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Characterization of cancer subtypes associated with clinical outcomes by multi-omics integrative clustering

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ABSTRACT

Cancer patients show heterogeneous phenotypes and very different outcomes and responses even to common treatments, such as standard chemotherapy. This state-of-affairs has motivated the need for the comprehensive characterization of cancer phenotypes and fueled the generation of large omics datasets, comprising multiple omics data reported for the same patients, which might now allow us to start deciphering cancer heterogeneity and implement personalized therapeutic strategies. In this work, we performed the analysis of four cancer types obtained from the latest efforts by The Cancer Genome Atlas, for which seven distinct omics data were available for each patient, in addition to curated clinical outcomes. We performed a uniform pipeline for raw data preprocessing and adopted the Cancer Integration via MultIkernel LeaRning (CIMLR) integrative clustering method to extract cancer subtypes. We then systematically review the discovered clusters for the considered cancer types, highlighting novel associations between the different omics and prognosis.

1. Introduction

Cancer is a heterogeneous disease, whose characterization requires the comprehension of complex molecular and cellular phenotypes, together with their interaction with the environment. It is now widely recognized that cancer patients present heterogeneous phenotypes that can lead to different responses even to common treatments, such as standard chemotherapy. Therefore, precision medicine stands as an emerging approach for cancer treatment, with the aim to exploit molecular characteristics of individual patients in order to determine the best therapeutic intervention [1].

Recently, high throughput experimental technologies have been exploited to collect large omics datasets, spanning from genomics to transcriptomics, providing multiple omics data obtained from the same patient. Such multi-omics datasets provide a unique opportunity for a comprehensive characterization of molecular and clinical features of cancer patients [2]. To this end, the identification and characterization of cancer molecular subtypes, showing a significant correlation with patients' outcomes becomes a crucial aspect.

In this work, we performed the analysis of four cancer types obtained from the latest multi-omics dataset released by The Cancer Genome Atlas (TCGA) [3], providing seven omics data for each patient, in addition to curated clinical outcomes, namely: substitutions and small insertions/deletions, copy number alterations, methylations, gene expression profiles, microRNAs, reverse-phase protein microArrays and microbiome data.

We adopted a uniform pipeline for the preprocessing of raw data and exploited the Cancer Integration via MultIkernel LeaRning (CIMLR) integrative clustering method [4] to detect subtypes from such multi-omics datasets, particularly focusing on the molecular

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characteristics that could explain significant association to prognosis. We systematically review the four considered cancer types, highlighting the valuable molecular insights achieved by our multi-omics approach, shedding some light into the biology underlying the specific tumor heterogeneity.

2. Results

We performed multi-omics integrative clustering analysis in four cancer types, showing significant association to prognosis, namely overall survival (OS) and progression-free survival (PFS), over a 10-year period. The considered cancer types were (i) bladder urothelial carcinoma, (ii) endometrial carcinoma, (iii) sarcoma and (iv) thymoma.

Our selection of the four cancer types considered in this study was based on several criteria. Firstly, we focused on cancers for which there is no clear consensus on multi-omics subtypes. Secondly, we prioritized cancers for which we had full data available, including all seven omics data types and survival data. Finally, we selected cancers for which new omics data, such as microbiome data, could potentially have a significant impact on our understanding of the disease.

2.1. Bladder urothelial carcinoma

Urothelial carcinoma of the bladder is one of the major causes of morbidity and mortality worldwide, with 430,000 new cases and more than 165,000 related deaths per year [5]. At diagnosis, 75% of the patients present non-muscle-invasive bladder cancer (NMIBC), while 25% of the patients have muscle invasive bladder cancer (MIBC), with an associated high risk to develop metastatic disease. Heterogeneity in disease response to therapy suggests that different subtypes might exist within and between NMBIC and MIBC [6]. The most comprehensive attempt to classify MIBC was proposed by Robertson and colleagues who generated separate clustering for each of 7 omics and finally integrated mRNA, lncRNA and miRNA expression clusters using the Cluster of Clusters method. This effort allowed the identification of 5 different subtypes, dominated by mRNA data: luminal-papillary, luminal-infiltrated, luminal, basal/squamous, and neuronal [7]. We applied CIMLR to the classification of 332 MIBC cases from TCGA, simultaneously integrating all available data types. Our analysis classified MIBC patients in 6 different clusters (C1-6) showing significantly different OS and PFS (see Fig. 1, Supplementary Figs. 1 and 2 and Supplementary Table 1). C1, showing the longest OS and PFS, is almost totally composed of luminal-papillary tumors and shows distinct features of this histological subtype, such as high mutational rate of FGFR3 and low mutational rate of TP53 and RB1. Moreover, C1 shows high expression of BMP5, a marker of sonic-hedgehog (SHH) signaling in luminal-papillary subtype, and high expression of BMP7, EEF1A2 and SOX15, which were identified to be downregulated in carcinoma in situ (CIS) lesions. As CIS lesions are associated with high risk of disease progression [8], this suggests that expression of such genes negatively correlates with bladder cancer aggressiveness. In addition, our multi-omics analysis revealed high demethylation and high expression of DMBT1 and MSMB genes (Fig. 1A, Supplementary Table 1). DMBT1 was previously reported in bladder carcinoma and its expression correlates with tumor grade [9], while MSMB is described as a biomarker in prostate cancer, but not in bladder [10]. Furthermore, C1 tumors exhibit copy number loss and reduced expression of GAS1, an unfavorable prognostic marker in several cancers. Finally, C1 shows high methylation level and consequently low expression of CDO1 and IGF1; CDO1 promoter is methylated in multiple human cancers [11], while IGF1 axis promotes tumorigenesis and confers resistance to treatment in cancer [12]. Hence, C1 summarizes several features from various datasets that, independently, have been linked to good prognosis, thus validating our approach.

Focusing on C6, which shows the worst OS and PFS (see Fig. 1B and Supplementary Fig. 1), we observed heterogeneous histological subtypes within the cluster (Fig. 1C) and a similar gene expression profile to C3. The two clusters show the highest levels of expression of GAS1, CDO1 and IGF1. In addition, they also have a high expression of RSPO2, a secreted glycoprotein that is known for its role in the stimulation of Wnt/β-catenin signaling and has been reported as a cancer driver [13]. Particularly, aberrant RSPO2 expression levels were associated with worse prognosis in bladder cancer [14]. C3 and C6 also share increased protein levels of RICTOR and MYH11 by RPPA. Expression of RICTOR is associated with poor clinical outcomes and resistance to treatment [15]. Notably, high levels of RICTOR and MYH11 were already identified in bladder cancer patients with poor outcome [7]. We then analyzed differences between C6 and C3, that may explain different outcomes of the

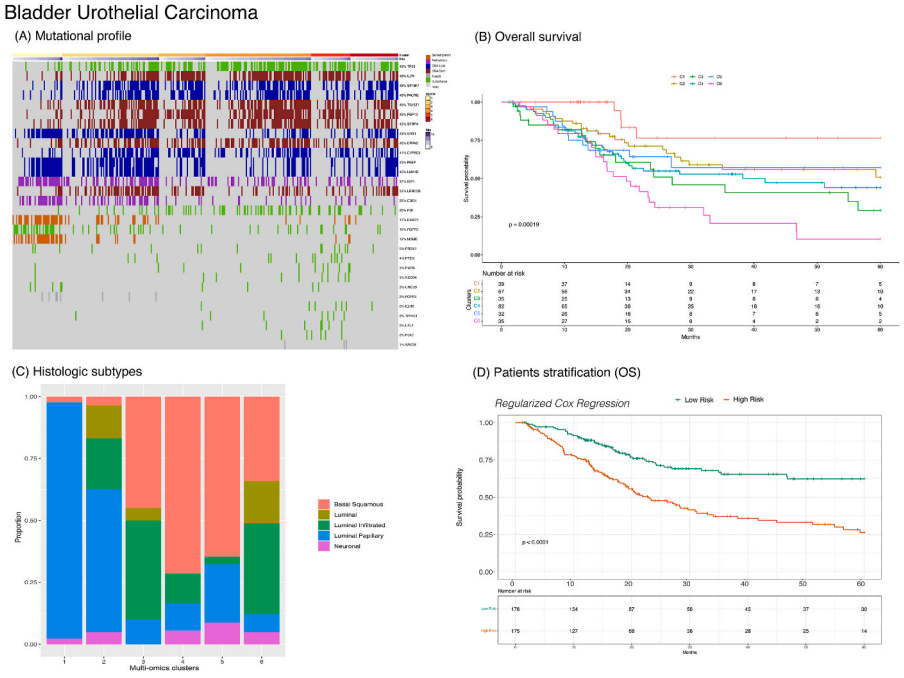

Fig. 1. Clustering analysis of 332 bladder urothelial carcinomas. In panel (A) we show the mutational profiles of the significantly different genes among the clusters (the reported percentages correspond to the proportion of patients in the dataset who have a mutation in the specific gene). In panel (B) we report the overall survival Kaplan-Meier curve comparing the discovered clusters. In panel (C) we show the histological subtypes per cluster. Finally, in panel (D) we report the Kaplan-Meier curve obtained by stratifying the patients based on regularized Cox regression considering the microbiome.

patients in the two subgroups: interestingly, we identified higher levels of FN1 in C6. FN1 is involved in cell adhesion, motility and extracellular matrix formation, and its expression correlates with unfavorable prognosis in many cancers, such as breast cancer [16] and gastric adenocarcinoma [17]. Thus, our analysis yielded a comprehensive, multi-level portray of bladder cancer patients with a poor prognosis.

Finally, in the last few years, the role of microbiota in the regulation of tumor development has gained increasing attention [18], and alterations in the urinary microbiota have been found in bladder cancer patients in comparison to healthy individuals [19]. We sought to examine a possible correlation between microbiota taxa and survival probability. Comparing microorganisms' presence across our clusters, we found that bacteria from the Methylibium, Sphaerotilus and Sediminibacterium genera, which have been reported as potential biomarkers in lung cancer [20], are represented in C1 two times more than in C6. What we found is in line with the work of Mifuchi and colleagues, who described antitumor activity of Sphaerotilus in mice, suggesting that its involvement against tumor depends on macrophages activation [21] and highlights how microbiota may be used as a novel biomarker also in bladder cancer.

To further test the association between microbiome and prognosis, we performed regularized Cox regression analysis (see Methods) to stratify patients into high-risk vs low-risk groups considering the whole microbiome. We then associated these features to survival data. In particular, bacteria of the Shimia genus were found as the most relevant risk factor for both OS and PFS. Moreover, Criblamydia, Sodalis and Whispovirus were associated with poor prognosis and Microvirus with better prognosis. Finally, Methyloferula, Microvirga, Rufibacter were associated with bad prognosis and Anaplasma, Lymphocryptovirus, Saccharophagus were associated with good prognosis for progression free survival. We finally stratified patients based on the selected features, obtaining very significant prognostic groups (see Fig. 1D and Supplementary Fig. 2), highlighting novel associations between microbiome and prognosis.

Overall, multi-omics clustering applied to the available data allowed stratification of bladder cancer patients based on multiple phenotypic features, leading to a higher resolution and highly significant associations with survival. Moreover, we uncovered a novel microbiome-based biomarker that may play an important role in disease outcome prediction. These results demonstrate that multi-omics CIMLR analysis is able to extract several important characteristics from various heterogeneous datasets and merge the information into a single clustering that, in our opinion, better captures cancer heterogeneity.

2.2. Endometrial carcinoma

Endometrial carcinoma (EC) is the sixth most common cancer in women globally, with 417,367 new cases (2.2% of all sites) and 97,370 deaths (1% of all sites) in 2020 [22]. We considered a dataset comprising 393 tumors [23] and performed an integrated multi-omics clustering analysis, which identified seven different clusters (C1-7) (Fig. 2, Supplementary Figs. 3 and 4 and Supplementary Table 2). We compared our multi-omics stratification to the one by TCGA [23]. C1 is mostly of the CN Low subtype by TCGA, while C7 is mostly CN High. The other clusters are mixed, comprising CN Low, CN High, MSI and POLE TCGA subtypes at different frequencies (Fig. 2C). Thus, our clusters C2-6 are transversal to the TCGA ones and highlight novel molecular features and prognostic associations.

At the genomic level, the main alterations which significantly characterize the clusters are substitutions, with 67 genes significantly different among the clusters. The patients in C1 show the lowest mutational burden, when compared with all other clusters. Moreover, C1 is characterized by copy number gain of CTNNA3, EBF3 and FGF8 genes. C2 shows copy number gain of AIM2 and CNTN2 genes, while C7 has reduced substitutions in PTEN and a general increase in copy number alterations compared to the other clusters, especially C1 (see Fig. 2A). Interestingly, by comparing the two clusters with the most differences in prognosis, i.e., C1 and C7, we observe that they show an opposite genomic pattern (see Fig. 2A–B).

Next, to analyze multi-omics features specifically associated with the outcome, we merged the clusters with similar (good) prognosis (C1 to C6) into a single macro-cluster (MC). We then compared MC with C7, i.e., the cluster with the worst prognosis. With this analysis, we highlighted the main molecular differences which correlated with survival. Here below we review the most interesting findings, which, to the best of our knowledge, have not emerged from previous analyses, demonstrating the potential impact of multi-omics approaches.

We identified 6 mRNAs significantly over-expressed in C7 *vs* MC:

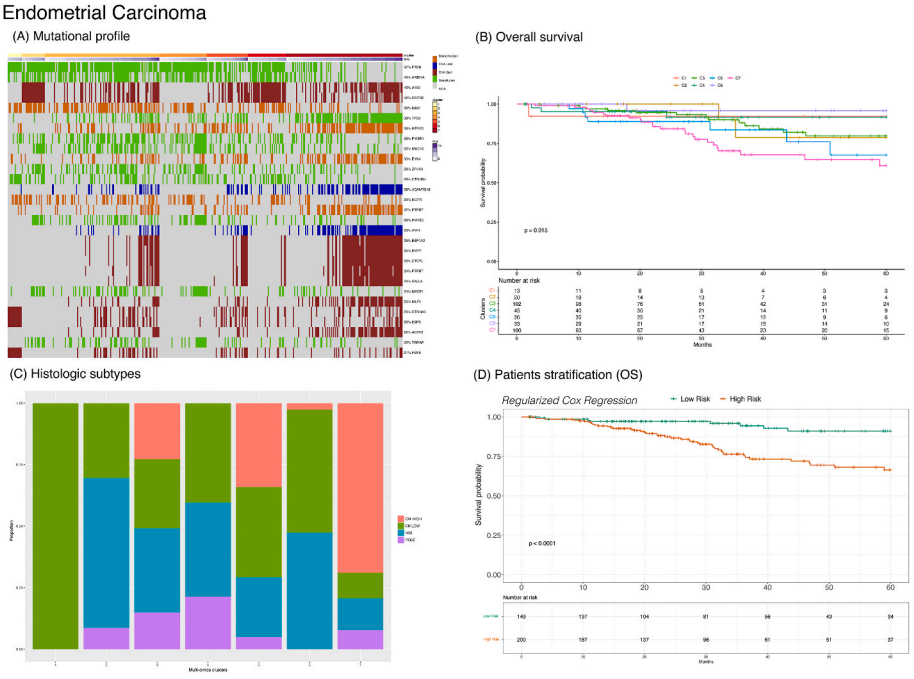

Fig. 2. Clustering analysis of 393 endometrial carcinomas. In panel (A) we show the mutational profiles of the significantly different genes among the clusters (the reported percentages correspond to the proportion of patients in the dataset who have a mutation in the specific gene). In panel (B) we report the overall survival Kaplan-Meier curve comparing the discovered clusters. In panel (C) we show the histological subtypes per cluster. Finally, in panel (D) we report the Kaplan-Meier curve obtained by stratifying the patients based on regularized Cox regression considering the expression of 6 genes (CTCFL, EEF1A2, LMO1, MAGEA11, SSX4, TKTL1) and log2 values for miR-3131.

CTCFL, *EEF1A2*, *LMO1*, *MAGEA11*, *SSX4* and *TKTL1*. Each of these genes have previously been associated with prognosis in different settings: SSX Family Member 4 (*SSX4*) is a transcriptional repressor, highly expressed in endometrial, ovarian and cervical cancer [24]. Its expression has been correlated with the clinical stage of multiple myeloma patients [25]. MAGE Family Member A11 (MAGEA11) acts as an androgen receptor coregulator that increases androgen receptor activity [26]. It is frequently expressed in human cancers, increases during tumor progression, and correlates with poor prognosis [27]. LIM Domain Only 1 (LMO1) modulates gene expression programmes by regulating the assembly of transcriptional complexes [28]. It has been associated with progression, metastasis and apoptosis of leukemia [29], colorectal cancer [30], lung cancer [31,32] and gastric cancer [33]. Transketolase Like 1 (TKTL1) regulates the nonoxidative pentose-phosphate-pathway (PPP) [34]. In endometrial carcinomas, *TKTL1* expression is significantly increased compared to benign endometrial tissue [35] and is associated with disease progression and worse prognosis [36–38]. Eukaryotic Translation Elongation Factor 1 Alpha 2 (EEF1A2) promotes binding of aminoacyl-tRNAs to ribosomes during protein biosynthesis [39]. *EEF1A2* shows high expression levels in approximately 30% of all primary ovarian tumors [40], as well as in breast [41], lung [42], prostate [43] and liver cancer [44]. In prostate cancer, *EEF1A2* expression correlated with tumor stage [45]. CCCTC-Binding Factor Like (*CTCFL*) is transiently expressed in pre-meiotic male germ cells [46]. Its silencing leads to senescence and death of cancer stem cells [47]. *CTCFL* mRNA level has been associated with poor survival in endometrial cancer [48]. High *CTCFL* expression was also detected in uterine mixed mesodermal tumors [49] and gastric cancer cells [50], where it provides invasive properties. Finally, in our analysis, miR-3131 was downregulated in C7. According to the miRDB [51], *miR-3131* targets *LMO1*, one of the 6 most upregulated genes in C7. Interestingly, *hsa-miR-3131* was significantly downregulated in gastric cancer patients compared with healthy subjects [52].

Expression of these 6 mRNAs plus one miRNA potentially represents a signature of poor outcome. In order to verify the predictive potential of the identified genes, we performed regularized Cox regression analysis (see Methods) to stratify patients into high-risk *vs* low-risk groups. In particular, we considered gene expression log2 values for the 7 transcripts discussed above, namely: *CTCFL*, *EEF1A2*, *LMO1*, *MAGEA11*, *SSX4*, *TKTL1* and *miR-3131*. We then associated these genes to OS and PFS data. The expression of 4 genes (*CTCFL*, *EEF1A2*, *LMO1* and *MAGEA11)* was found as a risk factor for both analyses, indicating that they are associated with poor prognosis. We finally stratified patients based on the 4 selected genes, which led to very significant prognostic groups (see Fig. 2D and Supplementary Fig. 4), highlighting the prognostic potential of our approach.

In conclusion, our analysis showed that C7 is uniquely characterized by the highest expression of CTCFL, EEF1A2, LMO1, MAGEA11, SSX4, TKTL1 (mRNA) and the lowest expression of miR-3131 (miRNA). It is interesting to note that CTCFL and EEF1A2 expression data correlated with the genomic analysis at the cluster level, in fact more than 50% of C7 patients showed copy number gain of these two genes. The differential expression of these targets, compared to the other clusters, may explain the worse prognosis observed in patients belonging to C7. Notably, all these features have been previously described in separate reports, but were never found, as a whole, associated with survival in endometrial cancer.

2.3. Sarcoma

Sarcoma is a heterogeneous disease generally classified based on its mesenchymal tissue of origin. Soft tissue sarcoma and primary bone sarcoma are the two main histological groups. Soft tissue sarcoma comprises six major subtypes including dedifferentiated liposarcoma (DDLPS), leiomyosarcoma (LMS), undifferentiated pleomorphic sarcoma (UPS), myxofibrosarcoma (MFS), malignant peripheral nerve sheath tumor (MPNST) and synovial sarcoma (SS) [53].

We adopted a multi-omics approach to analyze a dataset of 206 soft tissue sarcoma patients, including 80 LMS, 50 DDLPS, 44 UPS, 17 MFS, 10 SS and 5 MPNST [54]. Our analysis led to the identification of 4 clusters (C1–4) characterized by significantly different OS and PFS (Fig. 3B and Supplementary Fig. 5). C1 includes mostly LMS (50% of the patients in the cluster), MFS/UPS (12.5%) and DDLPS (12.5%). C2 includes MFS/UPS (50%) and DDLPS (48%). C3, which shows the best overall survival, consists mostly of LMS (95%), while C4, comprising 70% MFS/UPS, 20% LMS and 10% DDLPS, showed the shortest survival (Fig. 3B–C and Supplementary Fig. 5).

We performed enrichment analysis to assess the presence of differences among the clusters for each considered omic data. In terms of genetic alterations, such as fusions and substitutions, we did not appreciate substantial differences across clusters. However, clusters 3 and 4, which displayed very different survival curve trends, showed opposite profiles of gene methylation (Fig. 3A–B). Cluster 3 was hypermethylated in comparison to the other clusters, especially compared to cluster 4. Furthermore, C4 displayed not only the lowest gene methylation profile in terms of enrichment analysis, but also the lowest methylation degree, with an average methylation value of 0.30, compared to the other clusters (C1: 0.71, C2: 0.78, and C3: 0.90).

In particular, Runt-related transcription factor 2 (*RUNX2*) gene was methylated in 98% of C3 and in 10% of C4 patients. RUNX2 methylation profile correlated with its mRNA expression levels, as RUNX2 expression in C4 was twofold higher than in C3. Another molecular mechanism that could impact on RUNX2 expression was revealed by analyzing differences in miRNA expression across clusters: miR-320d expression was higher in C3 than in the other clusters, in particular cluster 4 displayed the lowest expression. Notably, RUNX2 is a target of miR-320 family [55]. These findings suggest that promoter methylation and miRNA expression could be a double inhibitory mechanism which positively impacts on the survival rate of cluster 3 by favoring RUNX2 downregulation. In support of this hypothesis, high RUNX2 expression has been correlated with poor response to chemotherapy in osteosarcoma [56].

A similar trend was displayed by expression of genes belonging to the WNT family, such as *WNT10B*, *WNT11*, *WNT2*, *WNT5A*, *WNT1*, *WNT7A*, which were upregulated in C4 compared to C3. *WNT10B* expression was in line with its methylation, as it was demethylated in approximately 60% of C4 and in less than 10% of C3 (Fig. 3A and Supplementary Table 3). Alterations of the components of the WNT signaling pathway have been documented in sarcomagenesis [57], and a reciprocal regulation between *RUNX* genes and the WNT pathway has been shown [58]. Based on these results, we can speculate an involvement of RUNX2 in metastasis formation in sarcoma regardless of the specific histological subtype, as it has been documented by an integrative multi-omics analysis of a colon cancer cell line [59]. Furthermore, we found that the protein expression of E-cadherin, whose loss is considered a hallmark of metastatic cells, is very low in C4.

To further dissect whether RUNX2 expression could directly impact on prognosis, we stratified patients in two groups based only on RUNX2 expression levels, and we found that the two macro-clusters ($RUNX2^{high}$ and $RUNX2^{low}$) displayed statistically different OS and PFS (Fig. 3D and Supplementary Fig. 6). Notably, these two macro-clusters comprised similar percentages of histological subtypes, highlighting the importance of molecular subtyping.

In conclusion, our multi-omics analysis identified RUNX2 as a new candidate prognostic factor that may impact on sarcoma outcome. This analysis also allowed us to dissect possible multi-level molecular mechanisms that may control RUNX2 expression, such as methylation and miRNA expression levels.

2.4. Thymic epithelial tumors

Thymic epithelial tumors (TETs) are extremely rare primary tumors

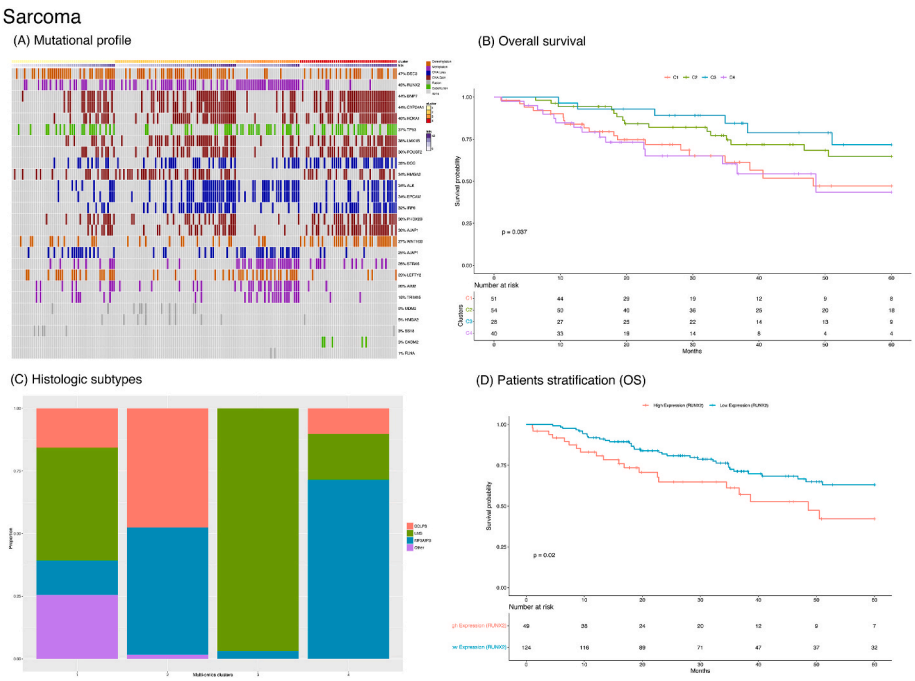

Fig. 3. Clustering analysis of 206 sarcomas. In panel (A) we show the mutational profiles of the significantly different genes among the clusters (the reported percentages correspond to the proportion of patients in the dataset who have a mutation in the specific gene). In panel (B) we report the overall survival Kaplan-Meier curve comparing the discovered clusters. In panel (C) we show the histological subtypes per cluster. Finally, in panel (D) we report the Kaplan-Meier curve obtained by stratifying the patients based on RUNX2 expression.

of the mediastinum, with an incidence of 0.15 cases per 100,000 person-years. TETs include thymoma, classified into five histological subtypes A, AB, B1, B2 and B3, and thymic carcinoma (TC), which is far less common but more aggressive [60]. Thymoma types A, AB and B1 have an excellent OS rate of more than 90% at 10 years, while TC shows a dismal 5-year survival of only 48% [61].

We analyzed a dataset providing multi-omics data for 87 TETs [60]. Our method identified three clusters (C1-3) showing significantly different survival, with C3 showing the worst OS and PFS compared to C1 and C2 (Fig. 4, Supplementary Figs. 7 and 8 and Supplementary Table 4).

About 80% of TC patients in this dataset were allocated to C3 by our algorithm, which may explain its short survival and clearly different mutational and transcriptional profiles (Fig. 4A–C, Supplementary Table 4). In particular, this cluster shows distinctive features of TC, such as significantly higher frequency of point mutations in CYLD. This gene acts as a tumor suppressor, through a negative regulation of NF-kB [62] and has been reported to be mutated in 19% of TC [63]. Moreover, C3 presents increased mRNA and protein expression of KIT, a well-known oncogene associated with the TC subtype [64].

In addition, our method was able to identify novel genomic alterations that may better characterize patients with a bad prognosis. Particularly, C3 presented significantly higher frequency of copy number gains in RGS7, that were associated with an increase of its

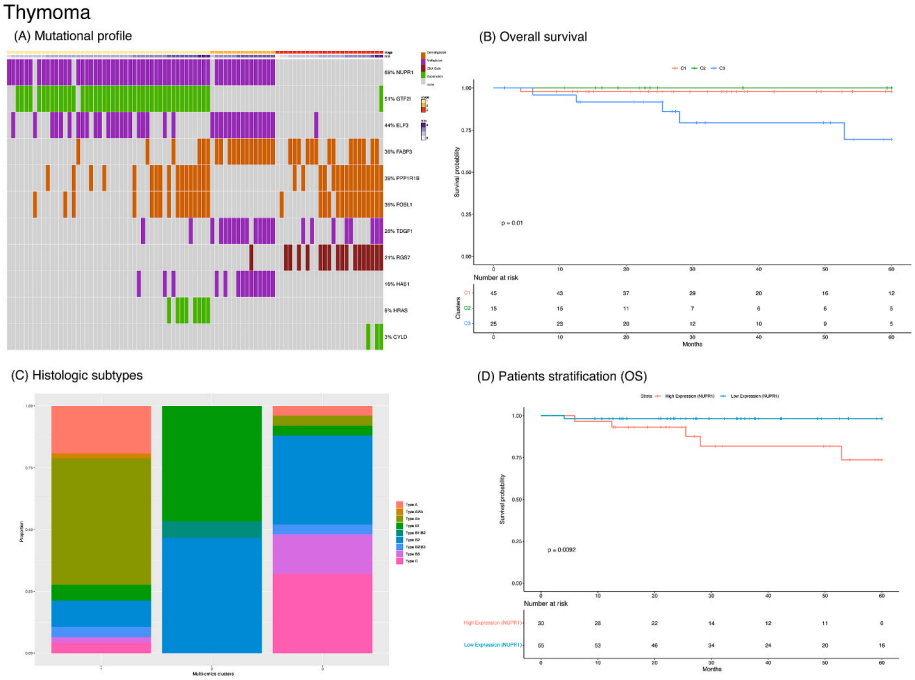

Fig. 4. Clustering analysis of 87 thymomas. In panel (A) we show the mutational profiles of the significantly different genes among the clusters (the reported percentages correspond to the proportion of patients in the dataset who have a mutation in the specific gene). In panel (B) we report the overall survival Kaplan-Meier curve comparing the discovered clusters. In panel (C) we show the histological subtypes per cluster. Finally, in panel (D) we report the Kaplan-Meier curve obtained by stratifying the patients based on NUPR1 expression.

expression. RGS7 is a member of the regulator of G-protein signaling (RGS) family that regulates downstream signaling of G-protein coupled receptors. At the best of our knowledge, RGS7 has never been reported as a thymoma marker gene. Other members of the RGS family play a role in cancer progression [65]. Furthermore, our analysis revealed different epigenetic modifications in patients of C3: specifically, we observed a global demethylation and, in particular, a complete absence of methylation in FOS Like 1 (FOSL1) and Nuclear Protein 1 (NUPR1) genes. These differences correlated with a higher expression of the two genes compared to C1 and C2. Both FOSL1 and NUPR1 have never been associated with thymoma, although FOSL1 was found upregulated in head and neck squamous cell carcinoma and correlated with a poor prognosis [66]. NUPR1 expression is higher in hepatocellular carcinoma samples than in normal tissue, and its silencing reduces tumor growth in vivo [67].

We focused our attention on NUPR1, as this gene showed consistent methylation and expression profiles both in clusters 1 and 2, which were different compared to cluster 3. Therefore, we verified if NUPR1 was predictive of prognosis, by stratifying the patients in two groups based on its expression levels, namely $NUPR1^{low}$ (patients with low NUPR1 expression) and $NUPR1^{high}$ (patients with high NUPR1 expression). Kaplan-Meier analysis showed that $NUPR1^{low}$ patients have better prognosis (Fig. 4D and Supplementary Fig. 8). Interestingly, the $NUPR1^{low}$ cluster comprises 5 samples of the TC subtype, which is normally expected to have poor outcome, that were instead stratified in the group with excellent survival, thus providing a finer classification than classical subgrouping.

While preliminary, these findings highlight the importance of methods tailored to the analysis of multi-omics data and show a potential mechanism based on epigenetics associated with prognosis in thymic epithelial tumors, by identifying specific multi-omics features that are directly associated with the prognosis of this disease. We leave to future work a further investigation and the validation of the potential role of NUPR1 as a prognostic factor for TETs.

3. Discussion

Cancer is a complex disease with heterogeneous phenotypes, making it difficult to treat with standard chemotherapy. Precision medicine has emerged as a new approach to cancer treatment, aiming to determine the best therapeutic intervention by exploiting molecular characteristics unique to each patient.

The translational relevance of cancer subtyping based on multi-omics data is now widely recognized, also thanks to the availability of the latest state-of-the-art high-throughput experimental technologies. These technologies have generated large omics datasets that include multiple omics data measured for the same patients, allowing for a comprehensive characterization of cancer heterogeneity. As a result, efforts to understand and classify cancer subtypes have now become more feasible.

We here focused on the analysis of four cancer types exploiting multi-omics data from TCGA, including seven omics data for each patient, and curated clinical outcome. We preprocessed the raw data with a uniform pipeline and applied the CIMLR integrative clustering method to detect subtypes based on molecular characteristics linked to prognosis. We systematically reviewed the multi-omics subtypes we discovered for the four cancer types, emphasizing the valuable insights our approach provided into understanding tumor heterogeneity and the underlying biology.

Our findings underscore the significance of computational efforts focused on leveraging multi-omics data for defining cancer subtypes. As the availability of such data increases, we anticipate that the predictive power of these approaches will improve. Subtyping can be a valuable tool for stratifying patients and predicting outcomes, leading to improved personalized treatment strategies.

Ultimately, our results suggest that integrating multi-omics data into clinical decision-making can enhance patient care and contribute to the ongoing efforts to better understand and treat cancer. This method can be applied to any cancer type, as more data become available.

4. Methods

4.1. Data collection and preprocessing

We considered data from the TCGA studies published within the PanCanAtlas initiative [3]. For each cancer type, we collected seven omics data types from cBioPortal [68,69] (https://www.cbioportal.org/), considering the following four cBioPortal datasets: Bladder Urothelial Carcinoma, dataset ID: blca_tcga_pan_can_atlas_2018; Endometrial Carcinoma, dataset ID: ucec_tcga_pan_can_atlas_2018; Sarcoma, dataset ID: sarc_tcga_pan_can_atlas_2018; Thymoma, dataset ID: thym_tcga_pan_can_atlas_2018. Specifically, for each dataset we considered: (1) substitutions and small insertions/deletions, (2) copy number alterations, (3) methylations, (4) gene expression profiles, (5) microRNAs, (6) reverse-phase protein microArrays (RPPA) and (7) microbiome data. In addition, we also retrieved curated clinical information, particularly, overall survival and progression free survival.

Substitutions reported information regarding presence/absence of somatic mutations in each patient. Copy number alterations provided log2 ratios between tumor and normal tissue for each gene. Methylations data consisted of beta-values measuring intensities in the range of 0 and 1. Expression data provided RNA expression counts per gene. microRNA reported expression counts. RPPA provided expression levels for a set of around 200 proteins. Finally, microbiome data consisted of estimates of microbial signatures in tissue and blood [70].

Each of the seven data matrices (patients x features) was normalized such that each value ranged between 0 and 1.

4.2. Multi-omics integrative clustering

We adopted the CIMLR (Cancer Integration via MultIkernel LeaRning) algorithm [4] to perform multi-omics integrative clustering considering the seven normalized data matrices described above.

CIMLR is a kernel-based machine learning algorithm that integrates multi-omics data for cancer subtype classification and patient stratification. The algorithm starts by transforming different omics data types into kernel matrices that capture the sample similarity based on their feature values. These kernel matrices are combined into a single integrated kernel matrix using a weighted sum approach, where the weight values are also learned. The integrated kernel matrix is then subjected to dimensionality reduction to extract the most informative features. CIMLR's kernel-based approach allows it to capture complex patterns and nonlinear relationships between the different data types. Additionally, the algorithm can also identify the most informative data types and features for cancer classification, which can help guide future experiments and research.

In our settings, the method first computed 55 gaussian kernels with different variance per data type, for a total of 385 kernels since we considered seven omics input data. Then, it computed a patient x patient similarity matrix, which recapitulates the kernels and provides a quantitative measure to assess the similarity between patients. We then performed k-means clustering on such similarity. The optimal number of clusters was estimated using the standard elbow method.

4.3. Survival analysis

We considered two prognostic outcomes provided by TCGA, namely overall survival (OS) and progression-free survival (PFS), over a 10-year period. For both survival metrics, we censored data points corresponding to patients who died within 1 month from diagnosis or that were over the age of 80 years also, in order to limit uncertain observations.

Associations between clusters and survival outcomes were assessed

by Kaplan–Meier analysis using a log-rank test p-value, with a threshold of 0.05 for statistical significance.

4.4. Differential analysis and features selection

We considered both categorical and continuous omics data. In particular, the considered categorical features were substitutions and small insertions/deletions (0 or 1 respectively to indicate absence and presence), copy number alterations (as GISTIC scores to indicate gain and loss copy number events), and methylations (beta-value >0.7 to indicate high methylation and <0.3 to indicate demethylation). The considered continuous features were gene expression profiles, microRNAs, RPPA and microbiome data.

For categorical features we performed proportions z-test to assess statistical differences, while for continuous features we performed analysis of variance (ANOVA). We corrected p-values for multiple hypothesis testing to account for false discoveries using the Benjamini-Hochberg procedure and selected features with FDR-adjusted p-value <0.05.

We exploited the power of multi-omics data by further filtering out genomics features with expression data. Particularly, we verified that copy number gains and demethylations were associated with significant overexpression of the relative gene, while copy number losses and methylations were conversely associated with reduced expression.

We finally filtered out features with a fold change <1.5 in each direction (over and under expression).

4.5. Regularized Cox regression analysis

We performed Regularized Cox regression analysis using the Coxnet algorithm [71,72] to identify significant variables for predicting patient outcomes. The method is a variant of the Cox proportional hazards model, which assumes that the hazard rate (i.e., the risk of an event occurring at any given time) for a particular individual is proportional to a linear combination of their covariates (predictors), with a baseline hazard that is common to all individuals. Regularized Cox Regression adds a regularization term to the likelihood function of the Cox model, which shrinks the estimates of the regression coefficients towards zero and selects the most relevant predictors.

The elastic net method with LASSO penalty [73] was used to minimize cross-validation error and select the most relevant variables (i.e., the ones with a regularized regression coefficient different from 0). Using the Cox model, we calculated a risk score for each patient. The score can be computed as the weighted sum of the covariate values for each patient, where the weights are the corresponding estimated coefficients from the Cox model. This allowed us to stratify them into two distinct risk groups: those with risk scores greater than the dataset mean, indicating a high-risk group with poor prognosis, and those with lower risk scores, indicating a low-risk group with a more favorable prognosis.

Author contributions

Conceptualization: VC, FM, MV, LM, DR. Methodology and Software: DR. Investigation: VC, FM, MV, LM, DR. Visualization: DR. Funding acquisition: AG, RP, DR. Supervision: AG, RP, LM, DR. Writing – original draft: VC, FM, MV, LM, DR. All authors read and approved the final manuscript.

Data and materials availability

All cancer data are publicly available from the relative original publication or from the cBioPortal repository (https://www.cbioportal.org/).

Software availability

CIMLR is available as an R package and in Matlab on GitHub (https://github.com/danro9685/CIMLR).

Declaration of competing interest

None Declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.compbiomed.2023.107064.

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