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ORIGINAL RESEARCH

Immunological metagene signatures derived from immunogenic cancer cell death associate with improved survival of patients with lung, breast or ovarian malignancies: A large-scale meta-analysis

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ABSTRACT

The emerging role of the cancer cell-immune cell interface in shaping tumorigenesis/anticancer immunotherapy has increased the need to identify prognostic biomarkers. Henceforth, our primary aim was to identify the immunogenic cell death (ICD)-derived metagene signatures in breast, lung and ovarian cancer that associate with improved patient survival. To this end, we analyzed the prognostic impact of differential gene-expression of 33 pre-clinically-validated ICD-parameters through a large-scale meta-analysis involving 3,983 patients ('discovery' dataset) across lung (1,432), breast (1,115) and ovarian (1,436) malignancies. The main results were also substantiated in 'validation' datasets consisting of 818 patients of same cancer-types (i.e. 285 breast/274 lung/259 ovarian). The ICD-associated parameters exhibited a highly-clustered and largely cancer type-specific prognostic impact. Interestingly, we delineated ICD-derived consensus-metagene signatures that exhibited a positive prognostic impact that was either cancer type-independent or specific. Importantly, most of these ICD-derived consensus-metagenes (acted as attractor-metagenes and thereby) 'attracted' highly co-expressing sets of genes or convergent-metagenes. These convergent-metagenes also exhibited positive prognostic impact in respective cancer types. Remarkably, we found that the cancer type-independent consensus-metagene acted as an 'attractor' for cancer-specific convergent-metagenes. This reaffirms that the immunological prognostic landscape of cancer tends to segregate between cancer-independent and cancer-type specific gene signatures. Moreover, this prognostic landscape was largely dominated by the classical T cell activity/infiltration/function-related biomarkers. Interestingly, each cancer type tended to associate with biomarkers representing a specific T cell activity or function rather than pan-T cell biomarkers. Thus, our analysis confirms that ICD can serve as a platform for discovery of novel prognostic metagenes.

Abbreviations: ATP, Adenosine triphosphate; CD, Cluster of differentiation; CRT, Calreticulin; EGA, European genome-phenome archive; GEO, Gene expression omnibus; HMGB1, High mobility group box 1; HSP, Heat shock protein; ICD, Immunogenic cell death; IFN, Interferon; IL, Interleukin; OS, Overall survival; PERK, Protein kinase RNA-like endoplasmic reticulum kinase; TCGA, The cancer genome atlas; TLR, Toll-like receptor; TNF, Tumor necrosis factor

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Introduction

Cancer is a complex disease where tumor progression is also determined by a dynamic interaction between cancer cells and non-cancer cells, such as immune cells.[1-4] However, this complexity renders the process of patient prognosis based on defined biomarkers extremely difficult. Ideally, biomarkers should reflect the complexity of a tumor mass across various cancer-types.[5] Several clinical and pathological indicators have been introduced for estimating patient prognosis[6,7] however, while such systems are valuable, they mostly rely on clinical parameters or cancer cell-related factors.[6] The recently emerging role of the cancer cell-immune cell interface in shaping tumorigenesis[2-4] and the appearance of anticancer immunotherapy[8,9] has increased the need to identify new integrated as well as broad sets of prognostic biomarkers based on the cancer cell-immune cell interface.

The availability of high-throughput microarray technologies has enabled the investigation of global gene expression profiles or 'transcriptome' of the tumor, which has revolutionized the search for prognostic markers.[5] Furthermore, transcriptomic analysis is capable of revealing 'multi-gene expression patterns' or 'metagenes' (i.e., aggregate patterns of gene expressions like a cluster of genes, exhibiting or stratified to exhibit collective high expression) as prognostic biomarkers.[10-13] Interestingly, recent progress in the fields of prognostic metagene biology in cancer has suggested that initial consensus-metagenes identified through biomarker discovery approaches may subsequently exhibit the ability to act as 'attractor'-metagenes for a

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further set of 'convergent'-metagenes.[11] More specifically certain consensus-metagenes (acting as attractor-metagenes), may have the ability to associate with, or, be 'converged' upon by, a further sets of co-expressed genes, with close but not necessarily identical relationship (which can be termed as convergent-metagenes).[11] Thus, this data-mining process has the ability to not only characterize defined biological process-specific metagenes but also help in discovery of new sets of co-expressing genes with putative prognostic impact. Owing to such developments, it has been proposed that apart from a single high-powered, prospective, randomized controlled trial, retrospective meta-analysis of publicly available microarray datasets from multiple clinical studies is a powerful (if not exhaustive) data-mining methodology for discovery as well as assessment of novel prognostic biomarkers.[7]

An emerging immunological process that has not yet been comprehensively exploited for a bottom-up approach of prognostic biomarker discovery, especially with respect to prognostic metagene biology, is ICD. Comprehensive preclinical studies have established ICD as an important predictor of potent anti-tumor immunity.[1-3,14,15] ICD is associated with danger signaling pathways mediating the extracellular emission of danger signals like surface calreticulin/heat-shock proteins, secreted ATP and secreted HMGB1.[14,16-24] These danger signals help in activating the innate immune system[14,22] which further boosts the adaptive immunity leading to anti-tumor immunity.[14,25-27] A significant advantage of using ICD for biomarker discovery is that it, uniquely, simultaneously integrates several immune-related pathways such as danger signaling, effector T cell infiltration/activity and others into a single paradigm.[1-3] Thus the probability that ICD, as a primary endpoint, may identify immune biomarkers and respective metagenes is high. A limited number of retrospective studies carried out in human cancer patients to ascertain whether ICD-associated parameters can be used as prognostic biomarkers, have yielded contradictory results.[28-35] Possible reasons for this could be restriction to a few ICD-derived biomarkers (mainly calreticulin, HMGB1 or CD73), the limited number of patients and variations related to cancer-types.

We therefore hypothesized that the known catalogs of ICD-associated parameters, characterized through extensive preclinical research and validated through a large-scale meta-analysis, could be prognostic for the overall survival (OS) of cancer patients. Through this *in silico* meta-analysis we further wanted to identify ICD-derived consensus-metagene signatures with a prognostic significance either across different cancer types (breast, ovarian and lung cancer) or for specific cancer-types. We also wanted to test whether these consensus-metagenes may have the ability to act as attractor-metagenes for convergent-metagenes, with similar prognostic impact and specific or broad immunological indications.

Results

Immunogenic cell death is a diverse source of 33 putative prognostic factors dealing with various levels of immune-complexity

An extensive literature survey was done for identifying pre-clinically validated ICD-parameters (searching PubMed, Scopus and Web of Knowledge, for relevant studies conducted in mice *in vivo* and/or with primary human immune cells *ex vivo*, until October 2014). Studies/specific results within them were considered eligible if they met all of the following criteria: (1) explored the association between ICD and danger signaling mechanisms/immunological processes, (2) carried out prophylactic/curative rodent vaccination experiments and/or experiments involving cancer cells-immune cells co-culture, (3) carried out experimental interventions (e.g. siRNA/shRNA), antibody-based depletion/blockade or whole-body/tissue/lineage-specific knock-out rodent models and (4) associated processes/molecular entities with ICD on the basis of proper untreated/negative/positive controls. Studies/specific results were excluded based on any of the following reasons: (1) not sufficient data reported, (2) letters/reviews/commentary/perspectives/case reports/conference abstracts/editorials or expert opinion, (3) studies/results where the experimental interventions/knock-down/knockout phenotypes did not affect ICD-based anticancer vaccination effect, and (5) association of a process/entity with ICD was not established due to lack of proper negative/positive controls.

This literature survey identified 22 papers that fulfilled the above eligibility criteria, from which we extracted 33 pre-clinically relevant parameters of ICD in cancer (Table 1, Fig. 1). It is noteworthy that majority of these ICD-parameters were established using rodent models.[2] Overall, 26 ICD-parameters were found to positively-regulate ICD (i.e. whose ablation abrogated ICD; or whose high expression associated with ICD; thus they can be putatively predicted to have 'good prognostic' implication) (Table 1, Fig. 1). Others were found to either negatively-regulate ICD (i.e., Four parameters, whose ablation enhanced ICD; or whose low/null expression associated with ICD; thus they can be putatively predicted to have "poor prognostic" implication) or exert context-dependent activity (4 parameters, for which context-dependent contradictory experimental data exists) (Table 1, Fig. 1).[1-3]

These 33 ICD-parameters were largely evenly distributed across various levels of ICD and represented various newly-emerging as well as classical immunological processes (Table 1, Fig. 1). For instance, ICD is associated with instigation of danger signaling[2] (here represented by *ATG5, BAX, CASP8, PDIA3, EIF2AK3, PIK3CA*) that mediates emission of certain danger signals[36] (*CALR, HMGB1, HSP90AA1*). At this step, some molecules may act as danger signal-degraders, thereby counteracting ICD (*ENTPD1, NT5E*).[14] ICD-associated danger signaling and other factors then go onto activate various levels of innate immune system[1,14] e.g., by positively regulating various innate immune effectors (*IL6, IFNA1, IFNB1, TNF, CXCR3*), the 'purinergic receptor-inflammasome-interleukin-1' axis (*P2RX7, CASP1, NLRP3, IL1B, IL1R1*) and toll-like receptor signaling (*TLR4, MYD88, LY96*). This productive 'priming' of the innate immune system paves way for effective activation of various adaptive immune mechanisms[1,14] e.g. T cell infiltration (*CD4, CD8A/B*) and T cell effector activity (*IFNG, IFNGR1, IL17A, IL17RA* and *PRF1*). Last but not least, ICD is expected to negatively regulate or blunt the immunosuppressive effects of various antiinflammatory innate (*IL10*) or adaptive (*FOXP3*) immune factors (Table 1, Fig. 1).[2,3] As evident, many of these ICD-derived parameters are actually also part of

Table 1. The main parameters of immunogenic cell death (ICD) characterized in human and murine experimental systems.

Molecule	Human Gene	Effect on ICD-associated anticancer immunity	Experimental System	Putative Prediction (based on experimental data) for Clinical Survival/ prognosis if molecules show high expression#
Molecules that can act as ICD-associated danger signals or danger signal-degraders				
CD39	*ENTPD1*	Overexpression compromised ICD[63]	Mice	*Poor*
CD73	*NT5E*	Overexpression compromised ICD[64]	Mice	*Poor*
CRT	*CALR*	Ablation compromised ICD[17-19,65]	Mice	Good
HMGB1	*HMGB1*	Ablation compromised ICD[22]	Mice	Good
HSP90	*HSP90AA1*	Inhibition compromised ICD[66]	Human	Good
Molecules participating in ICD execution as danger signaling components				
ATG5	*ATG5*	Ablation compromised Chemotherapy-induced ICD;[21,27] Ablation enhanced Hyp-PDT induced ICD;[16]	Mice Human	Context-dependent
BAX	*BAX*	Ablation compromised Chemotherapy-induced ICD and partially but not completely Hyp-PDT induced ICD[17,18]	Mice	Context-dependent
Caspase-8	*CASP8*	Ablation compromised Chemotherapy-induced ICD but not Hyp-PDT induced ICD[17,18]	Mice	Context-dependent
ERp57	*PDIA3*	Ablation compromised Chemotherapy-induced ICD but not Hyp-PDT induced ICD[67,68]	Mice	Context-dependent
PERK	*EIF2AK3*	Ablation compromised Chemotherapy-induced ICD and Hyp-PDT induced ICD; Considered a "core" component;[17,18,69]	Mice	Good
PI3K p110α	*PIK3CA*	Ablation compromised Chemotherapy-induced ICD and Hyp-PDT induced ICD; Considered a 'core' component;[17]	Mice	Good
Innate Immune Effectors associated with ICD				
CXCR3	*CXCR3*	Ablation compromised ICD[1]	Mice	Good
IFNα/β	*IFNA1, IFNB1*	Increased amount enhanced ICD[1,70]	Mice	Good
IL-10	*IL10*	Low IL10 associated with enhanced ICD[16,17,71-73]	Mice/Human	*Poor*
IL-6	*IL6*	High IL6 associated with enhanced ICD[16, 17,72,73]	Mice/Human	Good
TNF	*TNF*	High TNF associated with enhanced ICD[73]	Mice/Human	Good
'Purinergic Receptor-Inflammasome-interleukin1β axis' associated with ICD				
Caspase 1	*CASP1*	Ablation compromised ICD[74]	Mice	Good
IL1 Receptor	*IL1R1*	Ablation compromised ICD[74]	Mice	Good
IL1β	*IL1B*	Ablation compromised ICD[74]	Mice	Good
Nlrp3	*NLRP3*	Ablation compromised ICD[74]	Mice	Good
P_2X_7 Receptor	*P2RX7*	Ablation compromised ICD[74]	Mice	Good
Toll-like Receptor Signaling associated ICD				
Ly96	*LY96*	Ablation compromised ICD[22]	Mice	Good
Myd88	*MYD88*	Ablation compromised ICD[22]	Mice	Good
TLR4	*TLR4*	Ablation compromised ICD[22]	Mice	Good
T cell infiltration pattern associated with ICD				
$CD4^+$ T cells	*$CD4^+$*	Depletion of these cells compromised ICD[1, 22,25-27,71]	Mice	Good
$CD8^+$ T cells	*$CD8^+A$, $CD8^+B$*	Depletion of these cells compromised ICD[1, 22,25-27,71,75]	Mice	Good
Foxp3+ Treg cells	*FOXP3*	Decreased amount associated with enhanced ICD[72,73]	Mice	*Poor*
T cell effectors associated with ICD				
IFNγ	*IFNG*	Ablation compromised ICD[22]	Mice	Good
IFNγ Receptor	*IFNGR1*	Ablation compromised ICD[22]	Mice	Good
IL-17A	*IL17A*	Ablation compromised ICD[25]	Mice	Good
IL-17A Receptor	*IL17RA*	Ablation compromised ICD[25]	Mice	Good
Prf1	*PRF1*	Ablation compromised ICD[74]	Mice	Good

Abbreviations: CD – Cluster of differentiation; CRT – Calreticulin; ERp57 – Endoplasmic reticulum protein 57; HMGB1 – High mobility group box 1; HSP – Heat shock protein; Hyp-PDT – Hypericin-based Photodynamic Therapy; ICD – Immunogenic cell death; IFN – Interferon; IL – Interleukin; PERK – Protein kinase RNA-like endoplasmic reticulum kinase; PI3K – Phosphoinositide 3-kinase;
PRF – Perforin; TLR – Toll-like receptor; TNF – Tumor necrosis factor;
#'Good' means the high expression of a given molecule can be predicted to be associated with better survival/better prognosis and 'Poor' means vice-versa. In case of conflicting experimental data, the prognosis can be predicted to be context-dependent rather than conclusively good or poor.

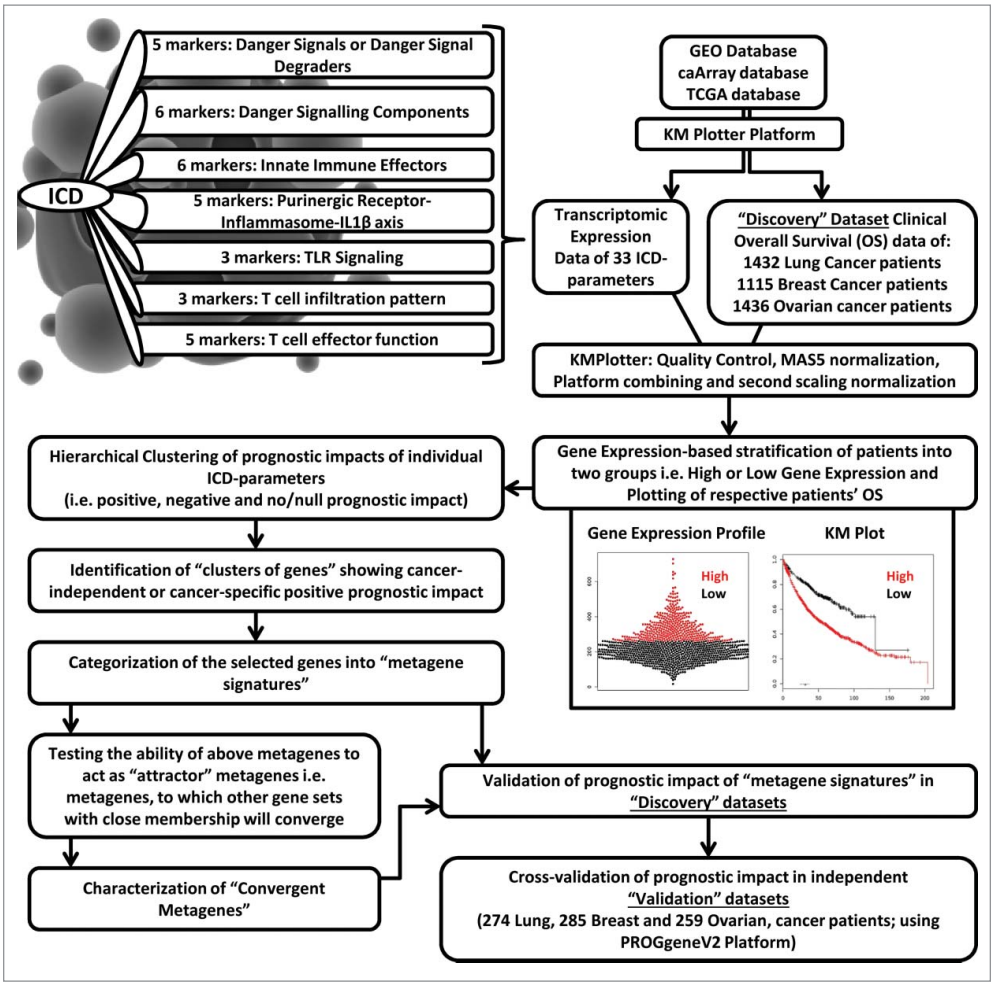


Figure 1. Schematic representation of the meta-analysis 'pipeline' for characterization and discovery of ICD-derived metagene signatures with prognostic relevance in cancer patients.

well-established classical immunological processes known to have prognostic impact e.g., innate immune effectors, T cell infiltrates and T cell effectors.[9,37]

Transcript levels of ICD-associated parameters exhibit a highly-clustered and largely cancer type-specific association with overall survival

Next, we evaluated whether the overall transcript-expression levels of various ICD-associated parameters [1-3] (Table 1) associate with OS of patients in 'discovery datasets' of non-small cell lung cancer (hereafter referred to as lung cancer; n = 1,432),[38] breast cancer (n = 1,115)[39] and ovarian cancer (n = 1,436) (Table S1) (Table 1, Fig. 1).[40]

To this end, the patients were first stratified into two groups on the basis of high or low expression of respective ICD-associated parameters (Fig. 1). The gene expression profiles of the respective ICD-parameters, and their median-based stratification into high or low expression, is depicted through the agency of bee-swarm scatter-plots for each gene per cancer-type in Figs. S1–S4. As visible, the overall gene expression profiles were rather stable thereby allowing appreciable sensitivity and providing a dynamic range for stratification. Thereafter, the OS of the two patient groups for respective ICD-parameters and cancer-types was estimated and is represented by various Kaplan–Meier (KM) plots in Figs. S5–S11. The KM plots data (Figs. S5–S11) was further summarized as a heatmap-based clustered representation of the respective Hazard Ratios (HRs) (Fig. 2A). As evident, the HRs patterns were very similar for various ICD-parameters between breast and ovarian cancers while lung cancer had a much more distinct HRs profile (Fig. 2A). Incidentally, certain ICD-parameters exhibited the highest (e.g., *CALR, IL6, PDIA3, HMGB1, BAX*) and lowest (*IL1R1, ENTPD1, NLRP3, IFNGR1, ATG5*) HRs exclusively in lung cancer patients.

Next, we decided to perform heatmap based-clustering of the prognostic impacts of various ICD-parameters for respective cancer-types based on 3 profiles (Fig. 1) i.e. positive (statistically significant association between high gene expression and prolonged OS), negative (statistically significant association between high gene expression and shorter OS) and null (statistically non-significant association between differential gene expression and OS). These analyses revealed that the prognostic impact of ICD-associated parameters, similar to the HRs profiles (Fig. 2A), exhibited a highly-clustered pattern (at statistical thresholds of both $p < 0.05$ and $p < 0.01$), which was strongly cancer type-specific (Fig. 2B-C). As many as 14 different prognostic clusters were formed by ICD-associated parameters

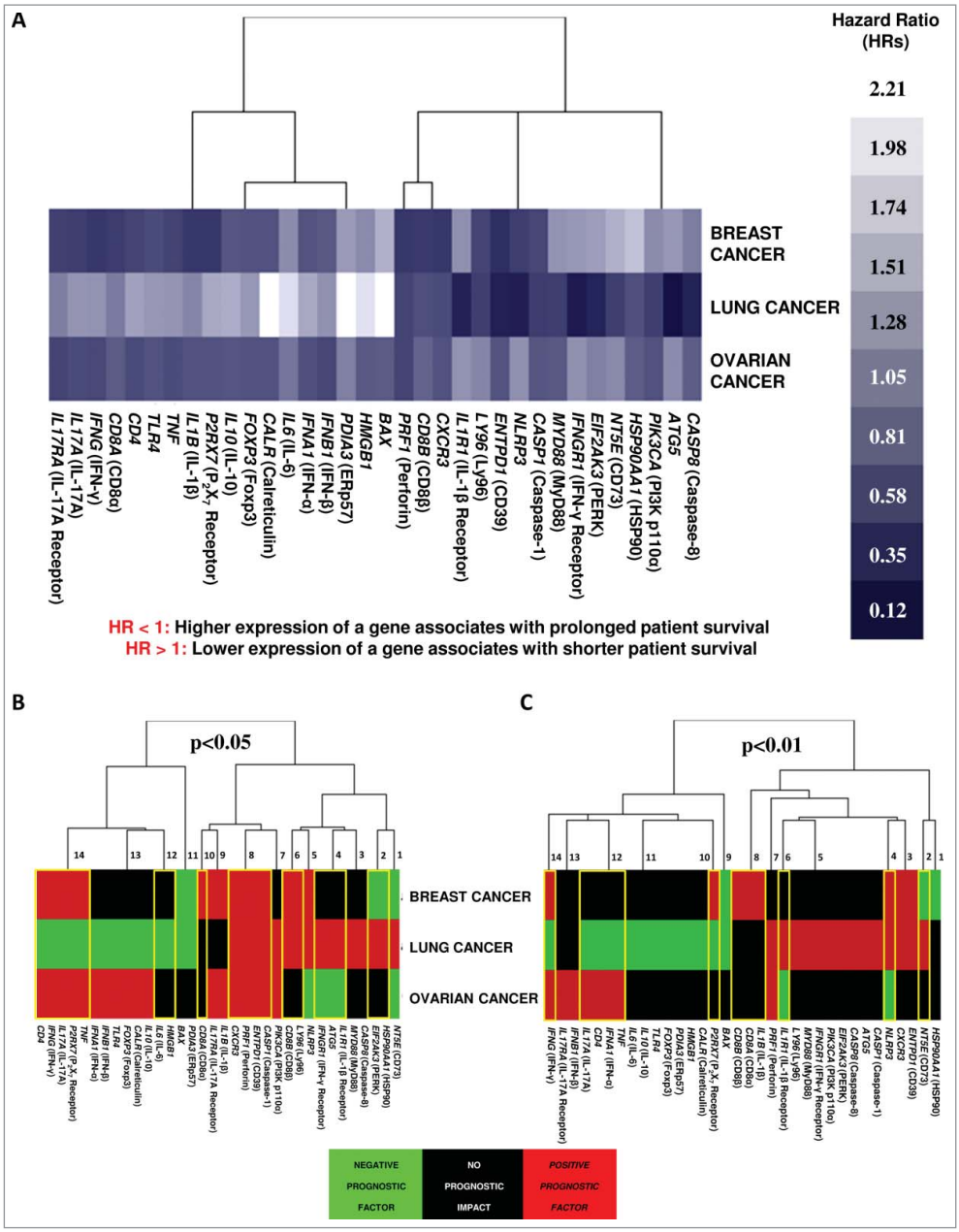


Figure 2. ICD-associated parameters show a cancer type-dependent, highly-clustered prognostic impact with variable overlap between different cancer types. (A) HRs obtained from KM Plot profiles of each ICD-associated parameter for each cancer type (see Figs. S5–S11) were pooled followed by hierarchical clustering represented through a heatmap. The legend within the graph explains the relationship between HR values and prognostic impact of each ICD-associated parameter. Subsequently, individual ICD-associated parameters were observed to show three types of prognostic profiles which were color coded i.e. high expression showing positive prognostic impact (red), high expression showing negative prognostic impact (green) and differential expression showing no conclusive prognostic impact (black). These profiles were then 'pooled' followed by either hierarchical clustering represented through a heatmap. Heatmaps for these respective prognostic profiles at shown for two different statistical significance thresholds i.e., $p < 0.05$ (B) and $p < 0.01$ (C). Alternate clusters in the heatmap are demarcated through yellow-lined boxes to improve interpretation.

relative to their impact on OS in breast/ovarian/lung cancer patients (Fig. 2B-C), a rather unexpected finding considering that, on the basis of available experimental data (Table 1), ICD-associated parameters were chiefly expected to cluster into three groups (i.e., positive/negative/context-dependent) irrespective of cancer type-differences.

Compared to other two cancer-types, lung cancer patients presented the highest propensity for a negative prognostic impact of high transcript-levels of ICD-associated parameters (at $p < 0.05$; Fig. 2B). Clusters 11-to-14 consisting of 15 ICD-associated parameters showed association with shorter OS when highly expressed in lung cancer patients (Fig. 2B). In contrast, in breast/ovarian cancer, no more than five ICD-associated parameters showed an association with shorter OS when highly expressed (Fig. 2B). On the flip-side, all three cancer-types presented nearly similar number of associations between prolonged OS and high transcript-levels of ICD-associated parameters (at $p < 0.05$, Fig. 2B; 18 for ovarian, 16 for lung and 15 for breast cancer). It is noteworthy though that only for lung cancer, the association of respective ICD-associated parameters with OS was largely insensitive to stricter levels of statistical significance thresholds from $p < 0.05$ (Fig. 2B) to $p < 0.01$ (Fig. 2C). In case of breast cancer and especially ovarian cancer,

making the statistical significance threshold stricter to $p < 0.01$ (Fig. 2C), largely ablated the significant associations (observed at $p < 0.05$) between ICD-parameters expressions and OS (Fig. 2B). Thus, ICD-parameters' ability to associate with prolonged or shorter OS in lung cancer might be more stable (than breast/ovarian cancers) with respect to statistical significance thresholds.

Across the three cancer-types, very few ICD-parameters (e.g., *CASP1, CXCR3, PRF1*) exhibited an association with prolonged OS when highly expressed, in a cancer type-independent manner (at statistical significance threshold of $p < 0.05$ but not at $p < 0.01$) and in a manner consistent with putative predictions-based on preclinical evidence (Fig. 2B–C; Table 1). Not a single ICD-parameter exhibited association with shorter OS across all three cancer-types when highly expressed (Fig. 2B–C). However, in order to allow higher coverage across all three cancer-types, we decided to continue with the statistical significance threshold of $p < 0.05$.

Cancer type-specific and -independent, ICD-derived consensus-metagene signatures are associated with prolonged survival in breast, ovarian and lung cancer patients

The above results raised an important question i.e. do the ICD-derived clusters of genes with individual cancer type-specific or independent prognostic impacts may act as a co-expressed entity i.e., consensus-metagenes? And if so, then do they preserve their positive prognostic impact when treated as a metagene signature? To address this question, cancer type-specific/-independent ICD-derived clusters of genes were defined by considering the genes showing both association with prolonged OS (Fig. 2B) and consistency with experimental evidence-based putative predictions (Table 1). Thus, following ICD-derived clusters of genes were delineated: breast-cancer specific (*TNF/CXCR3/P2RX7/CASP1/NLRP3/ IL1B/LY96/CD4$^+$/CD8$^+$A/ CD8$^+$B/PRF1/IFNG/IL17A/IL17RA*); lung cancer-specific (*HSP90AA1/ EIF2AK3/PIK3CA/CASP8/ATG5/CXCR3/CASP1/ NLRP3/IL1R1/LY96/MYD88/PRF1/IFNGR1*); ovarian cancer-specific (*CALR/PIK3CA/TNF/IFNA/IFNB1/CXCR3/P2RX7/CASP1/ IL1B/TLR4/CD4$^+$/PRF1/ IFNG/IL17A/IL17RA*); and cancer type-independent (*CXCR3/CASP1/PRF1*). Next we determined, through hierarchical clustering, whether the genes within these respective clusters had the tendency to exhibit aggregate patterns of co-ordinated expression. Hence, Pearson's correlations were calculated between the expression levels of all the clusters of genes in the respective cancer-types. As evident in Fig. 3A-F, all the genes in the respective clusters exhibited, largely, the tendencies to positively correlate with various other's expression levels and thus act as metagene signatures. Genes within the cancer type-independent metagene exhibited very similar co-expression patterns across breast (Fig. 3A), lung (Fig. 3B) and

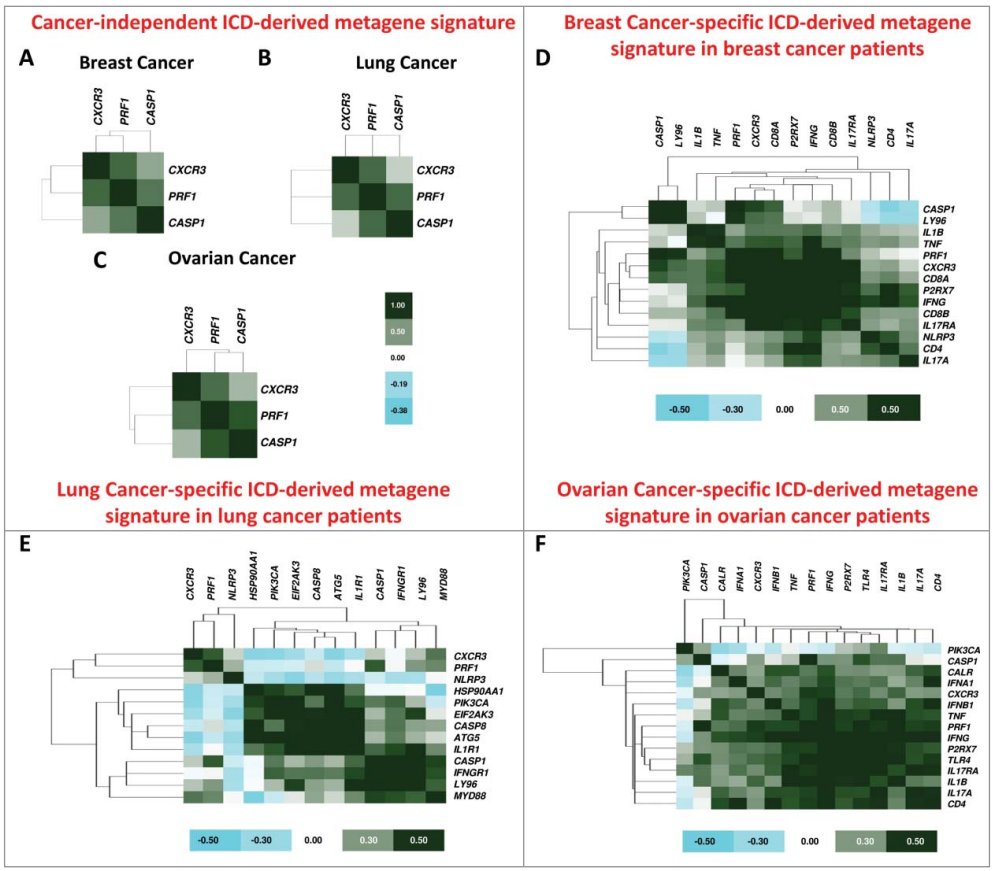


Figure 3. ICD-derived 'clusters-of-genes' with individual positive prognostic impacts largely behave like co-expressed metagene entities. Generation of gene co-expression profiles for establishing metagene profiles was accomplished by correlating the expression profiles of individual genes for respective cancer types with other genes as applicable and Pearson's correlation coefficient (r) was used for indicating tendency to co-express. Presented here are correlation/co-expression profiles for cancer type-independent metagene in breast (A), lung (B) and ovarian (C) cancer patients. Also presented are profiles for breast cancer-specific metagene in breast cancer patients (D), lung cancer-specific metagene in lung cancer patients (E) and ovarian cancer-specific metagene in ovarian cancer patients (F). The color code is represented as legend.

ovarian (Fig. 3C) cancers. Genes within breast cancer-specific (Fig. 3D) and ovarian cancer-specific (Fig. 3F) metagenes showed very high positive correlations/co-expression patterns (and relatively lower negative correlations), thereby indicating that in these two cancer-types, the immune or inflammatory reactions might operate in a much more unified manner.[41] On the other hand, the genes within lung cancer type-specific metagene (Fig. 3E), while showing considerable positive co-expression patterns also showed a certain degree of negative correlations (higher than breast/ovarian cancers) for very specific genes thereby further exposing the heterogeneity of inflammatory/immune reactions in this cancer-type. Overall, these analyses revealed the presence of various cancer type-specific and independent ICD-derived consensus-metagene signatures with considerable tendency to show co-expression.

Subsequent to the above metagene characterizations, we decided to estimate the prognostic impact of these individual metagene signatures across the 'discovery' datasets of all three cancer-types. High expression of breast cancer-specific ICD-derived metagene signature associated with prolonged OS in breast (Fig. 4A) and ovarian cancer patients (Fig. 4C), without having any significant prognostic impact in lung cancer (Fig. 4B). High expression of lung cancer-specific ICD-derived metagene signature associated with prolonged OS only in lung cancer patients (Fig. 4E) but not in breast (Fig. 4D) or ovarian (Fig. 4F), cancer patients. Furthermore, high expression of ovarian cancer-specific ICD-derived metagene signature associated with prolonged OS in ovarian cancer patients (Fig. 4I) and to a certain extent with prolonged OS in breast cancer patients (close but not significant; Fig. 4G). Remarkably, the cancer type-independent ICD-derived metagene signature showed a highly reliable association with prolonged OS when highly expressed in breast (Fig. 4J), lung (Fig. 4K) and ovarian (Fig. 4L) cancer patients.

Based on above trends, in histological terms, it is worth considering that, breast cancer and ovarian cancers are mostly of adenocarcinoma (ADC) histological-type[39,40,42] whereas lung cancer can be relatively more strongly subdivided into ADC and squamous cell carcinoma (SCC) across patients.[38,42] Thus, we analyzed whether the respective metagene signatures show association with prolonged OS in both patients with lung-ADC or lung-SCC. As apparent in Fig. 5, the stratification of patients based on histological sub-type of lung cancer did not largely affect the association between prolonged OS and higher expression of cancer type-independent/lung cancer-specific ICD-derived metagene signatures. This means that at least in this set-up, histological sub-type is not a very strong regulator of prognostic impact of ICD-derived metagene signatures.

Lastly, we also validated/cross-confirmed the prognostic impact of above ICD-derived metagene signatures in independent 'validation' datasets of all three cancer-types (Table S2). Our analysis showed that, the cancer type-independent ICD-derived metagene signature, when highly expressed, significantly associated with prolonged relapse-free survival (RFS) in breast (Fig. 6A) and prolonged OS in ovarian (Fig. 6C) cancer patients. Similarly, an association between high expression of this metagene signature and prolonged OS, was also visible in lung cancer patients (Fig. 6B) (as evident from the HR = 0.81, 95% CI = 0.65–1.00 and higher median survival of high expression 'cohort' over low expression cohort i.e. 2,444 vs. 1,662 d) and was nearly significant ($p = 0.053$). Of note, a dataset with RFS was used for breast cancer since datasets with OS data for >250 breast cancer patients independent of those used as 'discovery dataset' were not publicly available. Furthermore, high expression of breast cancer-specific, lung cancer-specific and ovarian cancer-specific ICD-derived metagene signatures considerably associated with prolonged RFS or OS in breast cancer patients (Fig. 6D), lung cancer patients (Fig. 6E) (as evident from the HR = 0.71, 95% CI = 0.50–1.00 and higher median survival of high expression 'cohort' over low expression 'cohort' i.e., 2,444 vs. 1,486 d) and ovarian cancer patients (Fig. 6F), respectively.

In conclusion, ICD is a promising source of cancer type-independent and —specific consensus-metagene signatures with high positive prognostic impact.

ICD-derived consensus-metagenes act as 'attractors' for highly co-expressed convergent-metagenes that are completely cancer type-specific in composition

Having established the presence of consensus-metagenes derivable from ICD, we wondered whether these can act as attractor-metagenes for novel sets of strongly correlating (ICD non-related) genes[11] with promising prognostic impact.[11] In order to address this probability, we first analyzed the sets of genes showing highly correlated expression with the genes composing the cancer type-independent ICD-derived metagene (i.e. *CXCR3, PRF1, CASP1*) across all three cancer-types (Fig. 1). Interestingly, hierarchical clustering showed that *CXCR3-PRF1-CASP1* collectively show highly correlated expression with *CD53, APOBEC3G* (apolipoprotein B mRNA editing enzyme), *CCR5* (chemokine C-C motif receptor 5), *LCP2* (lymphocyte cytosolic protein 2) in breast cancer patients (Fig. 7A), *PSTPIP1* (proline-serine-threonine phosphatase interacting protein 1), *CD2, CD247, SAMD3* (sterile alpha motif domain containing 3), *PTPN7* (protein tyrosine phosphatase, non-receptor type 7), *CCR5, ARHGAP9* (Rho GTPase activating protein 9), *IL12RB1* (interleukin 12 receptor, beta 1) in lung cancer (Fig. 7B) and *IL2RB* (interleukin 2 receptor, beta), *IL2RG* (interleukin 2 receptor, gamma), *CD2, GZMA* (granzyme A), *CCL5* (chemokine C-C motif ligand 5) in ovarian cancer (Fig. 7C). This additional and novel approach further reaffirms the observations above that for each cancer type, immunological signatures can be rather neatly differentiated between a broad cancer type-independent and a precise cancer type-specific clusters of genes.

The presence of these interesting sets of ICD non-related 'convergent' genes made us curious about their functional significance. To this end, we did a Gene Ontology (GO) Biological Process enumeration analysis for molecular networks for each of the 'convergent' sets of genes or metagenes. Interestingly, each convergent-metagene associated with different GO Biological Processes, in a cancer type-dependent fashion. For instance, in case of breast cancer, the convergent-metagene enumerated processes that were reminiscent of broad immunological/inflammatory reactions (Fig. 7D). On the other hand, other convergent-metagenes enumerated more specific processes such that lung cancer-specific convergent-metagene was

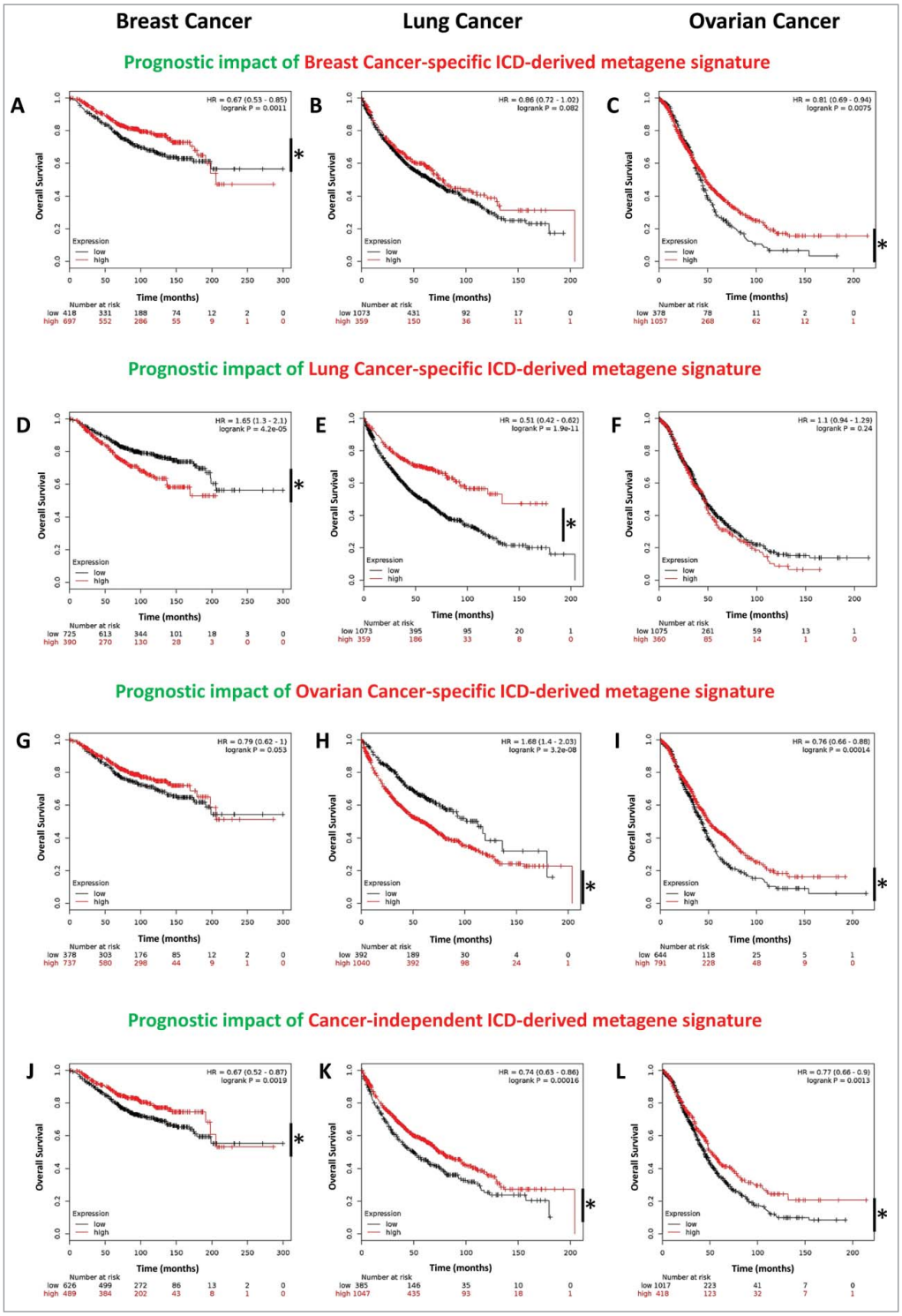


Figure 4. Cancer type-specific and -independent, ICD-derived metagene signatures show highly robust prognostic impact in 'discovery' datasets. KM plots of OS probability (plotted on Y-axis) of breast cancer patients (A, D, G, J), lung cancer patients (B, E, H, K) and ovarian cancer patients (C, F, I, L) are shown. The respective patients have been stratified into high (red lines) or low (black lines) expression-based 'risk-groups' by considering the mean of median transcript-expressions of *TNF, CXCR3, P2RX7, CASP1, NLRP3, IL1B, LY96, CD4*$^{+}$, *CD8*$^{+}$*A/B, PRF1, IFNG, IL17A* and *IL17RA* (i.e. breast cancer-specific ICD-derived metagene signature; A-C); *HSP90AA1, EIF2AK3, PIK3CA, CASP8, ATG5, CXCR3, CASP1, NLRP3, IL1R1, LY96, MYD88, PRF1* and *IFNGR1* (i.e., lung cancer-specific ICD-derived metagene signature; D-F); *CALR, PIK3CA, TNF, IFNA1, IFNB1, CXCR3, P2RX7, CASP1, IL1B, TLR4, CD4, PRF1, IFNG, IL17A* and *IL17RA* (i.e. ovarian cancer-specific ICD-derived metagene signature; G-I); and *CXCR3, CASP1* and *PRF1* (i.e., pan-cancer ICD-derived metagene signature; J-L). The patient follow-up duration is indicated in terms of months on the X-axis. Respective Log-rank test *p*-values and HR (with its 95% confidence interval in parenthesis) are displayed. Statistical significance (i.e. $p < 0.05$) is indicated through an asterisk (*). The numbers of patients at each point of follow-up are indicated below the respective KM plots.

associated with very specific T cell activation/motility-related processes (Fig. 7E) while the one specific for ovarian cancer was associated with IL-2 lymphocytic signaling (Fig. 7F).

We extended similar analysis to cancer type-specific consensus-metagenes and observed in general that they were relatively weaker than cancer type-independent consensus-metagene in

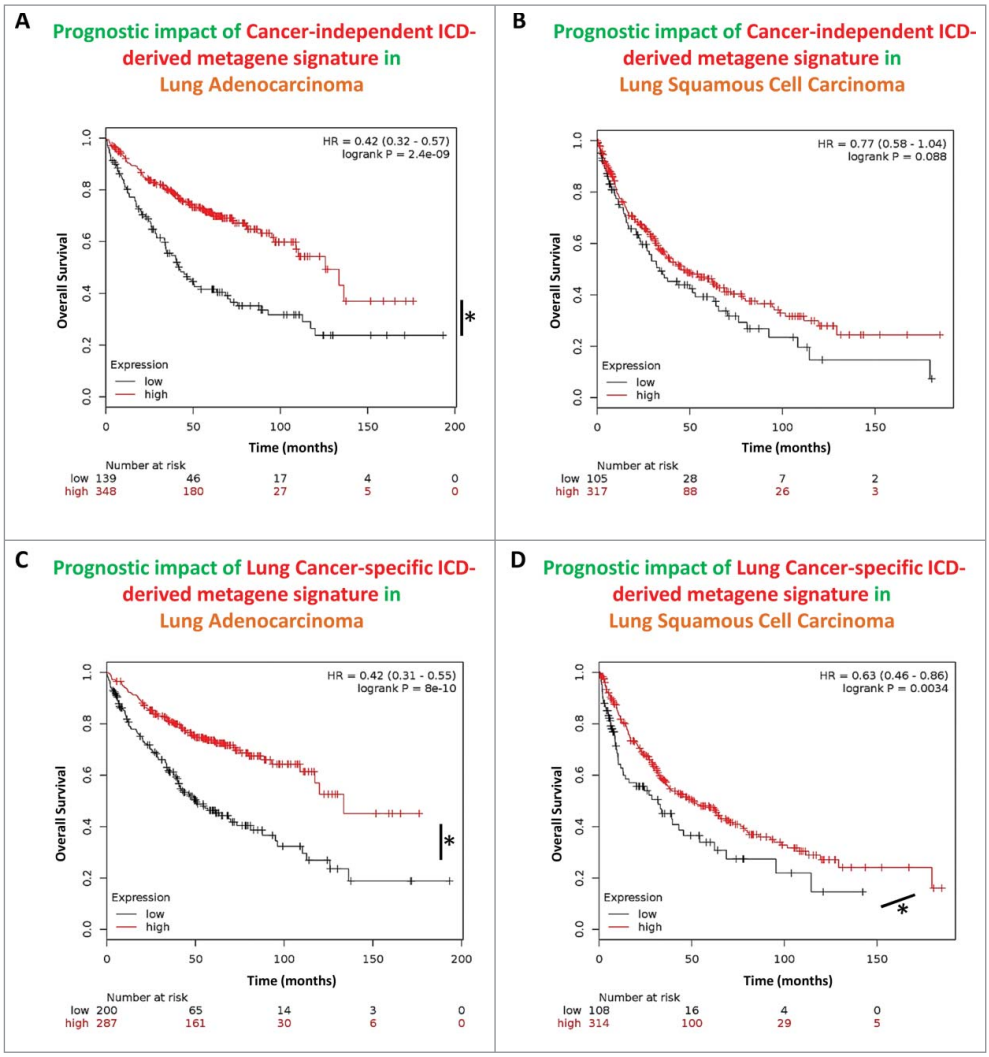


Figure 5. Lung cancer-specific and cancer type-independent, ICD-derived metagene signatures show robust prognostic impact in both Adenocarcinoma and SCC 'histotypes' of Lung Cancer. KM plots of OS probability (plotted on Y-axis) of patients having lung adenocarcinoma (A, C) or lung SCC (B, D) are shown. The respective patients have been stratified into high (red lines) or low (black lines) expression-based 'risk-groups' by considering the mean of median transcript-expressions of cancer type-independent ICD-derived metagene signature for lung adenocarcinoma (A) or SCC (B) and lung cancer-specific ICD-derived metagene signature for lung adenocarcinoma (C) or SCC (D). The patient follow-up duration is indicated in terms of months on the X-axis. Respective Log-rank test *p*-values and HR (with its 95% confidence interval in parenthesis) are displayed. Statistical significance (i.e., $p < 0.05$) is indicated through an asterisk (*). The numbers of patients at each point of follow-up are indicated below the respective KM plots.

'attracting' highly co-expressed genes with 100% coverage (across all genes in the metagene). In fact, with a coverage cut-off of 65%, convergent-metagenes could only be derived for breast and ovarian cancer-specific consensus-metagenes (Fig. 7G-J). Nevertheless, breast cancer-specific consensus-metagene 'attracted' *LILRB1* (leukocyte immunoglobulin-like receptor, subfamily B), *LCP2* (lymphocyte cytosolic protein 2), *SLAMF8* (SLAM family member 8) (Fig. 7G); which again together enumerated broad GO Biological Processes related to immunological/inflammatory reactions (Fig. 7H). Similarly, ovarian cancer-specific consensus-metagene attracted *FERMT3* (fermitin family member 3), *TAGAP* (T-cell activation RhoGTPase activating protein), *NCF1* (neutrophil cytosolic factor 1), *PTPRC* (protein tyrosine phosphatase, receptor type, C) (Fig. 7I); which together enumerated for GO Biological Processes related to immune cell differentiation/morphogenesis (Fig. 7J).

The observation that ICD-derived consensus-metagenes could act as 'attractors' for immunologically impactful convergent-metagenes, prompted us to see whether like their attractor-metagene counterparts, the respective convergent-metagenes could also be associated with positive patient prognosis. To address this question, we decided to test the prognostic impact of the respective convergent-metagenes derived above, in our 'discovery' and 'validation' datasets (Fig. 1). Interestingly, high expression of respective cancer type-specific convergent-metagenes derived either from cancer type-independent or -specific attractor-metagene, strongly associated with prolonged OS in breast (Fig. 8A, D), and ovarian (Fig. 8C, E) cancer patients. For lung cancer, the high levels of convergent-metagene derived from cancer type-independent attractor metagene also associated with prolonged OS (Fig. 8B, G). We further successfully validated these results in 'validation' datasets both for cancer type-independent attractor-metagene associated convergent-metagenes [for breast (Fig. 8F), lung (Fig. 8G) and ovarian (Fig. 8H) cancer patients] as well as to a larger extent for cancer type-specific attractor-metagene associated convergent-metagenes [for breast (Fig. 8I) and ovarian (Fig. 8J) cancer patients].

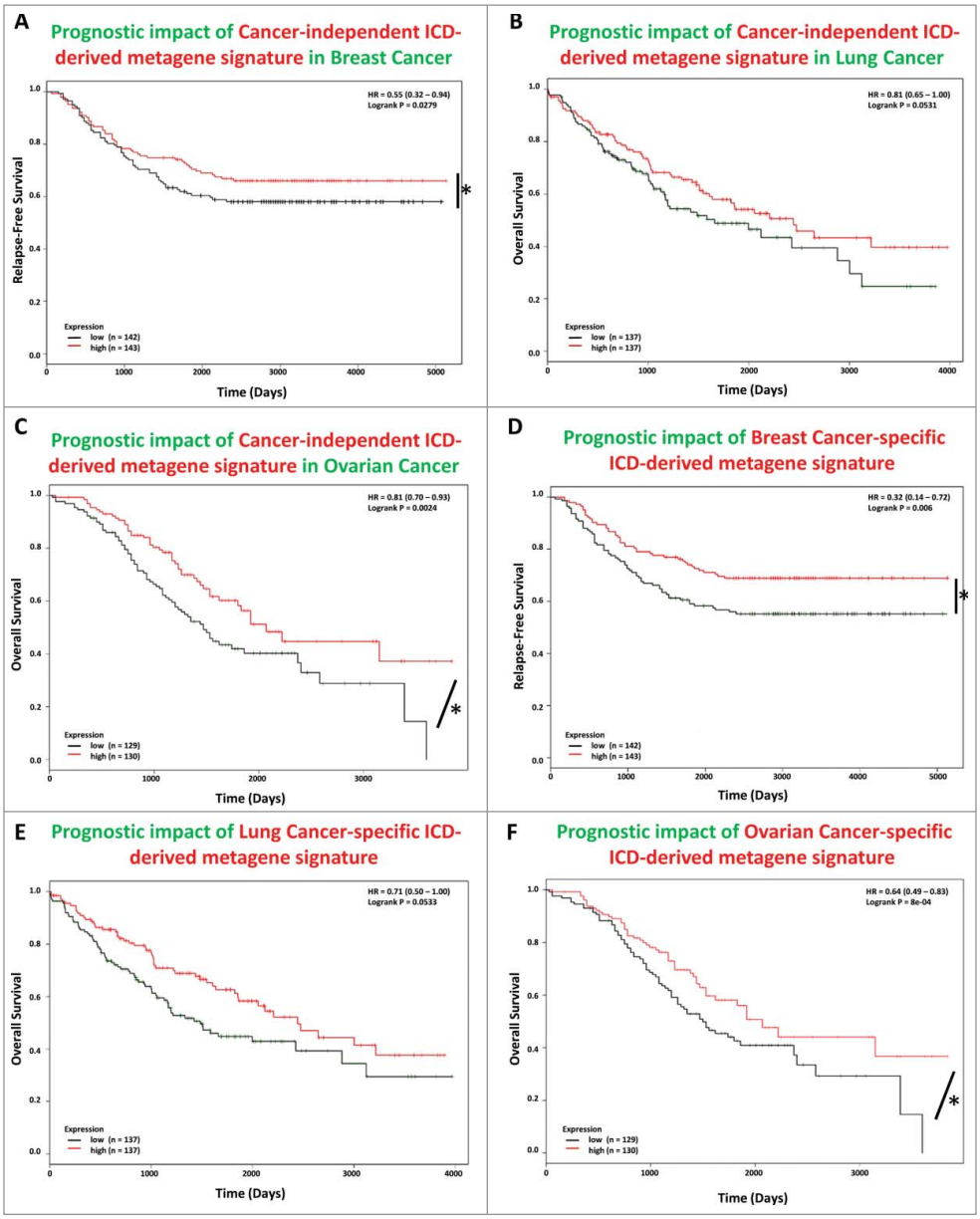
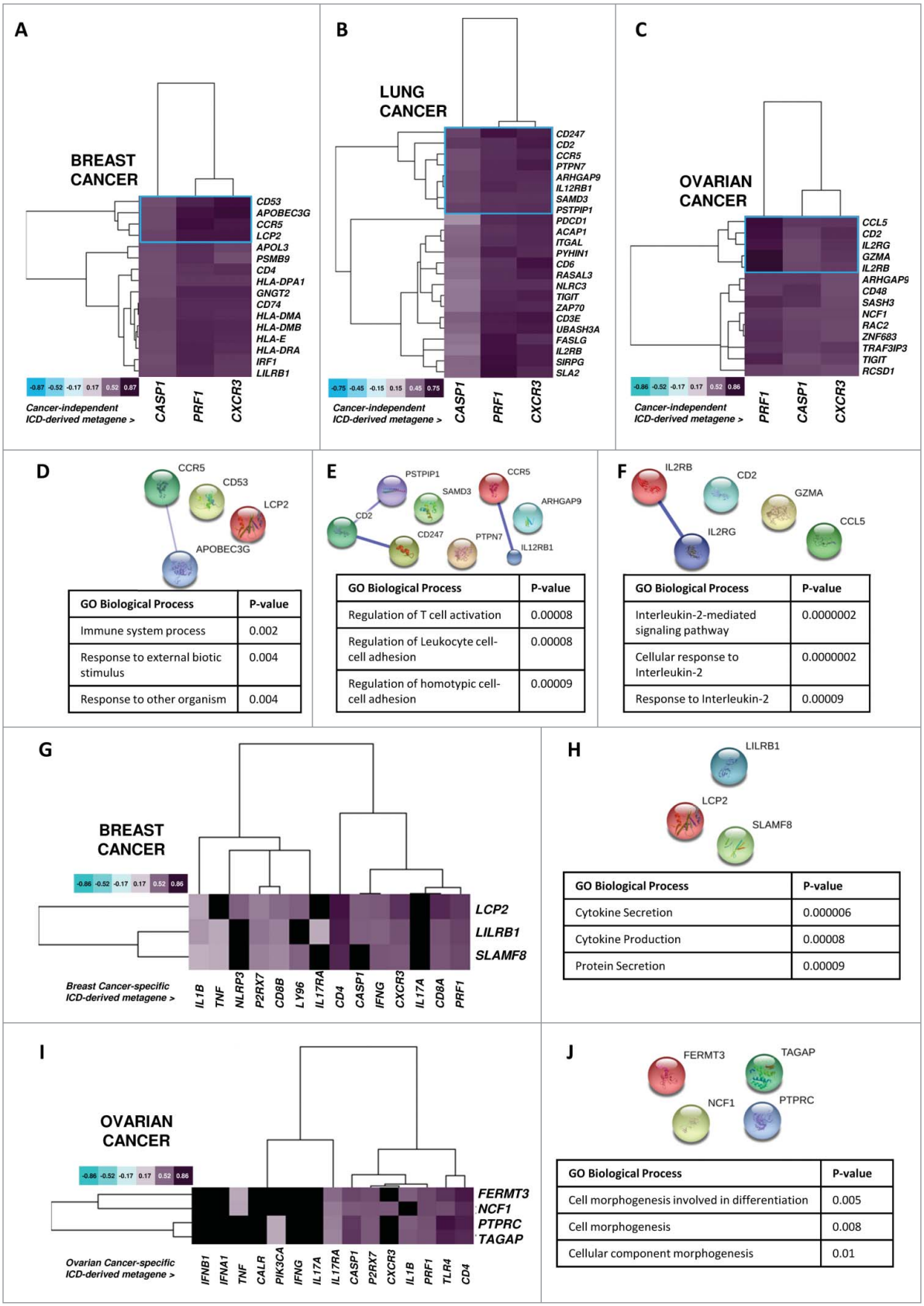


Figure 6. Cancer type-specific and -independent, ICD-derived metagene signatures show highly robust prognostic impact in 'validation' dataset. KM plots of OS probability (plotted on Y-axis) of breast cancer patients (A, D), lung cancer patients (B, E) and ovarian cancer patients (C, F) are shown. The respective patients have been stratified into high (red lines) or low (black lines) expression-based 'risk-groups' by considering the mean of median transcript-expressions of cancer type-independent ICD-derived metagene signature (*CXCR3, CASP1* and *PRF1*) for breast cancer (A), lung cancer (B) and ovarian cancer (C); breast cancer-specific ICD-derived metagene signature (*TNF, CXCR3, P2RX7, CASP1, NLRP3, IL1B, LY96, CD4*$^{+}$, *CD8*$^{+}$*A/B, PRF1, IFNG, IL17A* and *IL17RA*) in breast cancer (D); lung cancer-specific ICD-derived metagene signature (*HSP90AA1, EIF2AK3, PIK3CA, CASP8, ATG5, CXCR3, CASP1, NLRP3, IL1R1, LY96, MYD88, PRF1* and *IFNGR1*) in lung cancer (E); and ovarian cancer-specific ICD-derived metagene signature (*CALR, PIK3CA, TNF, IFNA2, IFNB1, CXCR3, P2RX7, CASP1, IL1B, TLR4, CD4, PRF1, IFNG, IL17A* and *IL17RA*) in ovarian cancer (F). The patient follow-up duration is indicated in terms of days on the X-axis. Respective Log-rank test *p*-values and HR (with its 95% confidence interval in parenthesis) are displayed. Statistical significance (i.e. $p < 0.05$) is indicated through an asterisk (*).

In conclusion, ICD-derived consensus-metagenes are capable of acting as attractor-metagenes for completely cancer type-specific convergent-metagenes with largely stable positive prognostic impact.

Discussion

The present study confirms that experimentally well-established process of ICD can serve as a platform for characterization of novel prognostic metagenes-based biomarkers (based on mRNA expression of largely immunologically-relevant genes). We believe that this study represents a comprehensive retrospective meta-analysis assessing the ability of ICD-associated parameters to act as prognostic biomarkers. Although ICD had coverage large enough to allow discovery of both cancer type-specific/-independent biomarker metagenes (at $p < 0.05$) yet not all individual ICD-parameters showed prognostic impact that was consistent with their experimental positioning. This shows that in a bottom-up approach of biomarker analysis based on a broad biological process, there is higher probability of uncovering specific metagenes as prognostic biomarkers rather than all the genes associated with that process.[43] This point is further validated by the observation that previously published studies, utilizing largely the same publicly available



GO Biological Process	P-value
Immune system process	0.002
Response to external biotic stimulus	0.004
Response to other organism	0.004

GO Biological Process	P-value
Regulation of T cell activation	0.00008
Regulation of Leukocyte cell-cell adhesion	0.00008
Regulation of homotypic cell-cell adhesion	0.00009

GO Biological Process	P-value
Interleukin-2-mediated signaling pathway	0.0000002
Cellular response to Interleukin-2	0.0000002
Response to Interleukin-2	0.00009

GO Biological Process	P-value
Cytokine Secretion	0.000006
Cytokine Production	0.00008
Protein Secretion	0.00009

GO Biological Process	P-value
Cell morphogenesis involved in differentiation	0.005
Cell morphogenesis	0.008
Cellular component morphogenesis	0.01

Figure 7. ICD-derived consensus-metagenes act as attractor-metagenes for highly co-expressed convergent-metagenes that exhibit strong cancer type-specific compositional profile. Convergent-metagene profiles were characterized by identifying the genes that were most highly correlating/co-expressing with genes composing the respective ICD-derived consensus-metagenes. Subsequently, Protein-protein networks and GO Biological Process enumeration were derived through the STRING database. Only the top three GO Biological Processes are shown (p-value <0.05). Presented here are correlation/co-expression profiles for cancer type-independent consensus/attractor-metagene in breast (A), lung (B) and ovarian (C) cancer patients; and respective protein-protein networks and GO Biological Process enumerations in breast (D), lung (E) and ovarian (F) cancer patients. Also presented are respective co-expression/network/GO Biological Process profiles for breast cancer-specific consensus/attractor-metagene in breast cancer patients (G-H) and ovarian cancer-specific consensus/attractor-metagene in breast cancer patients (I-J).

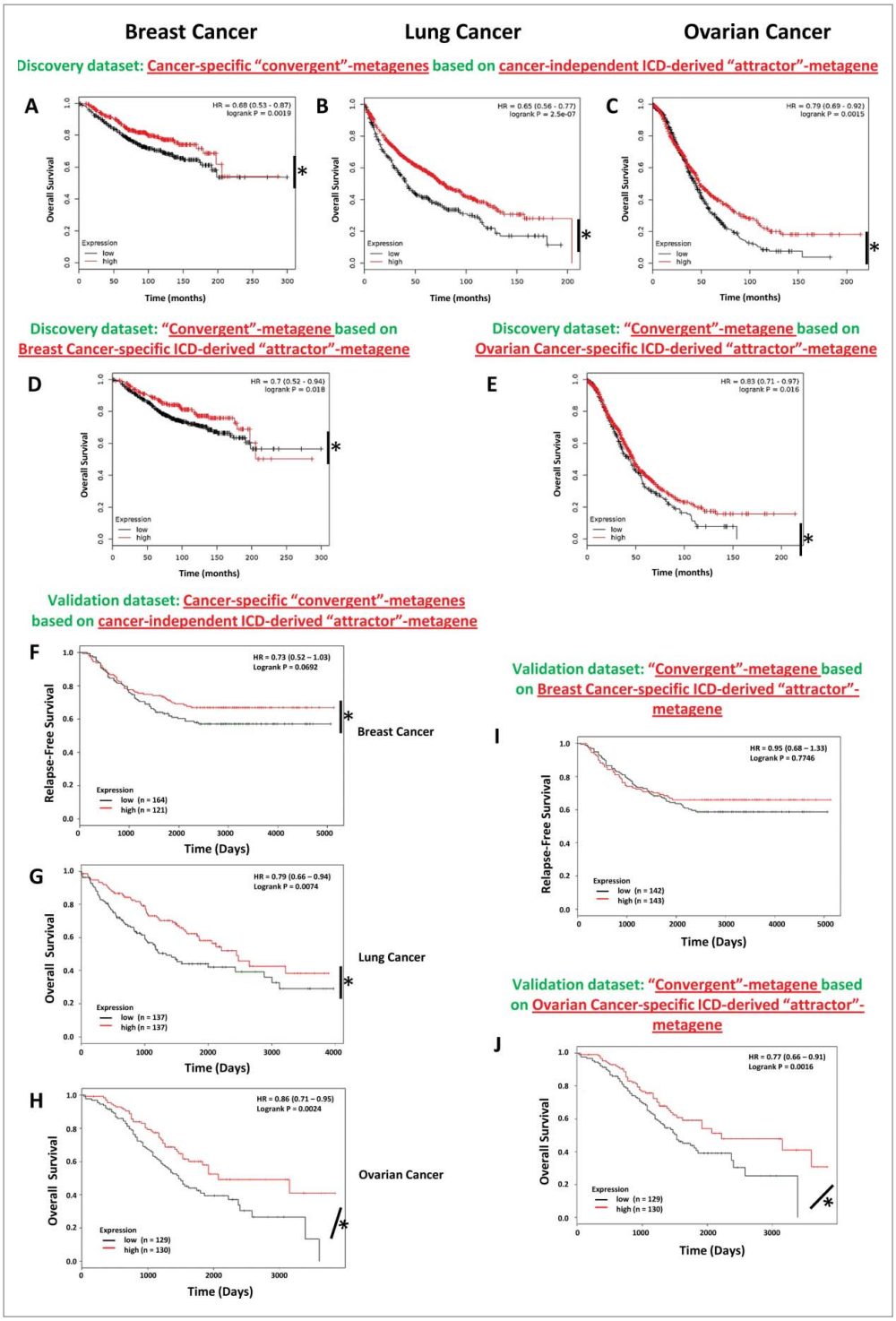


Figure 8. ICD-derived convergent-metagene signatures show highly robust prognostic impact in 'discovery' and 'validation' datasets. KM plots of OS probability (plotted on Y-axis) of breast cancer patients (A, D, F, I), lung cancer patients (B, G) and ovarian cancer patients (C, E, H, J) are shown. The respective patients have been stratified into high (red lines) or low (black lines) expression-based 'risk-groups' by considering the mean of median transcript-expressions of *CCR5, CD53, LCP2* and *APOBEC3G* (A, F); *PSTPIP1, CD2, CD247, SAMD3, PTPN7, CCR5, ARHGAP9, IL12RB1* and *APOBEC3G* (B, G); *IL2RB, IL2RG, CD2*, GZMA and *CCL5* (C, H); *LILRB1, LCP2* and *SLAMF8* (D, I); and *FERMT3, TAGAP, NCF1* and *PTPRC* (E, J); The patient follow-up duration is indicated in terms of months or days (as applicable) on the X-axis. Respective Log-rank test *p*-values and HR (with its 95% confidence interval in parenthesis) are displayed. Statistical significance (i.e., $p < 0.05$) is indicated through an asterisk (*).

datasets as we utilized, for a top-down approach of biomarker discovery, were able to delineate very few immune-pathway related genes with prognostic impact (namely *CD24* for lung cancer and *IFNG* for ovarian cancer).[38-40]

Moreover, this is the first study delineating ICD-parameters based attractor- and convergent-metagenes. Overall, while both ICD-derived cancer type-specific as well as -independent consensus-metagenes were associated with significantly prolonged survival of cancer patients when highly expressed; yet it was the cancer type-independent consensus-metagene that had the best ability to act as attractor-metagene for stable convergent-metagenes with absolute coverage. However, very interestingly, the resultant convergent-metagenes were found to be largely cancer type-specific. This clearly shows that every cancer type might

have a highly 'bifurcated' immunotranscriptome profile that is partly unique to that cancer and partly overlapping with a ' pan-cancer' immunoprofile — a conjecture that makes sense in light of the high intra-tumor heterogeneity.[41,44]

Despite these bifurcations and heterogeneity, there was a distinct presence of fingerprints of T cell activity/infiltration/effector functions within respective metagenes derived from ICD e.g. the presence of *PRF1, CD8$^+$A, CD8$^+$B, IL17RA, CD4$^+$, IL17A, IFNGR1* or *IFNG* across various consensus or attractor-metagenes and the presence of *CD247, CD2, PTPN7, PSTPIP1, IL2RG, GZMA, IL2RB* across various convergent-metagenes. This reaffirms the standing notion in the field of immune-prognostics that T cell activity/function-related biomarkers have a great probability of having a 'pan-cancer' positive prognostic impact in cancer patients.[5,6,37,41] However, what was peculiar was, that it was not always the same aspect of T cell activity/function that had a prognostic impact across all cancer types. For instance, T cell infiltration (*CD8$^+$B, CD8$^+$A, CD4$^+$*) and Th17 polarization (*IL17A, IL17RA*) markers associated with prolonged survival in breast cancer patients; on the other hand, T cell motility (*CD2, PSTPIP1*) and T-cell receptor (TCR)-associated signaling (*IFNGR1, CD247, PTPN7*) based markers showed positive prognostic impact in lung cancer patients while Th1/Th17 polarization (*CD4$^+$, IFNG, IL17A, IL17RA*), T cell-based cytotoxicity (*PRF1, GZMA*) and T cell proliferation (*IL2RG, IL2RB*) related biomarkers showed positive prognostic impact in ovarian cancer patients. This clearly shows that, at least on the mRNA-level, detecting only biomarkers of T cell infiltration may not be enough and other markers related to T cell polarization, motility, cytotoxicity and proliferation might need to be integrated in a cancer type-dependent fashion — a point partly reflected upon in the recently delineated tumor-associated/infiltrating immune cell-specific metagenes.[45] Nevertheless, it is interesting that despite starting with a broad immunological process like ICD for biomarker discovery, the end result was largely dominated by the classical T cell activity/function-related markers.

Importantly, this study also brings forth some conflicting/paradoxical observations on the prognostic impact of ICD-associated danger signals (e.g., higher levels of danger signal-degraders like CD39/CD73 associating with positive patients prognosis rather than negative as suggested by experimental models: discussed in Box S1) or prognostic consistency across immuno-receptor and respective ligands/receptors' associated signaling module(s) (e.g. *IL1B* and *IL1R1* or *IFNG* and *IFNGR1* showing contrasting prognostic impacts in the same datasets despite being *bona fide* ligand-receptor pairs; discussed in Box S2). Furthermore, we also observed the contradictory scenario where putative immunostimulatory factors (e.g., *IFNG* in lung cancer) are associated with negative prognosis while putative immunosuppressive factors (e.g. *FOXP3*/*IL10* in ovarian cancer) associate with positive prognosis (discussed in Box S3).

Our study, despite being very comprehensive on the level of retrospective meta-analysis of transcript-expression, has certain limitations. The meta-analysis was performed on publicly available datasets that have not explicitly taken into consideration tumor heterogeneity. While the number of cancer patients included in this study (>1,000 per cancer-type) may partly compensate for patient-to-patient tumor heterogeneity yet emerging evidence suggests that immunological tumor heterogeneity (e.g., tumor core vs. invasive margins) needs to be taken into consideration for prognostic analysis.[6] In this sense, it would be also important to differentiate between expression profiles of cancer cells versus immune cells — a point that could not be achieved in the current study. Even more importantly, differential expression of certain immune-genes may not be representative of their signaling context. For instance, various epigenetic, post-transcriptional or post-translational modifications play an important role in governing the immunological signaling outcome.[46] Moreover, differential gene expression-levels may not always reflect the differential enzymatic-activity, mutational status or compartmentalization (e.g. surface-exposure/secretion).[3,14] Similarly, differential gene expression analysis cannot give a comprehensive idea of signaling 'fluxes' (e.g., autophagy/ER stress). Thus, in near future, it is necessary to account for the prognostic impact of actual signaling context of a given molecule possibly by analyzing in the patient tumor biopsy samples, the concerned molecule's post-translationally modified/processed/mutated form or its delocalized activities.[47] These concerns can be addressed by carrying out well-strategized/well-supervised analysis in immunohistochemistry-based tissue microarray/proteomics settings, by using the cancer type-specific/-independent ICD-derived metagene signatures characterized in the current study. If validated successfully, the ICD-derived metagene signatures can be used to produce assays (e.g. qRT-PCR) crucial for patient risk assessment during clinical decision making as done in the case of other multigene classifiers e.g., Oncotype Dx (21-gene assay)/Mammaprint (70-gene assay).[48]

In reality, the currently delineated ICD-derived attractor/convergent-metagene signatures have to still clear a number of practical hurdles before they can be regarded as clinically-applicable *bona fide* prognostic biomarkers.[43] Most importantly, these signatures have to be rigorously validated in a prospective clinical trial.[43] It is also very important to consider that the metagene signatures derived in this study may not be used in a standalone manner. It would be greatly desirable to strengthen such emerging signatures by combining them with other traditional clinico-pathological parameters[7] thereby paving way for development of integrative models; which will increase our understanding of the complex cancer type-specific genotype-phenotype interplays.[7]

Nevertheless, our observations show that ICD can be useful as a platform for discovery of novel prognostic attractor- and convergent-metagenes. Moreover, the observations that immunological prognostic biomarkers may also largely function as predictive biomarkers[37] gives a future precedence to test whether the ICD-derived attractor- and convergent-metagenes characterized in this study could also be predictive of positive patient responses to clinically-applied ICD-inducing therapies. Besides, considering that ICD is currently an evolving concept, there is an interesting possibility that in future discovery of new molecular/immunological ICD determinants can open up opportunities for discovery of new prognostic/predictive biomarkers. In near future, as more large-scale data for other cancer-types beyond breast/lung/ovarian cancer becomes available, it would be crucial to characterize both other cancer type-specific ICD-derived metagene signatures as well as validate whether the cancer type-independent ICD-derived metagene signatures characterized here are applicable for other cancer-types.

Methods

Meta-analysis 'Pipeline' description

The current study consisted of a multistep sequential 'pipeline' (Fig. 1): (1) Extensive literature-search analysis to objectively delineate the most important ICD-associated parameters to be used for prognosis-estimation analysis (Table 1); (2) Analysis concerning the individual impacts of differential transcript-expression levels of various ICD-associated parameters on OS of breast, lung and ovarian cancer patients, in large 'discovery datasets' (Table S1); (3) Heatmap-based hierarchical clustering to delineate possible cancer type-specific and -independent ICD-derived consensus metagene signatures; (4) Estimation of the prognostic impact of high expression of these ICD-derived consensus metagene signatures on OS of respective patients in 'discovery datasets' (Table S1); (5) Cross-confirmation of the prognostic impact of these ICD-derived metagene signatures on OS/ RFS of respective patients in 'validation datasets' (Table S2); (6) In parallel, subsequent to step 3, testing the ability of ICD-derived consensus metagenes to act as attractor-metagenes for highly co-expressed convergent-metagenes; and (7) Estimating the prognostic impact of newly characterized convergent-metagenes in respective 'discovery' and 'validation' datasets (Fig. 1).

In silico *prognostic biomarker assessment in 'discovery and validation datasets'*

The data used in this manuscript originated from various publicly accessible databases like The Cancer Genome Atlas (TCGA)[49] or Gene Expression Omnibus (GEO).[50] The respective (Affymetrix) microarray gene expression data and clinical survival information from TCGA/caArray/GEO databases were analyzed through the KMPlotter platform[38,40,51] for breast cancer (n = 1115, derived from the following datasets: GSE1456, GSE16446, GSE20271, GSE20685, GSE20711, GSE3494 and GSE7390), ovarian cancer (n = 1436, derived from the following datasets: GSE14764, GSE15622, GSE18520, GSE19829, GSE23554, GSE26712, GSE30161, GSE3149, GSE9891, TCGA) and non-small cell lung cancer (n = 1432, derived from the following datasetdatasets: caArray, GSE14814, GSE19188, GSE29013, GSE31210, GSE3141, GSE37745, GSE4573, TCGA). These large datasets were considered as 'discovery datasets'. The available clinicopathological characteristics of the patients in these respective 'discovery' cohorts are described in Table S1. In case of prognostic analysis for individual transcript/genes, the respective patients were stratified into two risk-groups i.e., patient group showing high gene expression and patient group showing low gene expression by considering the median expression over the entire dataset (additionally, all percentiles between lower and upper quartile were computed and best performing threshold was used as final cut-off in a univariate Cox regression analysis). For each gene, the optimal probe set was utilized by scoring through the Jetset method that filters probe sets for specificity, coverage and degradation resistance.[52] Biased arrays were excluded. The effect of differential gene expression was estimated on the OS of the patients by using KM method. Hazard ratio (and its 95% confidence intervals) and logrank *P* values were calculated (*P* values less than 0.05 were considered to be statistically significant). Patients surviving over the follow-up threshold were censored. In case of metagene signatures mean of combined expression of respective gene-probe sets were utilized. Of note, the KMPlotter avoids batch effects through a double normalization of microarray chip-derived data i.e. first a MAS5 algorithm-based normalization on individual-chip level and a second scaling normalization to set the average expression on each chip to 1,000.[39] For the 'validation dataset' analysis, the cohorts consisting of >250 patients each for breast cancer (n = 285; GSE2034),[53] non-small cell lung cancer (n = 274; GSE41271)[54,55] and ovarian cancer (n = 259; GSE32062)[56] were analyzed as described above, using the PROGgeneV2 platform.[57] The available clinicopathological characteristics of the patients in these respective 'validation' cohorts are described in Table S2. Of note, all of the datasets used in this study were based on mRNA isolated from frozen tumor tissue/biopsy material or to a lesser extent, formalin-fixed paraffin-embedded samples.

Heatmap-based hierarchal clustering and metagene analysis

For generating gene co-expression profiles for metagene signatures, the expression profiles of individual genes for respective cancer types were correlated with other genes as applicable and Pearson's correlation coefficient (r) was used for indicating tendency to co-express. On the level of prognostic analysis-related clustering, three types of prognostic impacts for each gene were 'color-coded' through arbitrary relative integer values i.e., high expression showing statistically significant positive prognostic impact was red (+1), high expression showing statistically significant negative prognostic impact was green (−1) and differential expression showing no statistically significant prognostic impact was black (0). For characterization of convergent-metagene profiles, highly correlating/co-expressing genes were delineated on the basis of genome-wide Pearson's correlation coefficient analysis derived from cBioPortal[58] for respective cancer-types. Two stiff cut-off thresholds were set to delineate co-expressing genes — (1) an overall coverage cut-off of at least 65% of the genes in an attractor-metagene and (2) an overall Pearson's correlation coefficient cut-off of 0.6 for at least 40% of the genes within the attractor-metagene. Hierarchical clustering of these respective prognostic impacts, respective HRs and Pearson correlation-based gene co-expression profiles was implemented as described elsewhere[59] through Cluster 3.0[60] and visualized as a heatmap through TreeView[61] with Euclidean Distance as the similarity metric and a centroid linkage clustering criteria. Last but not least, for GO Biological Process enumeration, the network of respective genes were created using the STRING database[62] (confidence view protein-protein networks) and the top 3 GO Biological Processes were enumerated with a *p*-value cut-off of 0.05.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed. Q4

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