stage IC, 20 patients from the Japanese cohort and 7 patients from the UK cohort were Stage IC due to iatrogenic rupture (Stage IC-1) while 21 patients from both the Japan and UK cohort had surface

involvement or positive cytology (Stage IC-2, IC-3; Table 1). Further information on Stage IC disease were not available for the Singapore cohort.

The proportion of women diagnosed with late-stage disease were similar in the UK and Singapore cohorts at 48.2% and 49%, respectively and lower in the Japanese cohort (34.2%). Optimal debulking surgery, defined as no or < 1 cm residual disease after cytoreductive surgery [21], was achieved in more than 80% of patients from all three cohorts (72.9% with no residual disease; 9.2% with optimal debulking). A total of 24 samples (25%) from Singapore did not have accessible surgical data at the time of analysis (Table 1). Treatment practices appeared to be similar across the three countries. In total, 197 (77.3%) patients received chemotherapy in the adjuvant setting (Table 1) mostly with a platinum-based regimen (189 of 197 patients; 95.9%) while 137 of 197 (69.5%) received combination of a platinum–agent and taxane chemotherapy (Table 1). A total of 38 (14.9%) women did not receive any chemotherapy. Patterns of first line chemotherapy administration were similar across the three sites. Six patients from the UK cohort received radiotherapy as part of adjuvant treatment (7.2%) (Table 1). The median follow-up for OS in the Singapore, Japan and UK cohorts were 60.5, 49 and 41.6 months, respectively (Table 1).

**Immune related gene expression signatures (irGES) identifies four novel molecular subtypes of ovarian clear cell carcinoma**

Whole tumour immune-related gene expression profiling was conducted on archival FFPE samples using the NanoString platform. Nine samples did not pass quality control and were excluded from the analysis (Figure 1 and supplementary material, Table S1). To exclude batch artefacts from the three different cohorts due to FFPE storage, specimen age and processing, the Singapore cohort was used as the discovery set. Unsupervised clustering of the Singapore cohort using gene expression data was performed using the consensus clustering which revealed four distinct clusters (Figure 2; and supplementary material, Figure S1). The four clusters were named Pro-angiogenic (ProA), Antigen-presentation (AP), CTLA4-high and PD1-high relative to the dominant genes or pathways; we termed these as the irGES subtypes. In the ProA subtype, we observed increased overexpression of genes associated with stromal cell types relative to the other subtypes. The stromal markers in ProA include those of activated myofibroblasts (*MFGE8, MCAM),* vascular endothelial cells *(VEGFC)* and pericytes *(PDGFRB),* as well as enrichment of pathways and gene ontology groups defining extracellular matrix production/ remodelling (*FN1*, *COL3A1*), and immunosuppression (*TGFB2*) (supplementary material, Figure S1). The AP

subtype was characterized by overexpression of cell-cell adhesion markers (*CD58, CD164*) that enhance the binding of antigen presenting cells to T cells, thereby regulating T cell activation. Other genes highly expressed in this subtype included *CD46*, which encodes a protein that regulates the complement pathway, as well as *NF-KB1* and *ATF1*. Evidence of adaptive immune responses were observed among the genes significantly overexpressed within the CTLA4-high subtype, including overexpression of HLA class II markers (HLA-DPA, HLA-DPB1) and T-cells and granulocyte trafficking (*CXCL12, CX3CL1, CCL11, CCL13*). Overexpressed genes in the PD1-high subtype not only indicated upregulation of PD-1 (*PDCD1*), but also identified overexpressed NK cell markers that orchestrate cell migration and homing in the form of chemokines (*CR2, CXCR1, XCL2, CCR4*) (supplementary material, Figure S1).

To ascertain the reproducibility of the identified subtypes, we validated the subtypes in two independent cohorts – Japan ($n$ = 76) and UK ($n$ = 83) (Figure 2). Core samples from the Singapore cohort (72 with the least ambiguous subtype classification) were used to generate the immune-related signatures (Figures 1,2) to classify the OCCC samples from the two validation cohorts. In the ~~two~~ validation cohorts, 91.8% of the OCCC samples could be assigned to one of the four subtypes with 13 samples (Japan: 6; UK: 7) remaining unclassified (Figure 1). In total 242 samples from Singapore ($n$ = 96), Japan ($n$ = 70) and UK ($n$ = 76) were successfully classified (Figures 1, 2). Among them, 42.5% of women were classified into AP ($n$ = 103), 28.9% into CTLA4-high ($n$ = 70), 17.7% into ProA ($n$ = 43), and 10.7% into PD1-high ($n$ = 26) (supplementary material, Table S3). Unclassified samples (Figure 2, grey samples) were subsequently removed from the downstream analysis, yielding a final analysis of 242 irGES profiles with their related clinical and prognostic outcomes.

**irGES profiling correlates with distinct clinical features.**

Correlation of irGES profiles with patient demographics

Information on patient characteristics were subsequently correlated with irGES profiles and we observed no discernible differences in age of disease onset and stage of disease based on irGES profiles between the three cohorts (supplementary material, Figure S2).

Clinical outcomes based on irGES profiles

Kaplan–Meier analyses of overall survival (OS) and disease-free survival (DFS) were generated using the corresponding survival information available. The PD1-high and CTLA4-high subtypes had poorer outcomes when compared to the other subtypes (Figure 3). The 5 year-DFS for the PD1-high patients was almost half that of the other subtypes at 28% and 50.5% respectively (Hazard Ratio, HR = 1.93; $p$ = 0.06) (Figure 3). In the CTLA4-high, 5-year DFS was

40.8% compared to 51.6% in the other subtypes (HR = 1.49; $p$ =0.063). Conversely, women categorised to the AP subtype had significantly better outcomes compared to the other subtypes, with a median DFS that was more than twice as long as for other subtypes (76.7 versus 34.2 months, $p$ = 0.019, supplementary material, Figure S3). There was no observable difference in the OS and DFS outcomes of women categorised to the ProA subtype compared with other subtypes (Figure 3 and supplementary material, Figure S3).

In a univariate analysis (supplementary material, Table S4), Stage I tumours were associated with better survival ($p < 0.001$), consistent with previous reports [3]. In multivariate analyses, the PD1-high subtype was an independent predictor of poorer OS, even after adjusting for age and stage, with a HR = 2.1 (95% CI 1.14 – 3.88; $p < 0.018$) (supplementary material, Table S5). Five-year OS and DFS rates in women of the PD1-high subtype were around two times poorer compared to the other subtypes (5-year OS: HR 2.1, $p$ = 0.07 and 5-year DFS: HR 1.9; $p$= 0.06), respectively; Figure 3). Both AP and ProA subtypes showed a non-significant trend toward improved OS after accounting for age and stage, with a HR of 0.71 ($p$ = 0.10) and HR of 0.74 ($p$ = 0.27), respectively (supplementary material, Table S5).

Of the 33 women with Stage IA/IB disease, 21 (63.6%) women received adjuvant chemotherapy. Of these, 10 women (30.3%) were classified to the AP, 4 (12.1%) to the CTLA4-high, 7 (21.2%) to the ProA, and none to the PD1-high subtypes (Figure 4 and supplementary material, Table S6).

The rates of first line chemotherapy were higher in patients with stage IC–IV disease with 164 (88.2%) receiving adjuvant chemotherapy while only 22 (11.8%) patients did not. 72/186 (38.7%) women were in AP, 51/186 (27.4%) women in CTLA4-high, 15/186 (8.1%) women in PD1-high and 26/186 (14.0%) women in ProA (supplementary material, Table S6). For the 186 Stage IC - IV OCCC, both the poor prognosis PD1-high and CTLA-4 high, appeared to derive significant OS benefit from adjuvant chemotherapy compared to the other subtypes (HR = 0.09, $p$ = = 0.027; and HR = 0.08, $p$ = 0.0005 respectively; Figure 4). DFS showed a similar benefit following adjuvant therapy for PD1-high (HR = 0.13, $p$ = 0.048) and CTLA4-high (HR = 0.21, $p$ = 0.01; Figure 4).

MMR status based on irGES profiles

It is recommended that all women with OCCC, endometrioid or mucinous ovarian cancer should be offered somatic tumour testing for mismatch repair deficiency (dMMR) [22]. IHC for four mismatch repair (MMR) proteins (MSH2, MSH6, MLH1, PMS2) was performed successfully on 240 out of 242 (99.2%) OCCC samples (Figure 5A). When compared within each cohort, the proportion of MMR deficient patients were similar across the three cohorts with 6% ($n$ = 6) from Singapore, 6% ($n$ = 4) from Japan, and 5% ($n$ = 4) from UK (Figure 5A,B). The patterns of MMR protein loss were variable in our study. Two patients had loss of both MLH1/PMS2, whereas one had loss of only PMS2. Loss of both MSH2 and MSH6 occurred in five patients while the remaining five patients had loss of MSH6 protein only (supplementary material, Table S7). A total of fourteen women (5.8%) from Singapore, Japan and UK were observed to have loss of MMR proteins with all subtypes included except for ProA which showed 100% retained MMR expression across the three cohorts (Figure 5C). Strikingly, our data showed a significant enrichment of MMR-deficient tumours in the CTLA4-high subtype ($p$ = 0.016) compared to the other subtypes (Figure 5D). Eight (57.1%) MMR-deficient patients were categorised as CTLA-4 high, three (21.4%) as AP and three (21.4%) as PD1-high (supplementary material, Table S7).

**Discussion**

Of our 255 women with available clinical data, more than half the patients (54.6%) had Stage I disease ($n$ = 139) consistent with prior studies [23,24). Despite the small numbers, our study demonstrated overall good survival outcomes in Stage I OCCC of 83% 3 year-OS (supplementary material, Figure S4) similar to studies published previously [5,24].

Several studies have reported significant variation in prognosis of Stage I disease [25,26]. Our study identified that the PD1-high subtype conferred a less favourable prognosis with 3-year DFS of 64.2% and 3-year OS of 75% despite presenting with Stage I disease (supplementary material, Figure S4). The outcomes of Stage I PD1-high women appeared to be comparable to Stage IC patients with surface involvement, ascites or positive cytology which was shown to confer a 3-year DFS and OS rate of 56.4% and 71.9%, respectively in one study [25]. Historically, due to concerns relating to prognosis and limited data on outcomes with surgery alone, patients with low stage OCCC beyond FIGO stage IA/C1 would be recommended post-operative chemotherapy. Our data demonstrate that, women with Stage1C-IV OCCC who fall into either PD1-high or CTLA-4-high subtype would derive significant survival benefit with the addition of

adjuvant chemotherapy (Figure 4). However, we do not know whether women with Stage IC disease with surface involvement or positive cytology classified to the other irGES subtypes would derive benefit from adjuvant chemotherapy.

Few reports have investigated associations between expression of immune-related molecules and clinical features, particularly for OCCC. Mariya *et al* reported HLA Class Ⅰ expression and its association with T cell-infiltration and prognosis of EOC. They concluded that high expression of HLA class Ⅰ was a good prognostic marker of EOC. However, they also reported that OCCC had lower CD3 and CD8 T cells, which did not impact on patients survival outcome [27]. In addition, Tan *et al* categorized OCCC into two gene expression subtypes in relation to the individual epithelial-mesenchymal transition states, namely epithelial and mesenchymal OCCC. The later had a poorer prognosis and was enriched in immune-related genes such as genes associated with antigen processing and presentation [15]. Our study is the first to include both Asian and Caucasian women with OCCC, which could shine a light on the impact of racial differences in the immune microenvironment of OCCC. In our data, the PD1-high and CTLA4-high subtypes were associated with poorer 5-year DFS and OS outcomes (Figure 4).

In a case report by Bellone *et al*, an exceptional complete response to pembrolizumab was described in a woman with OCCC following disease refractory to several prior lines of treatment. This patient had increased aberrant expression of PD-L1 following a gain of function structural variant disrupting the 3'-region of the *PD-L1* (*CD274*) gene [28]. This was consistent with a eparate study which found marked elevation of aberrant *PD-L1* transcripts secondary to truncation of their 3′-UTR [29]. A different study found that OCCC patients with better prognosis had higher expression of HLA class I genes, and those with poorer outcomes had higher ratios of PD-1/CD8 or CTLA-4/CD8 [14]. These data support our results suggesting expression of antigen presentation genes may confer a better prognosis and overexpression of immune checkpoint molecules, particularly PD-1 and CTLA-4, may confer a poorer outcome in women with OCCC.

Our study found that dMMR was infrequent with 14 out of 240 (5.8%) OCCC demonstrating dMMR similar to other reports [30]. Leskela *et al* demonstrated the loss of both MLH1 and PMS2 proteins was the most frequently observed pattern of dMMR across all histological types of EOC [30]. However, this pattern was not observed in our OCCC cohorts where patterns of MMR protein loss were spread out with no particular pattern standing out. We also observed that the CTLA4-high subtype had the most women with deficient MMR status

(Figure 5 and supplementary material, Table S7). OCCC with microsatellite instability molecular profiles have been shown to be associated with a high number of CD8+ TILs with higher PD-1 expression compared to microsatellite stable (MSS) tumours [14], suggesting that perhaps patients of the CTLA4-high subtype may derive additional benefit from immune checkpoint inhibition.

Finally, our collaborative analyses from different ethnic backgrounds revealed similarities between OCCC from the Asian and Caucasian populations. Considering transcriptomes, there were great similarities, with the majority of tumours clustering within the four irGES subtypes (Figure 1). Clinically, the demographics of the women who presented with OCCC were fairly similar, with patients in the UK observed to be slightly older with a median age at diagnosis of 61 years compared to the Asian population (52 in Singapore; 57 in Japan; Table 1). The Stage of disease at diagnosis was also similar between the 3 cohorts although the Japanese population had a slightly higher proportion of patients with early-stage disease (Table 1). This data is reassuring as we aim to understand the drivers and susceptibilities of this rare disease; we are only able to do so through extensive collaborative efforts from around the globe. Despite their similarities, the irGES subtypes across the three cohorts were not fully concordant, in particular, we observed a higher incidence of the PD1-high subtype in our Singapore cohort compared to the rest. While this difference could be attributed to population differences, it could also arise from the difference in patient demographics or sample handling and processing. Nevertheless, the addition of our data to the taxonomy of OCCC warrants further exploration as it allows classification of women based on irGES into distinct prognostic groups and has the potential to predict treatment responses especially with immunotherapeutic strategies. However, the utility of irGES clustering in OCCC, will require further prospective validation.

In conclusion, our data affirms that OCCC are molecularly and clinically similar in the Asian and Caucasian populations. We also confirm that early-stage OCCC has a favourable prognosis, but women who fall into the PD1-high subtype have similar prognostic outcomes to women with Stage IC-2/3 disease. Future OCCC-specific studies are crucial and will require multi-group collaboration to translate our growing knowledge of OCCC molecular features into intelligently designed clinical trials.

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**Author contributions statement**

RYH, KH, CG, DSPT and AO supervised the study. RYH, TZT, DSPT, KH, CG and VH designed and conceptualized the study. JY performed sample collection and experiments. NN, MM and YI participated in data collection and methodology of the study. DL, CSH, M Yasuda, M Yano and JW processed and reviewed samples and FFPE. TZT performed the bioinformatics analysis. RYH, TZT, VH, DSPT, KH, CG, CSH, JW and MM analysed the data, interpreted the results and participated in writing, reviewing and editing of the manuscript. All authors approved the final version of the manuscript

**Data availability statement**

The NanoString Immune Panel profiling of the ovarian clear cell carcinoma has been deposited in Gene Expression Omnibus (GEO) with the accession ID GSE128990.

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## Figure legends

**Figure 1. Workflow of the sample processing pipeline.**

**Figure 2. Clustering of immune related gene expression signatures (irGES) from Singapore, Japan and UK into four immune subtypes**. Heatmap shows the clustering expression data derived from 96 Singapore samples and validated in the Japan and UK cohort. OCCC were robustly clustered or classified into four *k*-means groups; PD1-high, CTLA4-high, Antigen-Presentation (AP) and Pro-angiogenic (ProA). Average linkage hierarchical clustering using a Pearson correlation metric was used to cluster genes based on relative expression.

**Figure 3. Five-year clinical outcomes of women with OCCC in each irGES subset compared to the rest of the cohort**. Top row: 5-year overall survival and lower row: 5-year disease free survival of PD-1 high (blue) compared to the non-PD-1 high subset (black), CTLA4-high (green) compared to the non-CTLA4-high (black), Antigen-presentation subset (red) compared to the non - Antigen-presentation (black) and Pro-angiogenic subset (brown) compared to the non-Pro-angiogenic (black).

**Figure 4. Clinical outcomes of Stage 1 OCCC women based on their irGES profiles and receipt of adjuvant treatment**. Kaplan–Meier plots showing (upper row) overall survival and (lower row) disease free survival outcomes of women with stage IA/IB OCCC and stage IC - IV based on their irGES and whether or not they received adjuvant chemotherapy.

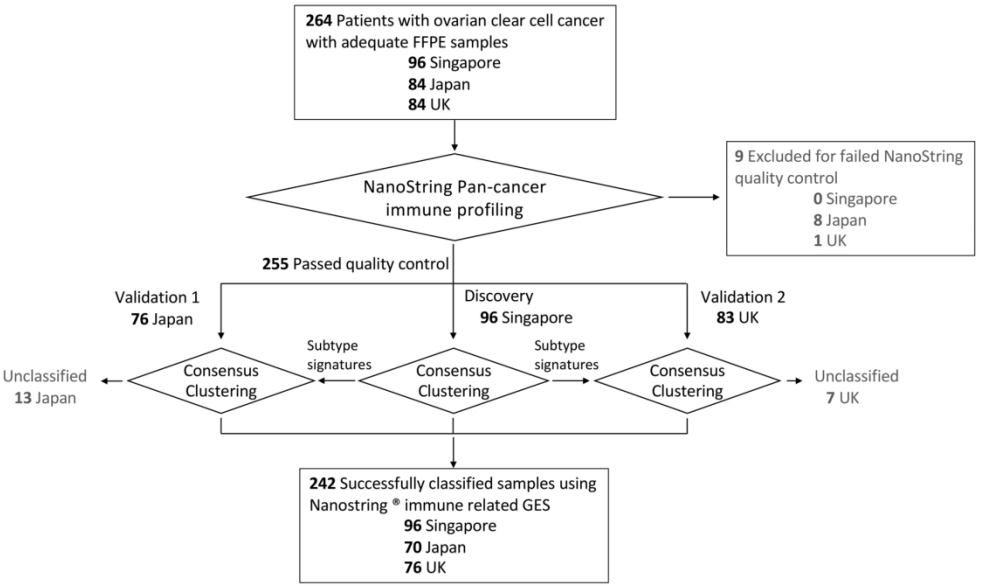
**Figure 5. Distribution of retained and deficient MMR proteins in OCCC**. (A) Bar chart showing frequency (*y*-axis) of combined, and of Singapore, Japan, and UK ovarian clear cell carcinoma cohorts (*x*-axis). (B) Bar chart showing frequency percentage (*y*-axis) of retained and deficient mismatch repair (MMR) proteins status assessed using IHC in individual cohorts (*x*-axis). (C) Bar chart depicting the combined analysis of the MMR protein status based on their irGES profiles. (D) Comparison of the MMR protein status specifically in the CTLA4-high subtype relative to the rest of the other subtypes in the combined cohort analysis. Numbers above or within bars indicate the number of samples or the percentage.

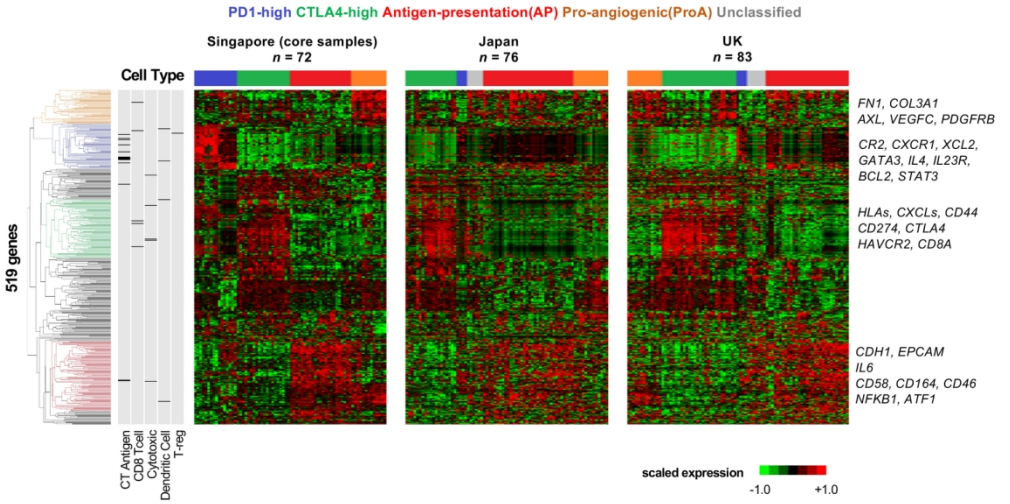
**TABLE**

**Table 1. Demographics of patients and their clinicopathologic characteristics from the three ovarian clear cell cohorts.**

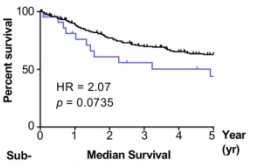
| **Parameter** | **Combined** | **Singapore** | **Japan** | **UK** |
|---|---|---|---|---|
| *n* | 255 (100%) | 96 (100%) | 76 (100%) | 83 (100%) |
| **Age** (Median yr) | 56 | 52 | 57 | 61 |
| **Follow up** (Median months) | 41 | 33.5 | 49 | 41.6 |
| **Stage** | | | | |
| I | 139 (54.6%) | 46 (48%) | 50 (65.8%) | 43 (51.8%) |
| IA/ IB | 40 (15.7%) | 16 (16.7%) | 9 (11.8%) | 15 (18.1%) |
| IC | 99 (38.9%) | 30 (31.3%) | 41 (54%) | 28 (33.7%) |
| IC-1 | 27 (10.6%) | NA | 20 (26.3%) | 7 (8.4%) |
| IC-2 | 8 (3.1%) | NA | 5 (6.6%) | 3 (3.6%) |
| IC-3 | 34 (13.3%) | NA | 16 (21.1%) | 18 (21.7%) |
| II, III, IV | 113 (44.3%) | 47 (49%) | 26 (34.2%) | 40 (48.2%) |
| NA | 3 (1.2%) | 3 (3.1%) | 0 | 0 |
| **Clinical outcomes** | | | | |
| Overall median survival (months) | 48.4 | 60.5 | 49 | 41.6 |
| Disease-free median survival (months) | 36.6 | 35 | 29.2 | 31.7 |
| **Debulking** | | | | |
| No residual disease | 186 (72.9%) | 60 (62.5%) | 66 (86.8%) | 60 (72.3%) |
| Optimal (< 1cm) | 23 (9.2%) | 7 (7.3%) | 2 (2.6%) | 14 (16.9 |
| Suboptimal (≥ 1cm) | 22 (8.6%) | 5 (5.2%) | 8 (10.5%) | 9 (10.8%) |
| NA | 24 (9.4%) | 24 (25%) | 0 | 0 |
| **Adjuvant therapy** | | | | |
| Yes | 197 (77.3%) | 66 (68.8%) | 64 (84.2%) | 67 (80.7%) |
| No | 38 (14.9%) | 10 (10.4%) | 12 (15.8%) | 16 (19.3%) |
| NA | 20 (7.8%) | 20 (20.8%) | 0 | 0 |
| **Therapy** | | | | |
| Platinum-containing | 184 (72.2%) | 66 (68.8%) | 62 (81.6%) | 61 (73.5%) |
| Taxol-containing | 137 (53.7%) | 59 (61.5%) | 45 (59.2%) | 33 (39.8%) |
| Radiotherapy | 6 (2.4%) | 0 | 0 | 6 (7.2%) |

NA, not available.

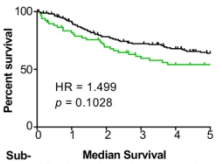




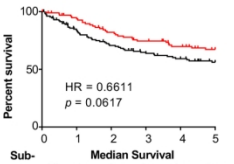
**Overall Survival** PD1-high CTLA4-high Antigen-presentation(AP) Pro-angiogenic(ProA) Rest of the immune subtype



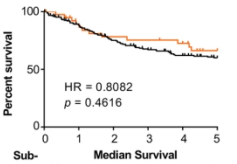

| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | --- | 89 | 77.2 | 69.8 | 65.3 | 62.8 |
| PD1 | --- | 80.2 | 58.8 | 55.8 | 50.2 | 43.9 |




| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | --- | 90.9 | 78.2 | 72.2 | 67.9 | 63.8 |
| CTLA4 | --- | 81.9 | 69.4 | 59.6 | 54.2 | 54.2 |

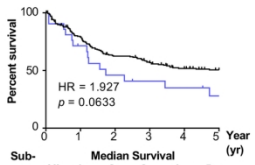



| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | --- | 83.9 | 70.5 | 63.9 | 59.2 | 56.2 |
| AP | --- | 93.8 | 82.1 | 74.5 | 69.8 | 67.2 |

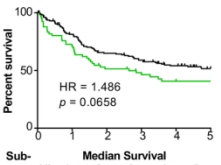



| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | --- | 88 | 75 | 67.1 | 62.1 | 60 |
| ProA | --- | 89.4 | 78.2 | 75.4 | 72.5 | 66.2 |

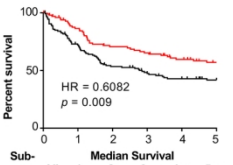
**Disease-Free Survival**



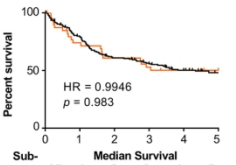

| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | --- | 79.1 | 62.5 | 56.8 | 51.8 | 50.5 |
| PD1 | 20.8 | 71.4 | 45.9 | 40.8 | 35 | 28 |




| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | --- | 81.4 | 65 | 57.6 | 54.1 | 51.6 |
| CTLA4 | 31.7 | 71.2 | 51.4 | 46.4 | 40.8 | 40.8 |




| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | 34.2 | 72.1 | 53.5 | 47.5 | 42.8 | 41.7 |
| AP | --- | 86.5 | 70.6 | 65.3 | 59.7 | 57.1 |




| Sub-type | All | 1yr | 2yr | 3yr | 4yr | 5yr |
|---|---|---|---|---|---|---|
| Rest | --- | 79.3 | 61 | 55.8 | 50.2 | 48.1 |
| ProA | --- | 74 | 60.8 | 52.9 | 50.2 | 50.2 |