**A patient stratification signature mirrors the immunogenic potential of high grade serous ovarian cancers**

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**Abstract**

High-grade serous ovarian cancer (HGSC) responds poorly to immunotherapy on the whole, and the diverse molecular and cellular factors that govern its immunogenicity are beginning to emerge. Using genomic strategies, we investigated the existence of HGSC subclasses that may be more resistant or susceptible to anti-tumor immunity. We developed an algorithm, CONSTRU, to identify gene expression states that influence the prognostic performance of an immune cytolytic activity signature (CYTscore). From the identified genes, we developed a patient stratification signature (STRATsig) capable of segregating HGSC populations into patient tertiles that varied markedly by CYTscore survival-protective effect. In multiple validation cohorts, measures of immune suppression, evasion and dysfunction varied significantly across STRATsig tertiles. Tumors comprising STRATsig tertile 1 (S-T1) showed no immune-survival benefit and displayed a hyper-immune suppressed state marked by activation of TGF-beta, Wnt/β-catenin and CD73/CD39 adenosine-mediated immunosuppression pathways, with concurrent T cell dysfunction, reduced potential for antigen presentation, and enrichment of cancer-associated fibroblasts. By contrast, S-T3 tumors exhibited a diminished immunosuppressive signaling, heightened antigen presentation machinery, lowered T cell dysfunction, and an immune-survival benefit that correlated with persistent TMB, consistent with anti-tumor immunoediting. These tumors also showed elevated activity of DNA damage/repair, cell cycle/proliferation and oxidative phosphorylation, and displayed greater proportions of Th1 CD4+ T cells. In these patients, predictors of immunotherapy response were prognostic of longer survival. STRATsig is a composite of parallel immunoregulatory pathways that mirrors the immunogenic potential of HGSC. The fraction of patients that classify as S-T3 may show heightened responsiveness to available immunotherapies.

**Statement of Significance**

A patient stratification signature can delineate novel HGSC subpopulations that differ significantly by immunogenic potential. Approximately a third of HGSC tumors display a hypo-immunosuppressed and antigenic molecular composition that favors immunologic tumor control.

**BACKGROUND**

Ovarian cancer is the leading cause of gynecologic cancer-related death in developed countries and the 5th leading cause of cancer death among women in the US1,2. The most common subtype of epithelial ovarian cancer (EOC) is high grade serous ovarian cancer (HGSC) which accounts for three quarters of all ovarian cancer cases and is largely responsible for the high rates of cancer-related deaths. The vast majority of HGSC patients present with Stage III or IV disease. A combination of aggressive surgery and platinum-based chemotherapy has remained the standard primary treatment over the last three decades, yet these strategies are associated with high rates of recurrence and low rates of long-term survival. Recurrent disease after surgery and chemotherapy is inevitable and incurable for 75% of patients, leading to a poor five-year survival of approximately 30% for advanced stage disease at diagnosis3. There remains a critical need for more effective treatments in both the upfront and recurrent settings.

Immunotherapy is an attractive treatment which has had success in other solid tumor disease sites such as skin, lung, endometrial, breast, colorectal, and urologic cancers4-8. There has been interest in the use of immunotherapy in HGSC, as the levels of tumor infiltrating lymphocytes (TILs) and specific TIL subsets have been significantly correlated with survival9. HGSC response to immunotherapy, however, has been disappointing thus far, with overall response rates (ORR) of 8-28%10,11 with most ORRs being < 15%. Interestingly, it has been reported that patients who do respond experience durable responses of greater than 6 months12, and multiple case reports describe remarkable, durable responses to single agent immunotherapy13,14. These findings may point to the existence of a fraction of HGSC with a molecular disposition capable of eliciting effective anti-tumor immunity.

Biomarkers such as tumor mutational burden (TMB), PD-L1 expression, deficiency in mismatch repair (MMR), and the presence of microsatellite instability (MSI) have emerged as clinically relevant predictors of immunotherapy efficacy15. HGSC, however, does not have a particularly high TMB16, rarely harbors MMR gene deficiency or high MSI17, and PD-L1, even when elevated, has not proven to be a reliable predictor of immunotherapy response in HGSC10. HGSC immunogenicity may depend on immune activating and suppressing signals that we do not yet fully understand18.

TIL abundance and activity, as determined by tumor histopathological assessment, has been associated with immune-mediated survival in many solid tumor types19-21, including HGSC22. Recent reports by us and others23-26 have leveraged large-scale tumor gene expression profiling studies to identify immune gene signatures that quantify the relative abundance and functional orientation of distinct TIL populations. A number of these signatures, and those that reflect TIL biology in particular, have demonstrated robust associations with both patient survival and immunotherapy response in a variety of cancer types, sparking interest in their use as clinical biomarkers of immune checkpoint inhibitor (ICI) efficacy27-29. Recently, a quantitative gene-based measure of immune cytolytic activity was devised for studying relationships between anti-tumor immunity, gene mutations and mechanisms of tumor resistance30. Known as the CYT gene signature (CYTscore), it is based simply on the transcript levels of two key cytolytic effectors, Granzyme A (GZMA) and Perforin 1 (PRF1), which are both dramatically upregulated upon effector cell activation. In a series of reports on the prognostic power of CYTscore, significant associations with patient overall and disease-free survival were observed in multiple cancer types31-35, indicative of its utility as a biomarker of anti-tumor immune effectiveness.

The discovery of survival-associated biomarkers in patient populations has historically depended on analyses that, by default, are “all-inclusive” with respect to the population. In subsequent secondary analyses conducted within a population’s subgroups (such as early stage vs. late stage), insights into the clinico-prognostic relevance of a biomarker may be further elucidated. However, approaches that aim to uncover *de novo* patient subgroups, with no prior annotation, that discriminate between most significant and non-significant biomarker-survival associations, have not been described. Such strategies could reveal novel patient subgroups that are *selection-optimized* for a biomarker, and for which actionable clinico-prognostic insights may be revealed.

In this study, we hypothesized that HGSC exists in varying states of immunoregulation that are not yet clinically distinguishable, and that these states may be discoverable through a bioinformatics approach that can stratify patients according to differences in CYTscore-driven, immune-mediated survival benefit. Here, we demonstrate the existence of previously unrecognized subpopulations of HGSC that differ by potential for immunologic tumor control.

**RESULTS**

**CONSTRU identifies genes that alter the prognostic performance of the CYTscore signature**

Gene expression profiles of primary HGSC were analyzed for associations between immune effector cell function and patient overall survival (OS). We used a previously published gene signature, CYTscore, that quantifies intratumoral effector cell cytolytic activity30, to assess the potential survival benefit of anti-tumor immunity in multiple large, independent HGSC patient populations (**Table 1**). First, cases were stratified into tertiles based on tumor CYTscore values, then survival differences among the tertiles was assessed by logrank test (**Additional File 1:** **Fig. S1A-F**). Cox models were then used to assess CYTscore prognostic effect when analyzed as a continuous variable while adjusting for patient age, International Federation of Gynecologic Oncology (FIGO) stage and tumor debulking status (**Additional File 1: Fig S1G**). While associations were not consistently observed, higher CYTscore values tended to correlate incrementally with improved OS, reminiscent of previous reports associating immune gene signatures with HGSC survival36-39.

Multiple studies have shown that the prognostic and predictive power of immune signatures may be restricted to distinct tumor subgroups with favorable immunogenic properties27,40,41. We therefore considered the possibility that a hidden subclass of HGSC might exist for which effector cell activity would associate robustly with survival benefit. To uncover such a subtype, we developed a statistically guided data mining approach to screen genome-wide expression profiles for genes whose expression levels impact the prognostic performance of the CYTscore. Termed CONSTRU (Computing Prognostic Marker Dependencies by Successive Testing of Gene-Stratified Subgroups), the algorithm measures how the relative expression state for each gene (e.g., low, intermediate or high) in a typical RNAseq or microarray data matrix enhances or antagonizes the statistical association between a signature score and patient survival (**Fig. 1A**). In its current implementation, each gene of a HGSC data matrix was used to stratify patients into tertile groups based on the gene’s relative expression level (see Methods). Then within each tertile, a multivariable Cox model was fitted to compute the significance (p-value) and directionality (hazard ratio) of the association between the CYTscore and patient survival. This analysis was performed iteratively for each gene in the matrix. As shown in **Fig. 1B**, the output is an array of gene tertile-based Cox model statistics that can be ranked by a parity score to enable the sorting of genes into two desirable categories:

1) Genes whose *low* expression is uniquely conditional for a significant positive association between the CYTscore and OS (i.e., “LowerT” genes)

2) Genes whose *high* expression is uniquely conditional for a significant positive association between the CYTscore and OS (i.e., “UpperT” genes)

We applied CONSTRU to two cohorts OV1 and OV2; the results were compared for top-performing genes. In each cohort, the parity score was used to assign percentile ranks across all genes. Using different upper and lower bound percentile-rank thresholds, we identified high-ranking UpperT and LowerT genes common to both cohorts (**Fig. 1C-E**). For these genes, we evaluated the relationship between their gene tertiles (T1, T2, T3) and corresponding CYTscore-survival hazard ratios (HRs). The significance of these relationships was tested using a third independent HGSC cohort, OV3 (**Table 1**). As shown in **Fig. 1C**, using the top 2-percentile cutoff, 4 and 11 genes were found to overlap in the OV1 and OV2 UpperT and LowerT gene lists, respectively. As designed, the UpperT gene HRs (left panel) were smaller (i.e., associated positively with survival) in upper tertile T3 than the same genes’ HRs in the lower tertile T1, whereas the LowerT gene HRs (right panel) were smaller in T1 as that of T3. When assessed in the independent OV3 cohort, these relationships were reproducible, whereby the hazard ratio distributions for these genes remained significantly different between the T3 and T1 tertiles of the selected genes (UpperT genes, *P* < 0.001; LowerT genes, *P* < 0.001). Furthermore, these relationships remained significant irrespective of increasing numbers of UpperT and LowerT genes, as selected using the 4th and 6th percentile cutoffs (**Fig. 1D, E**). These findings support the hypothesis that in HGSC, the relative low or high expression states of certain genes are reproducibly associated with the prognostic performance of an effector cell cytolytic activity signature.

**A patient stratification signature (STRATsig) delineates transcriptomic subtypes that differ by CYTscore prognostic performance**

We next sought to integrate the unique properties of CONSTRU-selected genes for the development of a multi-gene signature for patient stratification. We first examined the internal correlation structure of UpperT and LowerT gene groups. The majority of genes showed positive correlation with one another within groups, while negative correlation predominated between groups. For each tumor, we leveraged this correlation structure by computing UpperT and LowerT gene signatures scores, defined as the geometric mean of genes comprising each signature. We ultimately selected UpperT and LowerT genes by the 4th-percentile cutoff (**Fig. 1D**) on the basis of the tradeoff between being highest-ranking genes and being sufficiently numerous in gene count so as to be robust against errors inherent to individual gene measurements (n=13 UpperT genes, n=27 LowerT genes). The UpperT and LowerT signatures were evaluated in the training and test cohorts for their abilities to stratify patients into tertiles that differed by CYTscore prognostic power. In the combined training dataset (OV1 and OV2 combined), higher CYTscore values associated significantly with longer overall survival only in the upper tertile of patients stratified by the UpperT signature (**Fig. 2A**) and the lower tertile of patients stratified by the LowerT signature (**Fig. 2B**). In the test cohort (OV3), these same relationships were observed (**Fig. 2D, E**) illustrating the reproducibility of the gene signatures.

The two signatures were then combined into a single stratification signature by subtracting the LowerT score from the UpperT score (to generate the ratio of UpperT to LowerT). As shown in **Fig. 2C and F**, this combined signature (termed STRATsig) stratified cases of the training and test cohorts into tertiles that differed markedly with respect to CYTscore prognostic significance. Higher STRATsig scores defined a patient upper tertile (T3) conditional for a significant positive CYTscore-survival association, while no positive association was observed in STRATsig tertiles T1 and T2, though an inverse CYTscore-survival association was observed in T1. To determine the reproducibility of these observations, we examined three independent HGSC cohorts for which expression profiles and clinical outcomes were available (OV4, OV5 and OV6). For each cohort, we allowed the expression data to be processed and normalized according to the authors’ published methods. We then computed their STRATsig and CYTscore tertiles and tested for CYTscore-survival associations. In all three cohorts, CYTscore achieved statistical significance only in patients comprising STRATsig (S)-T3 (**Fig. 2G-I**). Additionally, we further confirmed this finding by using individual gene surrogates for STRATsig and CYTscore in an analysis involving >3,700 HGSC cases from the Ovarian Tumor Tissue Analysis (OTTA) consortium study38,42 (**Additional File 1: Fig. S2**). Together, these findings confirm the broad reproducibility of the S-T3 CYTscore-survival relationship.

Next, we tested whether the CYTscore-survival association of S-T3 was influenced by conventional prognostic factors. We merged STRATsig and CYTscore assignments and clinical annotations of OV4, OV5 and OV6 (**Fig. 3A**), and examined CYTscore in the STRATsig tertiles after separating cases based on FIGO stage, surgical debulking status and patient age (**Fig. 3B-G**). In S-T3, the CYTscore remained significantly associated with OS independent of stage, debulking status and age. In parallel, we performed multivariable Cox regression analysis to evaluate CYTscore as a continuous variable in the presence of stage, debulking status and age. For this analysis we used the ComBat empirical Bayes method43 for batch correction to combine tumor expression profiles of OV4, OV5 and OV6 into one data set (n=646). We used this integrated data set (referred to hereafter as the test group) to re-compute CYTscore and STRATsig values. Consistent with initial findings, the CYTscore remained significantly associated with survival in S-T3 (*P* < 0.0001) after adjusting for stage, debulking status and age, but did not achieve significance in the first or second STRATsig tertiles (*P* = 0.64 and *P* = 0.14, respectively) (**Table 2**). Sub-optimal surgical debulking was the only variable which remained significantly associated with poor survival in all three STRATsig tertiles.

Finally, we considered the impact of HGSC molecular subtypes – Immunoreactive (IMR), Differentiated (DIF), Proliferative (PRO) and Mesenchymal (MES). While STRATsig tertiles varied with respect to molecular subtype composition (**Additional File 1: Fig. S3A-F**), the CYTscore survival associations could not be explained by molecular subtype (**Additional File 1: Fig. S3G, H**). These findings suggest that the CYTscore-survival association observed in S-T3 occurs independent of currently understood prognostic variables in HGSC.

**STRATsig tertiles differ by pathways of immune suppression and evasion**

The CYTscore-survival association that exists in S-T3 but not in lower tertiles may reflect tertile-dependent differences in immune regulatory potential. Therefore, we tested whether biological pathways or processes operative in tumors might vary with respect to the tertiles. For each of the six cohorts, genes were ranked by their correlation with STRATsig values, and gene ontology enrichment analysis44 was performed on the top 2% of the most positively, or negatively, correlated genes (**Fig. 4A**). In parallel, we used integrated training and test groups (ie, OV1, OV2 and OV3 in training group (n = 880); OV4, OV5 and OV6 in test group (n = 647)) to quantify activation levels of 54 cancer-related pathways within both STRATsig and CYTscore tertiles (**Fig. 4B**). From these analyses, a number of significant pathways associated with immune suppression emerged as common themes that could reproducibly differentiate S-T1 and S-T3 populations. Enrichment for *TGF-beta signaling*, *Wnt signaling*, and *angiogenesis* was associated with higher expression in S-T1, in all six cohorts (**Fig. 4A**). Similarly, pathway activation scores for *TGF-beta*, *Wnt, angiogenesis* and *hypoxia/adenosine-mediated immune suppression* were significantly higher in S-T1 relative to S-T3 (**Fig. 4B, D**). Importantly, these differences remained significant within the CYT-Hi fractions of S-T1 and S-T3 (**Fig. 4B**, right panel). The CYT-Hi groups, which have uniformly high CYTscore values that do not differ across STRATsig tertiles (**Additional File 1: Fig. S4A, B**), display significant survival differences, particularly when comparing S-T1 CYT-Hi to S-T3 CYT-Hi, where the average median survival times are 3.2 years and 7.2 years, respectively (**Additional File 1: Fig. S4C, D**).

The inferior survival of S-T1 CYT-Hi may reflect a heightened immunosuppression that potentiates a more severe immune dysfunction. To test this hypothesis, we employed a transcriptomic measure of T cell dysfunction29 to compare the CYTscore groups of S-T1 to those of S-T3. As shown in **Fig. 4C** (top panel), in both training and test sets T cell dysfunction scores were significantly higher in the CYTscore (CYT)-Mid and Hi groups of S-T1 as compared to their counterparts in S-T3. This finding associates T cell dysfunction with the reduced patient survival of the S-T1 CYT-Hi group.

Next, we assessed the enrichment of pathway activation in S-T3. Significant observations included growth and energy metabolism pathways, including *cell cycle* and *proliferation*, *DNA repair*, and *oxidative phosphorylation* (**Fig. 4A-B, D**). While these observations are consistent with previous reports correlating proliferation41, genomic instability45 and oxidative phosphorylation46 to immune-mediated survival, the exact mechanism through which these pathways contribute to tumor immunogenicity is not yet clear. A common mechanism of immune evasion in HGSC47 in ovarian cancer is the transcriptional repression of antigen processing and presentation machinery (APM) genes, resulting in lowered immunogenicity. As such, we compared the immunogenic potential of the CYTscore groups in S-T1 and S-T3 using an APM gene signature predictive of ICI response in melanoma48. The APM signature was significantly elevated in S-T3 CYTscore groups as compared to their cognate S-T1 CYTscore groups (**Fig. 4C**, middle panel), demonstrating an association between elevated APM expression and the immune-mediated survival benefit of S-T3 CYT-Hi. At a more granular level, IRF1, a major transcriptional activator of APM genes, was also significantly overexpressed in the CYTscore groups of S-T3 as compared to S-T1 (**Fig. 4C**, bottom panel) and this association extended to a number of core APM genes including TAP1, TAPBP, TAPBPL, PSMB8, PSMB9, PSMB10, PSME1 and PSME2 (**Additional File 2: Table S1**). In a similar vein, individual genes representative of immunosuppressive pathways also showed significant and reproducible differences between S-T1 and S-T3 (**Additional File 1: Fig. S5**), particularly with respect to overexpression in S-T1 CYT-Hi relative to S-T3 CYT-Hi including TGF-beta (TGFB1, TGFB3), drivers of adenosine-mediated immune suppression (NT5E (CD73), ENTPD1 (CD39)), Wnt pathway ligands (WNT7A, WNT4), IL10, and drivers/markers of cancer-associated fibroblast activation (INHBA, WNT7A, TGFB1, FAP, PDGFRA, PDGFRB)49-51 (**Additional File 2: Table S1**).

Together, these findings suggest that tumors comprising the lower STRATsig tertile are molecularly configured toward a hyper-immune suppressed state marked by the parallel activation of multiple immunosuppressive pathways, with concurrent T cell dysfunction and impaired antigen presentation. By contrast, tumors comprising the upper STRATsig tertile exhibit alterations consistent with a diminished immune suppression, a more effective antigen presentation and reduced T cell dysfunction.

We next sought to determine if other immunogenic factors, such as tumor mutational burden (TMB) or cellular components of the TME, vary across STRATsig tertiles. We examined the relationship between TMB and the STRATsig tertiles using the OV1 cohort, which was previously characterized by whole-exome sequencing52. We hypothesized that tumors which have evolved toward a hyper-immune suppressed state would be less subject to immunological constraints that would otherwise limit the accumulation of antigenic mutations. Consistent with this hypothesis, TMB levels and the levels of *loss-prone* mutations were significantly higher in S-T1 tumors as compared to S-T3 tumors (TMB: Mann–Whitney *P* = 0.002; Loss-prone TMB: *P* < 0.00001) (**Fig. 4E, F**). Niknafs and colleagues recently showed that TMB can be resolved into two immunogenic mutational classes, termed “loss-prone” and “persistent”, with differing immunological and clinical relevance53. Loss-prone mutations were defined as those that occur in diploid chromosomal regions, and are thus amenable to editing by cancer cells via chromosomal loss. By contrast, persistent mutations were defined as those that attract anti-tumor immune responses, but cannot be edited without lethal consequences (e.g., mutations occurring in haploid regions in linkage with essential genes) or because they exist in multiple copies that are unlikely to be simultaneously edited within a cell. Unlike loss-prone mutations, persistent mutations were shown to accumulate as tumors evolve under immune selective pressure, and the fraction of TMB consisting of persistent mutations (termed ‘pTMB’) was shown to be a more robust predictor of immunotherapy response in patients than TMB alone53. On this basis, we compared pTMB distributions across STRATsig tertiles and within CYTscore tertiles. While pTMB did not differ between S-T1 and S-T3 (**Fig. 4G**), we did observe an association with CYTscore that occurred only in S-T3, where pTMB was significantly higher in CYT-Hi tumors as compared to CYT-Lo tumors (Mann–Whitney *P* = 0.03; **Fig. 4H**). Thus, the survival advantage of patients in S-T3 CYT-Hi is associated not only with greater potential for cytolytic activity, but also higher pTMB.

We next investigated the cellular composition of tumors by computing abundance scores for 39 cell types (**Additional File 1: Fig. S6**). Notably, we found that for most of the effector and regulatory cell types examined, no significant associations with STRATsig tertiles were observed. Several cell types, however, did exhibit significant and reproducible tertile associations. Most notably, hematopoietic stem cells (*P < 0.001*), monocytes *(P < 0.001*) and cancer-associated fibroblasts (CAFs) (*P < 0.001*) displayed greater abundance scores in S-T1 tumors, while Th1 CD4+ T cells (*P < 0.001*) exhibited greater abundance scores in S-T3 tumors (**Additional File 1: Fig. S6A-C**). These findings suggest that certain cell populations may contribute to the STRATsig tertile phenotypes.

**STRATsig is an integrator of immunosuppressive pathways and regulates the prognostic power of multiple signatures of anti-tumor immunity**

Given the CYTscore-survival association specific to S-T3, we questioned which was more important: the stratification of patients by STRATsig, or the stratification of patients by immunosuppressive pathway activation scores. We also examined the relevance of the CYTscore itself, in light of other known prognostic and predictive immune effector genes54,55. In both the training and test groups, CYT-survival associations were observed for tertiles derived from pathways such as *Wnt beta catenin signaling* (*P* < 0.01) and *Angiogenesis* (*P* < 0.01) (**Fig. 5A**). However, no individual pathway was able to recapitulate a tertile-specific CYT-survival association of equal or greater significance than that of S-T3 (*P* < 0.0001). With respect to the CYTscore itself, the prognostic attributes of its genes PRF1 and GZMA were not particularly unique. The expression levels of a number of genes with roles in immunological rejection54,55 including CD8A, STAT1, CCL5, CXCL9 and CXCL10 were also associated with survival in S-T3, and with similar or greater statistical significance than that of PRF1 or GZMA (**Fig. 5B**). Moreover, whereas many genes involved in immune response were highly significantly associated with favorable survival in S-T3, no genes were identified as significantly associated with favorable survival in S-T1 after FDR adjustment (**Fig. 5C**). We then compared CYTscore prognostic power to that of two well-characterized immune activation signatures predictive of patient immunotherapy responses: the Immunologic Constant of Rejection (ICR) 25,26,56 and the T cell-inflamed Gene Expression Signature (TCIGEP) 27,57,58. In the STRATsig tertiles of the training and test groups, the prognostic performances of the ICR and TCIGEP signatures were remarkably similar in effect size and significance to that of CYTscore (**Fig. 5D**).

Together, these observations suggest that STRATsig may function as an integrator of multiple parallel immunoregulatory pathways, rather than as a proxy of a single immunoregulatory pathway, and that alternate markers of effector cell function, other than PRF1 and GZMA, also associate with patient survival in the S-T3 population.

**DISCUSSION**

Here, we present a new perspective on the immunological control of high grade serous ovarian cancer, which our findings suggest depends not only on measures of infiltrating effector cells and tumor mutational burden, but on a continuum of tumor-intrinsic immunoregulatory signaling. We demonstrate that the latter can be quantified by a patient stratification signature that serves as a determinant of the potential for immune-mediated survival benefit. Using population tertiles to dissect the meaning of this signature, we found that approximately a third of HGSC tumors are molecularly configured toward a hyper-immune suppressed state (STRATsig tertile 1, S-T1) characterized by the heightened activation of multiple immunosuppressive pathways, with concurrent T cell dysfunction and a reduced potential for antigen presentation. By contrast, the one third of tumors comprising the opposite end of the spectrum (S-T3) appear to exist in a state of diminished immunosuppressive signaling, which coincides with a more intact antigen presentation, a reduced T cell dysfunction, and an immune-mediated patient survival benefit (ie, CYTscore) that positively correlates with persistent TMB.

In comparisons of S-T1 vs. S-T3, as well as S-T1 CYT-Hi vs. S-T3 CYT-Hi, we observed significantly greater activation scores for TGF-beta, Wnt/β-catenin and CD73/CD39 adenosine-mediated immunosuppression pathways in S-T1 tumors. This association was further accompanied by significantly higher T cell dysfunction scores, and significantly lower expression of antigen presentation and processing genes in S-T1. In HGSC, activation of the TGF-beta and Wnt/β-catenin signaling pathways are known to produce pleiotropic effects. Not only do they promote tumor progression by inducing epithelial-mesenchymal transition (EMT)59,60, stem cell self-renewal61 and chemoresistance62, but they also drive T cell dysfunction and exclusion by regulating lymphocyte differentiation, expansion and survival63,64 and by promoting Treg accumulation, dendritic cell tolerance, and myeloid immune-suppressive functions65,66. TGF-beta has also been shown to directly down-regulate GZMA and PRF1 (ie, the components of our CYTscore) in cytotoxic T cells67. CD73 and CD39 are ectonucleotidases that convert ATP to free adenosine. The accumulation of extracellular adenosine in the TME impairs the recruitment and activation of CD8+ T cells and NK cells, and promotes the immunosuppressive functions of tumor associated macrophages68. In HGSC this pathway has been reported to promote tumor immune escape and is associated with poor prognosis69,70.

When we analyzed the relative abundance estimates of different cell types in STRATsig tertiles, we found that the fraction of cancer associated fibroblasts (CAFs) was significantly higher in S-T1 as compared to S-T3. CAFs are known to have an immunomodulatory secretome that can suppress effector cell function by inhibiting the activation and survival of cytotoxic T cells, and by promoting the recruitment and activation of immunosuppressive myeloid cells and Tregs71. Interestingly, CAFs, which are intimately linked to ovarian cancer progression72, are frequently activated by TGF-beta73-75 or Wnt76 signaling pathways in HGSC and other tumor types, and this process can be driven by WNT7A51, a LowerT gene belonging to our STRATsig signature.

In S-T3, where we observed a significant reduction in the immunoregulatory pathways described above, we observed a significant increase in the Th1 CD4+ T cell fraction, as compared to S-T1. This increase was also observed in S-T3 CYT-Hi relative to S-T1 CYT-Hi, and links a Th1-polarized CD4+ T cell population with patient survival benefit that is associated with heightened effector cell activity. This finding could reflect the importance of a helper T cell population in the recruitment and activation of effector cells capable of restraining HGSC clinical progression. Recently, it was reported that a CD4+/CD25+/FOXP3- T cell population with an exhausted Th1-like polarization, and that comprised up to 13% of CD4+ TIL, was highly correlated with HGSC progression-free survival77. Whether or not our finding reflects the activity of this cell subtype warrants further investigation. In another recent report, a tumor-reactive progenitor CD8+/TCF1Lo tissue-resident memory T cell population (TRMstem cells) was shown to be the predominant CD8+ T cell subtype associated with HGSC survival, as well as sustained antigen recognition78. An understanding of how these cells function in the context of the STRATsig tumor backgrounds differentiated in our study could yield important clinical insights.

We also observed in S-T3, but not other tertiles, that pTMB was significantly higher in CYT-Hi as compared to CYT-Lo, thus linking an immune-mediated survival advantage in HGSC patients to higher neo-antigen load, consistent with previous observations79. By contrast, the reduced survival experienced by S-T3 CYT-Lo patients may result from an ineffectual anti-tumor immunity that owes, in part, to suboptimal neo-antigen load.

These findings, together with the immunoregulatory differences described above, corroborate the view that tumors of the S-T3 class share a *hypo-*immunosuppressed and antigenic molecular composition permissive of immunologic tumor control. By extension, it is plausible that this subpopulation of patients may share a propensity for response to immune checkpoint blockade. While immune checkpoint inhibitors (ICI) have proven efficacious for a number of solid tumor types4-8, clinical trials in HGSC have been widely disappointing10. However, it has been reported that patients who do respond may experience durable responses of 6 months or more12-14. A biomarker that could distinguish these patients at diagnosis could guide treatment strategies. Several lines of evidence suggest that patients of the S-T3 class may have a heightened potential for ICI response. First, the Immunologic Constant of Rejection (ICR)25,26,56 and the T cell-inflamed Gene Expression Signature (TCIGEP)27,57,58 are well-characterized immune activation signatures that are both prognostic of cancer survival outcomes, and predictive of ICI clinical efficacy in multiple tumor types. In our study, we found that both signatures were highly significantly associated with survival in S-T3 patients, but not those of S-T2 or S-T1. Second, we found that an APM signature48 predictive of ICI response in melanoma, was significantly elevated in tumors of S-T3 as compared to S-T1, across all CYTscore groups. Third, NikNafs et al recently demonstrated that a new measure of TMB, one based on persistent mutations (i.e., pTMB), is a more significant predictor of immunotherapy response than TMB alone53. In our study, pTMB was associated with CYTscore only in S-T3, and was significantly higher in the survival-advantaged CYT-Hi population.

The discovery of the STRATsig signature was enabled by a new algorithm, CONSTRU, designed to uncover the existence of previously unrecognized patient subpopulations that differ with respect to the performance of a classifier. While our application was specific to a prognostic immune signature, in theory, the algorithm is amenable to any type of continuous or catagorical variable, including individual genes inherent to the data set that may or may not be associated with the outcome/attribute in question when assessed on the population as a whole. In this sense, any gene or gene signature believed to reflect a pathway related to cancer progression could be used by CONSTRU to define a population of patients for which it may be most applicable as a survival-associated biomarker. Furthermore, important biological insights into the nature of the tumors comprising that population may be revealed by the analysis of the genes most correlated with the stratification signature.

A central function of CONSTRU is to measure how gene expression levels influence the effect of a gene or gene signature on survival. Conceptually, this is akin to measuring variable interactions in Cox proportional hazards models, which was the approach used in a recent report to uncover genes that contribute to T cell dysfunction29. In CONSTRU, gene expression is binned into quantiles to enable control of the size of patient subpopulations where interactions may be discovered. In our study, the use of gene tertiles allowed the standardization of relative expression increments (ie, low, intermediate and high) across all genes, resulting in the delineation of patient subpopulations of equal and adequate size and power for downstream statistical comparisons. Future upgrades to CONSTRU will include refinements to the approach, such as methods to define optimal cutpoints for each gene and the ability to select stratifcation signature quantiles optimized simultaneously for the interaction in question and the population under study.

There are several limitations to this work. In HGSC, the tumor compartment in which TIL reside, intraepithelial versus stromal, is a strong determinant of TIL prognostic power, with intraepithelial TIL being most associated with survival80-82. In the tumor expression profiles analyzed in our study this information is lost. Thus, the CYTscore likely reflects the admixture of intraepithelial and stromal TIL which could obscure its prognostic power. Whether or not TIL compartment bias is related to the different CYTscore-survival associations observed between S-T1 and S-T3 remains to be determined. Intrinsic TIL heterogeneity is another limitation. T-cell hot and cold microcompartments are known to co-exist within HGSC tumors83 and this heterogeneity impacts the TIL survival association. In a multi-center survival study involving more than 3,000 HGSC cases84, substantial heterogeneity in CD8+ TIL was observed across multiple core samples from the same patient. The significant effect of CD8+ TIL on survival observed in the study was determined by a scoring system that, for each patient, identified TIL hotspots in the available cores, then assigned a score equivalent to the maximum TIL score (ie, number of TIL per high-power field counted within a hotspot) observed among all the cores for a patient. In our study, multiple cores per patient could not be assessed. Only a single tumor specimen per patient was profiled for gene expression. Therefore, it is likely that some cases in our study are innately T-cell hot, but misclassified as CYT-Lo due to the chance selection of a T-cell excluded tumor specimen. Finally, tertiles as a means to study patient populations, though advantageous for research purposes, lack precision for clinical applicability. Future work to address these limitations will be prerequisite for clinical translation.

Additional questions linger. If patients classified as S-T3 were to prove more responsive to immune checkpoint inhibitors, what then could be offered to patients of S-T1 and S-T2, where immunosuppressive pathways dominate? New research efforts aimed at targeting alternative immunosuppressive pathways (other than the PD-1/PD-L1 axis) may be key to reaching these patients. Indeed, work is already underway to therapeutically inhibit the WNT/beta-catenin85 and TGF-beta86 pathways in HGSC.

In summary, we show that the tumor-intrinsic potential for immunological control of HGSC can be measured in part by a patient stratification signature that reflects the functional output of parallel regulatory pathways in immune suppression, evasion and dysfunction. At one end of this continuum, lies a tumor phenotype of eased immunoregulation that supports a TMB/APM/TIL-linked immune-mediated survival advantage. How to best translate these findings into the clinical setting is the subject of ongoing investigations.

**MATERIALS AND METHODS**

**Expression data acquisition, processing and annotation**

Six curated data sets of tumor expression profiles and corresponding clinical data from high-grade serous ovarian cancer (HGSC) patients were analyzed. Protocols for patient consent and sample acquisition were approved by Institutional Review Boards at each site. The OV1 data set comprises 431 HGSC cases profiled on the Affymetrix U133A platform as part of the early TCGA initiative52 and accessible via Gene Expression Omnibus (GEO) accession number GSE82191. The 431 cases represent the subset of 527 cases annotated as high-grade (grade 2 or 3) serous histology with >3 months follow-up, and not identified as redacted in Table S1 of the TCGA clinical update report87. Data was normalized by the RMA method88 as implemented in the R package *affy*89 provided by Bioconductor90.

OV2 consists of 227 HGSC cases associated with the Australian Ovarian Cancer Study and profiled on the Affymetrix U133 Plus 2.0 platform91 with GEO accession GSE9891. The 227 cases represent the subset of 285 cases annotated as high-grade serous histology with accompanying overall survival data and >3 months follow-up. Data was normalized by RMA.

OV3 is a batch-corrected compilation of three smaller data sets GSE314992, n=110; GSE1476493, n=66; and GSE3016194, n=45) profiled on the Affymetrix U133A92,93 or U133 PLUS 2.094 platforms, and consists of 221 HGSC cases in total. The 221 cases are the subset of 255 cases annotated as high-grade serous histology with accompanying overall survival data. Data sets were normalized by RMA92,94 or MAS5.093. Original CEL files for GSE314992 were accessed at <https://bioinformatics.mdanderson.org/Supplements/ReproRsch-Ovary/Modified/DressmanArchive/index.html> and corrected for run date batch effects as recommended by Baggerly and colleagues 95. The ComBat empirical Bayes method43 was used to correct for batch effects. Updated corresponding clinical data were obtained via curatedOvarianData v3.18 (Bioconductor).

OV4 consists of 174 HGSC cases profiled on the Agilent-014850 Whole Human Genome Microarray 4x44K G4112F platform36 with GEO accession GSE53963. Data were normalized by Linear/LOWESS normalization as reported36.

The OV5 data set comprises 260 HGSC cases associated with the Japanese Serous Ovarian Cancer Study Group96 and profiled on the Agilent-014850 Whole Human Genome Microarray 4x44K G4112F platform with GEO accession GSE32062. Data were normalized by the scaling method as published96.

OV6 consists of 212 HGSC cases associated with the ICON7 multicenter clinical trial96 and profiled on the Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip97 with GEO accession GSE140082. Data were normalized by quantile normalization as reported97. Patient survival data and/or other clinical annotations for OV1, OV2, OV3 and OV5 were provided by the Bioconductor curatedOvariandata package98. Updated survival data for OV1 was used as published in Table S1 of the TCGA clinical update report87. For OV4 and OV6, patient survival data and other clinical annotations were obtained at GSE53963 and GSE140082, respectively.

The OTTA (Ovarian Tumor Tissue Analysis) consortium data set is derived from an international multi-site HGSC cohort consisting of 3,769 tumor specimens annotated for survival and other clinical characteristics 38,42. The tumor specimens were profiled on the NanoString n-Counter platform for the expression of 513 genes, and the normalized gene expression profiles were retrieved from GEO accession GSE132342. Details regarding NanoString data quality assurance, monitoring for batch effects, single-patient normalization by reference genes and reference pools, and metrics for sample inclusion are described in 42.

**Derivation of gene signatures**

Gene identities corresponding to array probes or probe sets were standardized as follows. The Ensembl BioMart (Ensembl Genes 102 DATABASE, Human genes (GRCh38.p14) DATASET)99 was used to annotate all human genes with Hugo Gene Nomenclature Committee (HGNC) approved gene names and symbols, as well as corresponding probe or probe set IDs for the Affymetrix, Agilent, and Illumina array platforms. Gene name and symbol updates were performed using the HGNC Multi-symbol checker tool (<https://www.genenames.org/>). Gene signature scores were obtained by computing the mean of the log2 expression values of the genes comprising a gene signature. The cytolytic activity signature (CYTscore) was initially defined as the mean of PRF1 (214617\_at) and GZMA (205488\_at) as described by Rooney and colleagues30.

The APM gene signature predictive of ICI response in non-small cell lung cancer and melanoma48 was defined as the sum of the log2 z-scores of the 8 genes: B2M, CALR, NLRC5, PSMB9, PSME1, PSME3, RFX5 and HSP90AB1. T cell dysfunction scores were computed as described by Jiang29 and Fu100 using the Tumor Immune Dysfunction and Exclusion (TIDE) web platform (http://tide.dfci.harvard.edu/).

**The CONSTRU algorithm and STRATsig candidate gene ranking by parity score**

CONSTRU (Computing Prognostic Marker Dependencies by Successive Testing of Gene-Stratified Subgroups) is an algorithm designed for use with tumor gene expression profiling data, to empirically discover groups of tumors designated by discrete gene expression-based partitions for which a continuous or categorical variable will significantly associate with patient survival. The algorithm takes as input a tumor-gene expression data matrix, with tumors (columns) annotated with survival time and event, and available prognostic variables. The expression data for each gene (rows) is used to organize tumors into groups based the gene’s relative expression level. In this implementation, tumors are grouped according to gene expression tertiles, thereby representing a standardized measure of low, intermediate or high expression. For each gene tertile, a multivariable Cox proportional hazards regression model is fitted to the data, and the significance (p-value) and directionality (hazard ratio) of the association between the CYTscore (continuous; mean expression of GZMA and PRF1) and patient OS is computed with adjustment for other prognostic factors, including patient age (continuous), FIGO stage (low (stage I or II), high (stage III or IV), NA), and surgical debulking status (optimal, suboptimal, NA).

Once the Cox model statistics for CYTscore are computed for each tertile of each gene, the algorithm outputs a text file displaying the tertile-specific statistics for all genes. This file is then used to rank genes according to their tertile-specific Cox statistics using a parity score developed to distinguish genes with largest difference between lower (T1) or upper (T3) tertiles with respect to CYTscore prognostic power. The parity score uses CYTscore-survival Cox p-values and hazard ratios according to the following formula: *([T1]-log2P/HR) – ([T3]-log2P/HR)* to generate a combined measure of significance (-log2P) and effect size (HR, hazard ratio) for each tertile (T1, T3) and calculates the delta between them. The larger this delta, the greater the difference between T1 and T3 with respect to CYTscore significance and effect size. Higher parity scores equate with genes that have greater CYTscore-survival associations in T1 (termed LowerT genes), while lower parity scores reflect genes with greater CYTscore-survival associations in T3 (termed UpperT genes). The parity scores are then assigned percentile ranks as a function of all genes used in the analysis, thus allowing a standardized approach for comparing genes across data sets.

As the goal is to stratify patients based on relationships between gene expression levels and the prognostic significance of a gene signature, genes having expression patterns correlated with the gene signature are poor candidates, as the tumor groups defined by the resulting gene tertiles will each exhibit a compressed distribution of gene signature values. For example, if a gene is positively correlated with CYTscore, then the gene’s lower tertile will be enriched for tumors with low CYTscore values, while the gene’s upper tertile will be enrich for tumors with high CYTscore values. Therefore, after running CONSTRU, genes were filtered based on their inherent positive or negative correlations with CYTscore. We used an empirical threshold of Pearson’s correlation coefficient of >0.15 or <-0.15 to exclude genes from analysis. When applied to the OV1 and OV2 data sets, a common set of 8,048 probe sets corresponding to 7,866 genes remained for parity score percentile rank comparisons between OV1 and OV2. The OV1 and OV2 datasets were selected for use in the training process for the following reasons: 1) they share a large common set of gene probes with identical sequence (i.e., 22,277 probe sets), 2) patient overall survival rates are not significantly different between OV1 and OV2, and 3) in terms of sample size, they are among the largest HGSC whole-genome expression profiling data sets published to date.

The parity score was used to select UpperT and LowerT genes identified within the 4th-percentile cutoffs of both OV1 and OV2. The UpperT gene signature was defined as the mean of C8orf33 (218187\_s\_at), CDC42EP4 (218062\_x\_at), DDX21 (208152\_s\_at), DNAJC9 (213092\_x\_at, 213088\_s\_at), MEGF6 (213942\_at), NCAPD3 (212789\_at), RAF1 (201244\_s\_at), RTF1 (212302\_at), TPD52 (201691\_s\_at), TUBGCP4 (211337\_s\_at), UBP1 (218082\_s\_at), UQCRB (209066\_x\_at) and ZNF250 (213858\_at). The LowerT gene signature was defined as the mean of ALB (211298\_s\_at), AMACR (209424\_s\_at), APBB2 (40148\_at), BAG2 (209406\_at), BDH2 (218285\_s\_at), CAMK2N1 (218309\_at), CAV2 (203323\_at), CDC14B (221556\_at), DNAJB4 (203811\_s\_at), EVA1B (220134\_x\_at), FAT4 (219427\_at), GALC (204417\_at), HAS2 (206432\_at), HOXA9 (214651\_s\_at), MFGE8 (210605\_s\_at), NPTXR (213040\_s\_at), OSR2 (213568\_at), PCGF1 (210023\_s\_at), PCOLCE2 (219295\_s\_at), PEPD (202108\_at), PID1 (219093\_at), PLAGL1 (207943\_x\_at), POGLUT2 (219479\_at), STAM2 (209649\_at), TRPC1 (205803\_s\_at), WDFY3 (212602\_at) and WNT7A (210248\_at). STRATsig scores were computed by subtracting the LowerT score from the UpperT score. For non-Affymetrix data sets, the probes corresponding to these genes were used to compute STRATsig scores; when a gene was represented by more than one probe, the probe expression values were averaged prior to computing LowerT and UpperT scores. In the case of OV4 and OV5, probes representing 2 of the 27 genes of the LowerT signature, EVA1B and POGLUT2, were not available for inclusion in the STRATsig calculation.

**Pathway enrichment**

In each of the six data sets (OV1-OV6), gene expression patterns were assessed for correlation (Pearson) with STRATsig, and the top 2% of genes most positively or negatively correlated with STRATsig were identified. The DAVID (Database for Annotation, Visualization and Integrated Discovery) Knowledgebase v6.8 and gene functional annotation tools44 were used to uncover significantly enriched pathways and gene ontologies in the 2nd-percentile gene lists (i.e., the positively or negatively correlated gene lists). The integrated training group (OV1, OV2, OV3) and test group (OV4, OV5, OV6) were used to compute activation levels of 54 cancer-related pathways in the context of STRATsig tertiles (T1, T2, T3) and CYTscore tertiles (Lo, Mid, Hi). For this work, single-sample gene set enrichment analysis (ssGSEA) was performed using the GSVA (v1.42.0) R package101 with the ‘gsva’ function kernel density parameter set as ‘Gaussian’ kernel. Gene sets used to compute pathway activation scores were obtained from multiple sources, including the 24 hallmark pathways frequently altered in cancer102, 21 non-redundant cancer and immune related pathways obtained from the IPA Knowledgebase v6.8, and a number of additional oncogenic and tumor immune-related pathways including Hypoxia/Adenosine Immune Cell Suppression, Immunogenic Cell Death, NOS1 Signaling, PI3Kgamma signaling and the SHC1/pSTAT3 pathway as described by Lu and colleagues in 2017103, mechanical barrier genes as defined by Salerno and colleagues in 2016104, a proliferation gene signature described by Miller and colleagues in 201641 and genes upregulated by MAPK mutation as described by Bedognetti and colleagues in 2017105.

**Enrichment of cell type abundance**

To investigate the cellular composition of the TME, we considered multiple deconvolution methodologies that employ a system of linear equations to assign weighted sums to genes based on the contribution of different cell types to a given gene’s expression, including CIBERSORT106, EPIC107, TIMER108, quanTIseq109 and xCell110. For this study, we utilized xCell in the immunedeconv (v2.1.0) R package111, which computes abundance scores for 64 cell types, as it was the least restrictive method with respect to handling log transformed quantile normalized data.

**Tumor mutational burden (TMB) analysis**

TMB estimates for tumors comprising the OV1 data set were originally computed from the multi-center mutation calls of the TCGA pan-cancer atlas112 using TCGA whole-exome sequencing data. The mutation calls were obtained from the GDC knowledge base maintained at the NCI Genomic Data Commons (https://gdc.cancer.gov) by Niknafs and colleagues53. TMB values computed from the nonsynonymous mutations, as well as the corresponding “loss-prone” and “persistent” TMB calculations performed by Niknafs, et. al. 53, were obtained from the supplemental files of that publication.

**Assignment of HGSC molecular subtypes**

Tumors were assigned to HGSC molecular subtypes (IMR, DIF, PRO and MES) using the consensusOV 1.16.0 R package 113. ConsensusOV is a random forest classifier trained on HGSC tumors with strong subtype agreement between different subtyping methods113. For each tumor, the classifier computes margin scores that reflects the degree of confidence with which the tumor belongs to each of the four subtypes (IMR, DIF, PRO and MES). In practice, the tumor is assigned to the subtype corresponding to the highest margin score. However, in instances where a tumor’s margin scores are similar across subtypes, the accuracy of subtype assignment is questionable. To address this, we ran the classifier on each data set six times, and compared each tumor’s margin scores for inconsistencies in subtype assignment. For each tumor, the margin scores for each subtype were averaged across the six runs and compared. When the difference between a tumor’s top two subtype margin scores was <0.1 (i.e., a 10% difference in confidence) the tumor was not assigned to a subtype. Accordingly, 13.11% of tumors in the combined training and test groups were deemed ineligible for downstream subtype analyses.

**Statistical Analyses**

Survival analyses, including Cox proportional hazards regression and logrank tests, were performed as implemented in the R survival package (https://cran.r-project.org/web/views/Survival.html) or SigmaPlot 12.0. Overall survival (OS) was defined as the time of diagnosis to death or last clinical follow up at 8 years. This standardized threshold was employed for two reasons: 1) to more accurately compare survival dynamics across different patient populations, particularly when data sets included >20 years survival for some patients, and 2) to minimize the effects of age-related mortality. In Cox models, variables were treated as categorical or continuous as described in table footnotes, and Wald test p-values were reported with hazard ratios and 95% confidence intervals. Significant differences between distributions were assessed by t-test or the Mann-Whitney rank sum test if test for normality failed.

**TABLES AND FIGURES**

**TABLE 1. HGSC data sets**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **TRAINING** | | | **TEST** | | | |
|  | **OV1** | **OV2** | **OV3** | **OV4** | **OV5** | **OV6** |
| Tumor No. | 431 | 227 | 221 | 174 | 260 | 212 |
| Average Age (Range) | 59.7 (30-89) | 60.4 (23-80) | 61.8 (38-85)\* | 63.2 (24-89) | NA | 59.3 (35-80) |
| No. FIGO Stage 1-2 | 35 | 19 | 4 | 8 | 0 | 21 |
| No. FIGO Stage 3-4 | 396 | 208 | 215 | 166 | 260 | 191 |
| No. Optimal Debulking# | 183 | 122 | 77 | 123 | 103 | 154 |
| No. Suboptimal Debulking^ | 43 | 79 | 77 | 48 | 157 | 57 |
| Gen Profiling Platform | Affymetrix | Affymetrix | Affymetrix | Agilent | Agilent | Illumina |
| \* only 20% of cases annotated for age; # optimal (<1 cm of residual disease); ^ suboptimal (>1 cm residual disease) | | | | | | |

**TABLE 2. Multivariable Cox regression for associations with overall survival (OV4-6)**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **STRATsig T1 (n = 216)** | | | **STRATsig T2 (n = 214)** | | | **STRATsig T3 (n = 216)** | | |
|  | **No. (%)** | **HR (95% CI)** | **P-val** | **No. (%)** | **HR (95% CI)** | **P-val** | **No. (%)** | **HR (95% CI)** | **P-val** |
| **Age < 62 yo** | 45 (21) | Ref | - | 81 (38) | Ref | - | 75 (35) | Ref | - |
| **Age ≥ 62 yo** | 72 (33) | 0.70 (0.42-1.15) | 0.15 | 70 (33) | 1.48 (0.92-2.36) | 0.1 | 43 (20) | 0.74 (0.42-1.29) | 0.28 |
| **Low (Stage I-II)** | 10 (5) | Ref | - | 7 (3) | Ref | - | 12 (6) | Ref | - |
| **High (Stage III-IV)** | 206 (95) | 0.17 (0.02-1.23) | 0.08 | 207 (97) | 0.45 (0.06-3.29) | 0.43 | 204 (94) | 0.37 (0.09-1.54) | 0.17 |
| **Optimal Debulking** | 117 (54) | Ref | - | 134 (63) | Ref | - | 129 (60) | Ref | - |
| **Suboptimal Debulking** | 97 (45) | 1.85 (1.23-2.80) | 0.003 | 78 (36) | 2.52 (1.66-3.84) | <0.0001 | 87 (40) | 2.02 (1.27-3.21) | 0.003 |
| **CYTscore, continuous** | Ref | 0.96 (0.82-1.13) | 0.64 | 72 (34) | 0.91 (0.79-1.03) | 0.14 | 64 (30) | 0.70 (0.60-0.81) | <0.0001 |

**Supplemental Table 1: Differential gene expression between STATsig T1 and T3 CYTscore groups.**

**FIGURE LEGENDS**

**Fig. 1. Description of CONSTRU workflow and strategies for gene ranking and selection. (A)** Graphical depiction of CONSTRU workflow illustrating the iterative process of ranking cases into gene expression tertiles, then computing CYTscore-survival statistics for each tertile of each gene. **(B)** Illustration of gene ranking by parity score and partitioning of LowerT and UpperT genes. **(C-E)** Assessing reproducibility of top UpperT and LowerT genes. For each gene selected by parity score percentile rank thresholds (2%, 4%, and 6%), and overlapping between OV1 and OV2, the CYTscore hazard ratios (HR) are plotted for each gene’s tertile, in each data set. The OV3 data set was used to assess the general reproducibility of the gene tertile-specific CYTscore-survival associations observed in OV1 and OV2. \*, *P* <0.05 & >0.01; \*\*, *P* <0.01 & >0.001; \*\*\*, *P* <0.001.

**Fig. 2. Stratification signatures and the reproducibility of tertile-specific CYTscore-survival associations.** UpperT, LowerT and the combined (STRATsig) gene signatures were used to stratify cases into population tertiles for assessment of CYTscore-survival associations by Kaplan-Meier analysis. **(A-C)** Shown are results for the OV1 + OV2 combined cohort stratified by **(A)** the UpperT signature, **(B)** the LowerT signature and **(C)** the combined signature. **(D-F)** Results are show for cases of the OV3 cohort stratified by **(D)** the UpperT signature, **(E)** the LowerT signature and **(F)** the combined signature. Similar results are shown for the validation cohorts **(G)** OV4, **(H)** OV5 and **(I)** OV6. CYTscore tertiles (Lo, Mid, Hi) were determined using all cases of a cohort as input. Heat maps of genes hierarchically clustered by average linkage clustering (with Pearson correlation as distance metric) are shown. Red indicates above-mean expression; blue denotes below-mean expression. Black and gray vertical bars to the right of heat maps denote UpperT and LowerT genes, respectively. Logrank p-values are shown.

**Fig. 3. The S-T3 CYTscore-survival association is independent of debulking status, stage and patient age.** **(A)** The integrated test group comprising cohorts OV4, OV5 and OV6 was used to assess relationships between tertile-specific CYTscore-survival associations and known prognostic factors. Cross-tertile Kaplan-Meier plots are shown for cases categorized as **(B)** optimally debulked, **(C)** sub-optimally debulked, **(D)** FIGO stage III, **(E)** FIGO stage IV, **(F)** younger age (<62) and **(G)** older age (>62). Logrank p-values are reported. With respect to treatments, all patients of each cohort received standard of care platinum based chemotherapy and surgery, while none received immunotherapy. Half of OV6 patients received concurrent bevacizumab (a VEGF inhibitor); however, this did not impact performance of STRATsig or CYTscore when investigated as a co-variable.

**Fig. 4.** **STRATsig tertiles differ by pathways of immune suppression, T cell dysfunction, antigen presentation and TMB.** **(A)** Pathway enrichment analysis using the DAVID knowledgebase. For each cohort, the top 2% of STRATsig positively or negatively correlated genes were analyzed. **(B)** Pathway activation scores were computed by single-sample gene set enrichment analysis in the integrated training (OV1-OV3) and test (OV4-OV6) groups. The mean pathway activity heat map shows the average of pathway activity scores for each CYTscore group (Lo, Mid, Hi) within each STRATsig tertile. Red indicates higher pathway activity; blue denotes lower pathway activity. The 20 pathways with significant activity differences between STRATsig T1 and T3, or CYTscore Hi groups within STRATsig T1 and T3, specifically, are shown. Right 2 panels: blue reflects higher activity in T1; red indicates higher activity in T3. **(C)** Comparison of measures of T cell dysfunction, APM signature and IRF1 gene expression between matched CYTscore groups belonging to STRATsig tertiles T1 and T3. **(D)** Tumors were ranked by STRATsig (left to right, ascending), and the mean pathway activation score was computed within a sliding window of n=40 with a slide increment of +1. The running mean score for select pathways is shown plotted across the stratified training and test populations. **(E-H)** In OV1, the tertile-specific distributions of tumor mutational burden (TMB) defined as **(E)** conventional TMB, **(F)** loss-prone TMB or **(G)** persistent TMB (pTMB) are shown. **(H)** pTMB distributions are shown as a function of CYTscore groups within STRATsig tertiles. Mann–Whitney U test p-values are shown.

**Fig. 5. Analysis of pathway-based tertiles and survival associations using alternate immune genes and signatures. (A)** Tertile reconstruction by pathways. Pathway activation scores were used to stratify cases into tertiles, and the significance of the CYTscore-survival associations within these tertiles was assessed by multivariable Cox regression. Results were compared to that of STRATsig T3 (top). **(B)** CYTscore deconstruction and alternative immune markers. Genes comprising the ICR gene signature were assessed for survival associations in STRATsig tertiles by Cox regression. (**C**) All profiled genes comprising the training and test groups were analyzed for survival associations by Cox regression in the S-T1 and S-T3 populations. Plotted are genes with hazard ratios (HR) < 0.80 in S-T1 or S-T3. Black and orange circles correspond to genes positively associated with survival with adjusted P < 0.01 (Benjamini-Hochberg). Orange denotes significant genes in common to both training and test groups. No significant genes were identified in the S-T1 population. **(D)** Within STRATsig tertiles, CYTscore-survival associations were compared to the survival associations observed for the ICI-predictive immune signatures, ICR and TCIGEP.

**Additional File 1: Fig. S1. CYTscore prognostic performance in HGSC datasets. (A-F)** In datasets OV1-OV6, tumors were stratified into tertiles based on tumor CYTscore values, and survival differences among the tertile groups were assessed by logrank test. **(G)** The CYTscore was analyzed as a continuous variable in Cox models while adjusting for patient age (continuous), International Federation of Gynecologic Oncology (FIGO) stage (I, II, III, IV) and debulking status (0, 1), when available.

**Additional File 1: Fig. S2. Surrogate gene analysis of STRATsig and CYTscore in the OTTA consortium cohort reproduces the STRATsig T3 CYTscore-survival association.** In recent reports by the Ovarian Tumor Tissue Analysis (OTTA) consortium, FFPE tumor specimens and corresponding clinical data from 3,769 HGSC patients were collected at 20 sites across multiple countries, processed using standardized protocols, and analyzed for the expression of 513 informative genes using the NanoString n-Counter mRNA quantitation platform (Millstein, et. al., PMCID: PMC7484370; Talhouk, et. al., PMCID: PMC7572656)~~38,114~~. In this data set (GEO accession GSE132342) the reproducibility of the observed STRATsig and CYTscore associations was evaluated by proxy using individual genes comprising the signatures, as well as genes with greatest correlation to the signatures. Included among the 513 genes profiled were the STRATsig UpperT gene, DNAJC9, and the LowerT gene, APBB2. In our training and test group data sets, the average correlation between these genes and STRATsig were 0.26 and -0.45 for DNAJC9 and APBB2, respectively. While the genes comprising the CYTscore (GZMA and PRF1) were not represented in the study, the genes CCL5 and CD2 were included. In both our training and test groups, CCL5 and CD2 ranked within the top five genes most positively correlated with CYTscore, with average correlations to CYTscore of 0.91 and 0.90, respectively. **(A)** Using the ratio of DNAJC9 to APBB2 as a proxy to approximate the STRATsig vector, and the geometric mean of CCL5 and CD2 to approximate the CYTscore vector, we analyzed the OTTA data set for CYT-survival associations within the approximated STRATsig tertiles. CYTscore significance in STRATsig T1, T2 and T3 was *P* = 0.73 (CYT Lo vs. Hi: *P* = 0.32), *P* = 0.01 (CYT Lo vs. Hi: *P* = 0.06), and *P* = 0.01 (CYT Lo vs. Hi: *P* = 0.006), respectively. **(B)** As reported by the OTTA consortium (Millstein, et. al., PMCID: PMC7484370)~~38~~, CXCL9, a chemokine that recruits effector T cells, was identified as among the top five genes most significantly associated with overall survival in the OTTA data set. In our training and test groups, CXCL9 showed an average correlation to CYTscore of r = 0.80. Using CXCL9 as a proxy for CYTscore, CYTscore significance in T1, T2 and T3 was *P* = 0.003 (CYT Lo vs. Hi: *P* = 0.89), *P* = 0.00007 (CYT Lo vs. Hi: *P* = 0.00002), and *P* < 0.00001 (CYT Lo vs. Hi: *P* < 0.00001), respectively. **(C)** Next we reconstructed the vector for STRATsig using the two genes represented in the OTTA data set that were most positively and negatively correlated with the original STRATsig in our training and test groups. These genes, LPAR3 and FBN1, were not part of the original STRATsig gene list, but within the training and test groups, they showed average correlations to STRATsig of 0.37 and -0.54, respectively (i.e., more positively and negatively correlated to STRATsig than DNAJC9 and APBB2). Using the ratio of LPAR3 to FBN1 as a proxy for STRATsig, and CXCL9 as a proxy for CYTscore, CYTscore significance in T1, T2 and T3 was *P* = 0.06 (CYT Lo vs. Hi: *P* = 0.79), *P* < 0.00001 (CYT Lo vs. Hi: *P* < 0.00001), and *P* < 0.00001 (CYT Lo vs. Hi: *P* < 0.00001), respectively.

**Additional File 1: Fig. S3. HGSC molecular subtype composition in STRATsig and CYTscore tertiles.** Training and test group tumors were assigned to their respective subtypes using the *consensusOV* algorithm of Chen and colleagues113. Similar subtype survival rates were observed when comparing **(A)** training and **(B)** test populations, with IMR and DIFF tumors having better survival than MES and PRO tumors, consistent with published observations36,113. **(C)** The percentage of subtypes within tertile groups, and **(D)** across tertile groups, was examined. Analysis of STRATsig tertiles subdivided by CYTscore groups showed a strong positive relationship between the IMR subtype and CYTscore, a strong negative association between PRO and CYTscore, as well as a moderately negative association between DIF and CYTscore. Analysis of subtype composition in STRATsig tertiles of the **(E)** training group and **(F)** test group showed that STRATsig T1 comprised largely of MES (43-53%) and PRO (19-24%) subtypes, while STRATsig T3 consisted mostly of DIF (43-49%) and IMR (36%) tumors; T2 tumors were more admixed, with all subtype fractions ranging from 17-31%. **(C)** In STRATsig T3, CYT Lo tumors were predominantly DIF (59-62%) and PRO (24-28%), while the CYT Hi tumors comprised mostly of IMR (77-80%) and DIF (14-23%). The relationship between IMR and the STRATsig T3 CYT-survival association was investigated. **(G)** In IMR tumors, CYTscore was associated with survival in STRATsig T3 (log-rank test *P* < 0.00001), but not in T2 or T1, indicating that IMR status does not fully explain the STRATsig T3 CYT-survival association. **(H)** The STRATsig T3 CYT-survival association was also observable among the non-IMR subtypes (log-rank test *P* = 0.001).

**Additional File 1: Fig. S4. Comparison of CYTscore distributions within CYTscore groups and survival characteristics.** CYTscore distributions within matched CYTscore groups are shown compared across STRATsig tertiles for **(A)** the training group and **(B)** the test group. No significant differences between (cross-tertile) matched CYTscore groups were observed. Kaplan-Meier survival curves comparing STRATsig T3 CYT Hi cases to STRATsig T1 CYT Hi cases are shown for **(C)** the training group and **(D)** the test group. Logrank p-values are reported.

**Additional File 1: Fig. S5. Representative genes recapitulate pathway-tertile associations.** Expression distributions of representative genes associated with various pathways are shown as a function of tertile and CYTscore group. \*, In the training group, expression profiles for CD274 (PD-L1) were only available in OV2.

**Additional File 1: Fig. S6. Analysis of cell type proportions in STRATsig tertiles.** Cell type proportions were computed by xCell single-sample gene set enrichment analysis in the integrated training (OV1-OV3) and test (OV4-OV6) groups. **(A)** The mean cell type fraction heat map shows the average cell type fractions for each CYTscore group (Lo, Mid, Hi) within each STRATsig tertile. Red indicates higher cell fraction; blue denotes lower cell fraction. **(B)** Significant cell fraction differences between STRATsig T1 and T3; **(C)** CYTscore Hi groups within STRATsig T1 and T3, specifically. Blue reflects higher cell fractions in T1; red indicates higher cell fractions in T3. Size of the circle denotes adjusted p value with the smallest circle representing a p value of 0.05 and the largest representing a p value of 0.001.

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