Pathology - Research and Practice 248 (2023) 154674

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Transcriptome analysis of primary sporadic neuroendocrine tumours of the intestine identified three different molecular subgroups

Paola Mattiolo [a], Anastasios Gkountakos [a], Giovanni Centonze [b], Michele Bevere [a], Paola Piccoli [a], Serena Ammendola [a], Corrado Pedrazzani [c], Luca Landoni [d], Sara Cingarlini [e], Michele Milella [e], Massimo Milione [b], Claudio Luchini [a,f], Aldo Scarpa [a,f], Michele Simbolo [a,*]

[a] Department of Diagnostics and Public Health, Section of Pathology, University and Hospital Trust of Verona, Verona, Italy
[b] Pathology Unit 1, Pathology and Laboratory Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy
[c] Division of General and Hepatobiliary Surgery, Department of Surgical Sciences, Dentistry, Gynecology, and Pediatrics, University and Hospital Trust of Verona, Verona, Italy
[d] General and Pancreatic Surgery Department, Pancreas Institute, University and Hospital Trust of Verona, Verona, Italy
[e] Department of Medicine, Section of Medical Oncology, University and Hospital Trust of Verona, Verona, Italy
[f] ARC-Net Research Centre, University and Hospital Trust of Verona, Verona, Italy

ARTICLE INFO

Keywords:
Neuroendocrine
NET
Intestinal
Transcriptome

ABSTRACT

Background: Intestinal neuroendocrine tumours (I-NETs) represent a non-negligible entity among intestinal neoplasms, with metastatic spreading usually present at the time of diagnosis. In this context, effective molecular actionable targets are still lacking. Through transcriptome analysis, we aim at refining the molecular taxonomy of I-NETs, also providing insights towards the identification of new therapeutic vulnerabilities.
Materials and Methods: A retrospective series of 38 primary sporadic, surgically-resected I-NETs were assessed for transcriptome profiling of 20,815 genes.
Results: Transcriptome analysis detected 643 highly expressed genes. Unsupervised hierarchical clustering, differential expression analysis and gene set enriched analysis identified three different tumour clusters (CL): CL-A, CL-B, CL-C. CL-A showed the overexpression of *ARGFX*, *BIRC8*, *NANOS2*, and *SSTR4* genes. Its most characterizing signatures were those related to cell-junctions, and activation of mTOR and WNT pathway. CL-A was also enriched in T CD8 + lymphocytes. CL-B showed the overexpression of *PCSK1*, *QPCT*, *ST18*, and *TPH1* genes. Its most characterizing signatures were those related to adipogenesis, neuroendocrine metabolism, and splice site machinery-related processes. CL-B was also enriched in T CD4 + lymphocytes. CL-C showed the overexpression of *ALB*, *ANG*, *ARG1*, and *HP* genes. Its most characterizing signatures were complement/coagulation and xenobiotic metabolism. CL-C was also enriched in M1/2 macrophages. These CL-based differences may have therapeutic implications in refining the management of I-NET patients. At last, we described a specific gene-set for differentiating I-NET from pancreatic NET.
Discussion: Our data represent an additional step for refining the molecular taxonomy of I-NET, identifying novel transcriptome subgroups with different biology and therapeutic opportunities.

1. Introduction

Small intestinal neuroendocrine tumour is the most frequent cancer type of the small bowel [1]. Globally considered, intestinal neuroendocrine tumors (I-NETs) are the most common intestinal malignancy after adenocarcinoma. Despite slow-growing [2,3], it represents a malignant disease usually diagnosed at metastatic stage [4]. Many patients are asymptomatic, and the disease is often diagnosed either incidentally or following vague mass or transient obstruction-related symptoms, also including poorly defined abdominal pain. Due to excessive secretion of serotonin and related molecules, in about one third of cases I-NETs can cause a carcinoid syndrome, which usually occurs in patients with distant metastases [5]. Regarding the current therapeutic opportunities, the somatostatin analogues (SSA)-based treatment significantly

* Correspondence to: Department of Diagnostics and Public Health, Section of Pathology, Policlinico GB Rossi, Piazzale L.A. Scuro, 10, Piastra Odontoiatrica (II floor), Italy.
E-mail address: michele.simbolo@univr.it (M. Simbolo).

https://doi.org/10.1016/j.prp.2023.154674
Received 23 June 2023; Received in revised form 3 July 2023; Accepted 3 July 2023
Available online 7 July 2023

improves patient survival by controlling tumour progression in metastatic I-NETs [6], but the overall clinical benefits remain far from satisfactory [7].

Despite of some investigations based on molecular analysis, it is important to note that the genetic landscape of this tumour type remains elusive and poorly informative [8]. Recent genome sequencing-based studies revealed a small number of non-recurrent mutations affecting *APC* [8],*CDKN1B* [9], *CDKN2C* [10], and in sporadic cases *BRAF* [11], *KRAS,* and *TP53* [12]. Differently, recurrent alterations were identified in chromosome alterations. Particularly, loss of chromosome 18 was observed in about 50% of cases, and loss of chromosome 16 in up to 18% of cases, whereas gains were reported in chromosomes 4, 5, 7, 14, and 20, ranging from 10% to 30% of cases [10,13].

Of note, only a single study explored the transcriptome profile of I-NET so far. The cohort of this study included 10 primary small-intestine tumours, 2 lymph node metastases and 21 liver metastases. Interestingly, it identified 3 clinically relevant subgroups [14]. This study was recently revised using artificial neural networks (ANNs), showing new potential key pathways and genes networks involved in I-NETs biology, such as Notch signalling, inflammatory response, coagulation, *KRAS* signalling, and allograft rejection [15].

Here, we provided a transcriptome characterization of a cohort of 38 primary sporadic I-NETs, with the aim of refining the molecular taxonomy of these tumours, also providing new insights towards the identification of potential therapeutic vulnerabilities.

2. Materials and methods

2.1. Cases

A retrospective series (2000–2021) of 38 non syndromic primary I-NETs, surgically resected, were retrieved from the archives of the ARC-Net Biobank at Verona University Hospital. None of the patients received neo-adjuvant therapy. Reclassification according to WHO 2019 criteria [16] and confirmation of histological diagnosis was performed by expert gastrointestinal pathologists (CL, AS). Tumor stage was defined according to the 8th edition of the TNM classification of malignant tumors [17]. The study has been approved by the Verona Ethics Committee (no.55893/2017).

2.2. Nucleic acids preparation

DNA was obtained from FFPE tumour tissue by manual microdissection of 10 consecutive 4-μm sections for each case. RNA was prepared using ReliaPrep FFPE Total RNA Miniprep System (Promega, Milan, Italy), qualified using RIN analysis of Agilent RNA 6000 Nano Kit on Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified using Qubit RNA HS Assay Kit (Thermo Fisher) [18]. The extracted RNA was considered suitable with RIN$>$ 5 and concentration over 10 ng/μl.

2.3. Gene expression analysis by next-generation sequencing

The Ampliseq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) was used to analyze the expression status of 20,815 human genes. Libraries were prepared using AmpliSeq technology and 1 μg of retro-transcribed RNA for each multiplex PCR amplification. Clonal amplification was performed using the Ion Chef System (Thermo Fisher Scientific). Sequencing was run on the Ion S5XL (Thermo Fisher Scientific) loaded with Ion 540 Chip. The AmpliSeqRNA plugin was used to generate expression data (counts per transcript) for each sample. Counts were normalized and transformed using the "DESeq2" package for R [19]. The expression data were subjected to quality control using the workflow defined by Law et al. [20]. We then corrected the batch affect using ComBat, as reported in Johnson et al. [21]. Visualization and clustering were performed using the "ComplexHeatmap" package for R [22]. Differential expression analysis between subtypes was performed using Deseq2 algorithm. A gene was considered differentially expressed if it showed an adjusted P-value $<$ 0.05. We downloaded c2 from MSigDB [23,24], and determined the cluster-specific enriched gene sets using the normalized and batch corrected count matrix. Immune infiltrate was estimated for all samples using TIMER [25] and xCell immune deconvolution methods [26]. We applied gene set enriched analysis (GSEA) using GAGE [27] R package between clusters to get pairwise significantly up- and down-regulated genes / pathways. Then we used an approach based on the ssGSEA score for determining the signatures differently enriched by different clusters. We performed a z-score normalization of the pathways-scores in each cluster.

Table 1
Clinico-pathological features of the cohort of 38 intestinal neuroendocrine tumours subjected to molecular analysis.

	Total	A	B	C
Age				
median	50	36	62.5	40
range	(12–82)	(14–71)	(12–79)	(10–82)
Gender				
Female	12	4	4	4
Male	26	8	8	10
Site				
Colon	1	0	1	0
Duodenum	1	0	0	1
Ileum	35	12	11	12
Jejunum	1	0	0	1
Stage				
I	1	0	1	0
II	2	1	1	0
III	15	2	6	7
IV	20	9	4	7
Tumor size (cm)				
median	2	2	2	1.9
range	(0.7–6)	(0.7–6)	(1–3)	(0.7–5)
Ki-67 index				
median	1.5	1.5	1.5	1.25
range	(0.5–10)	(0.5–5)	(0.5–6)	(0.5–10)
Grade				
G1	31	10	10	11
G2	5	2	2	1
G3	2	0	0	2
Survival outcome				
Alive	31	10	10	11
Dead of disease	1	0	0	1
NA	6	2	2	2
Overall Survival				
median	56	61	56.5	58.5
range	(12–149)	(12–149)	(12–134)	(27–144)

Abbreviations: NA = not available

2.4. Statistical analysis

Data associations were assessed using the Fisher's exact test for categorical variables, and the Kruskal-Wallis test for continuous variables. Correction for multiple comparisons was performed according to Benjamini-Hochberg. Overall survival (OS) was assessed from the time of diagnosis to the time of death or last follow-up. The log-rank test was used to assess the survival difference between patient groups. Cox proportional regression analysis were used to assess the association between clinical-pathological features and OS. Data analysis was performed using the R environment for statistical computing and graphics (R Foundation, Vienna, Austria - Version 3.6.2) and MedCalc for Windows version 15.6 (MedCalc Software, Ostend, Belgium). All tests were two-sided and p-values $<$ 0.05 were considered as statistically significant.

2.4.1. Validation immunohistochemistry

For validating transcriptome analysis, we performed immunohistochemistry selecting specific markers of each cluster. The expression of

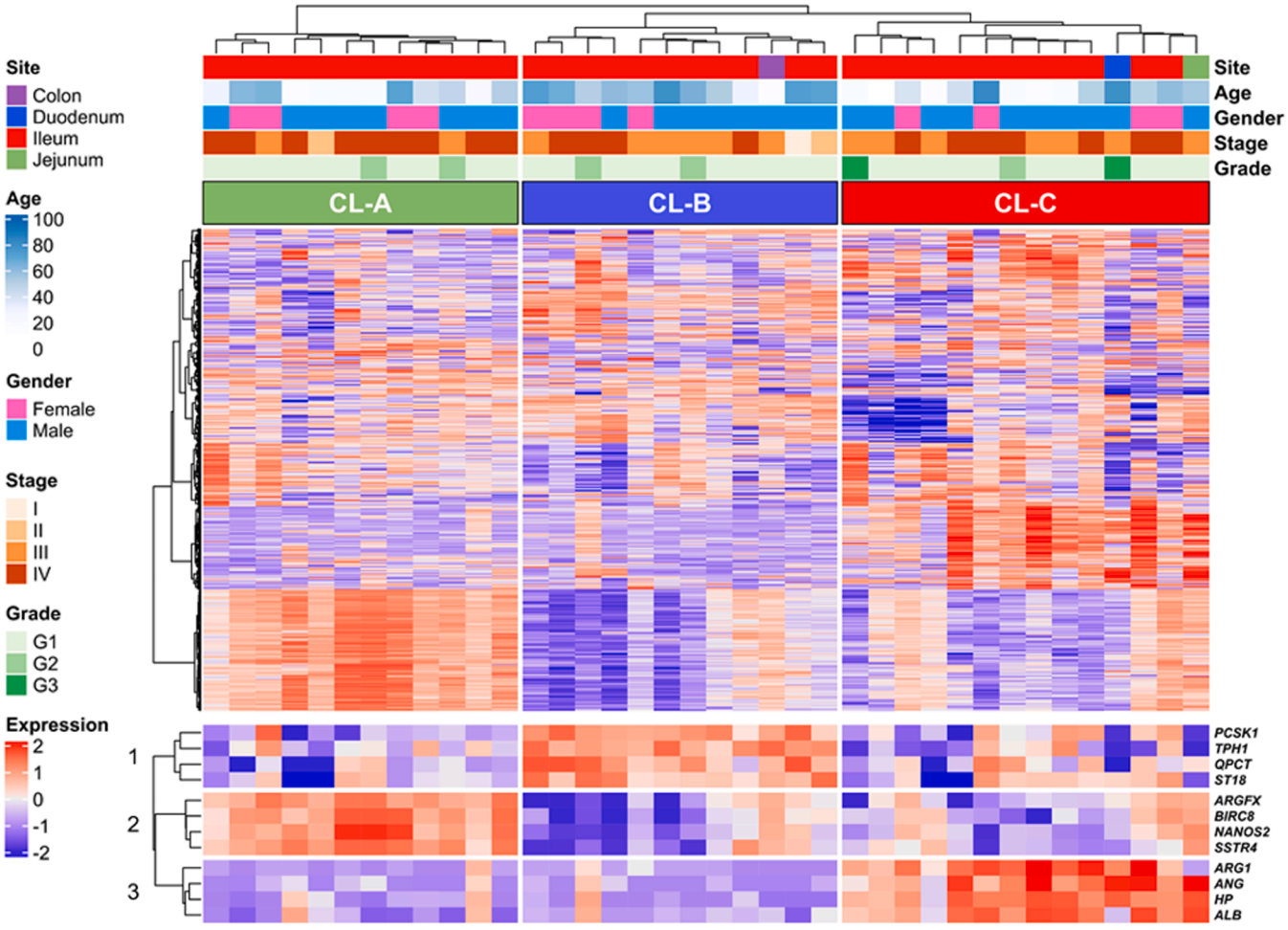

Fig. 1. Gene expression analysis of the cohort of intestinal neuroendocrine tumours (I-NETs). The unsupervised hierarchical clustering is displayed as a heatmap, where the 38 I-NET samples are arranged in columns. The expression values of the 643 genes identified are arranged in rows. Colour legend: red and blue indicate high and low expression, respectively.

these biomarkers was evaluated using a semi-quantitative (0−5) scoring system, as reported previously [28–30]: 0 = negative (no positive cells), 1 = rare (1–10 positive cells per high-power field, 400X), 2 = low (11–20 positive cells per HPF), 3 = moderate (21–30 positive cells per HPF), 4 = high (31–50 positive cells per HPF), 5 = very high (>50 positive cells per HPF).

3. Results

3.1. Clinicopathological features

The clinicopathological features of 38 I-NETs patients are summarized in Table 1. We collected 35 tumors from ileum, 1 from duodenum, 1 jejunum, and 1 from colon. All were primary tumors. The series comprised 12 females and 26 males with a median age of 50 years (range: 10–82 years). TNM stage was available for all samples: 1 stage I, 2 stage II, 15 stage III, and 20 stage IV. Tumor grade was G1 in 31 (81.5%), G2 in 5 (13.2%), and G3 in 2 cases (5.3%). The histological appearance was similar to all tumors, showing monomorphic cells with solid-trabecular and nested architecture, without formation of tumor necrosis. All neoplasms were positive for the neuroendocrine markers Chromogranin-A and Synaptophysin.

3.2. Unsupervised expression analysis identified three molecular clusters

Transcriptome analysis detected 643 genes as highly expressed. For clustering purposes, we considered all such most variable expression genes (HGVs). Then, we used the NbClust package to estimate the best number of clusters, which resulted to be 3 (k = 3). Subsequently, we applied the hybrid hierarchical k-means approach to all HGVs, performing a principal component analysis (PCA) and constructing a dendrogram showing the relationships between samples. To verify the resulting associations between samples, unsupervised consensus clustering was performed using ConsensusClusterPlus. The resulting consensus matrix confirmed the associations obtained by PCA and dendrogram.

The three clusters obtained were: cluster A (CL-A), including 12 samples, cluster B (CL-B), including 12 samples, and cluster C (CL-C), including 14 samples (Fig. 1). There were no statistically significant associations between the different clusters and clinicopathological variables. A pairwise differential expression (DE) analysis was performed among the three clusters (Supplementary Table 1). Among all HGVs, 162 genes were DE and highly exclusive for CL-A, 20 for CL-B, and 102 genes for CL-C. Among these, we identified 4 overexpressed genes for each cluster, as follows: i) cluster A: *ARGFX*, *BIRC8*, *NANOS2*, and *SSTR4*, ii) cluster B: *PCSK1*, *QPCT*, *ST18*, and *TPH1*, iii) cluster C: *ALB*, *ANG*, *ARG1*, and *HP*.

The survival analysis did not reveal any significant difference in terms of overall survival between the different clusters.

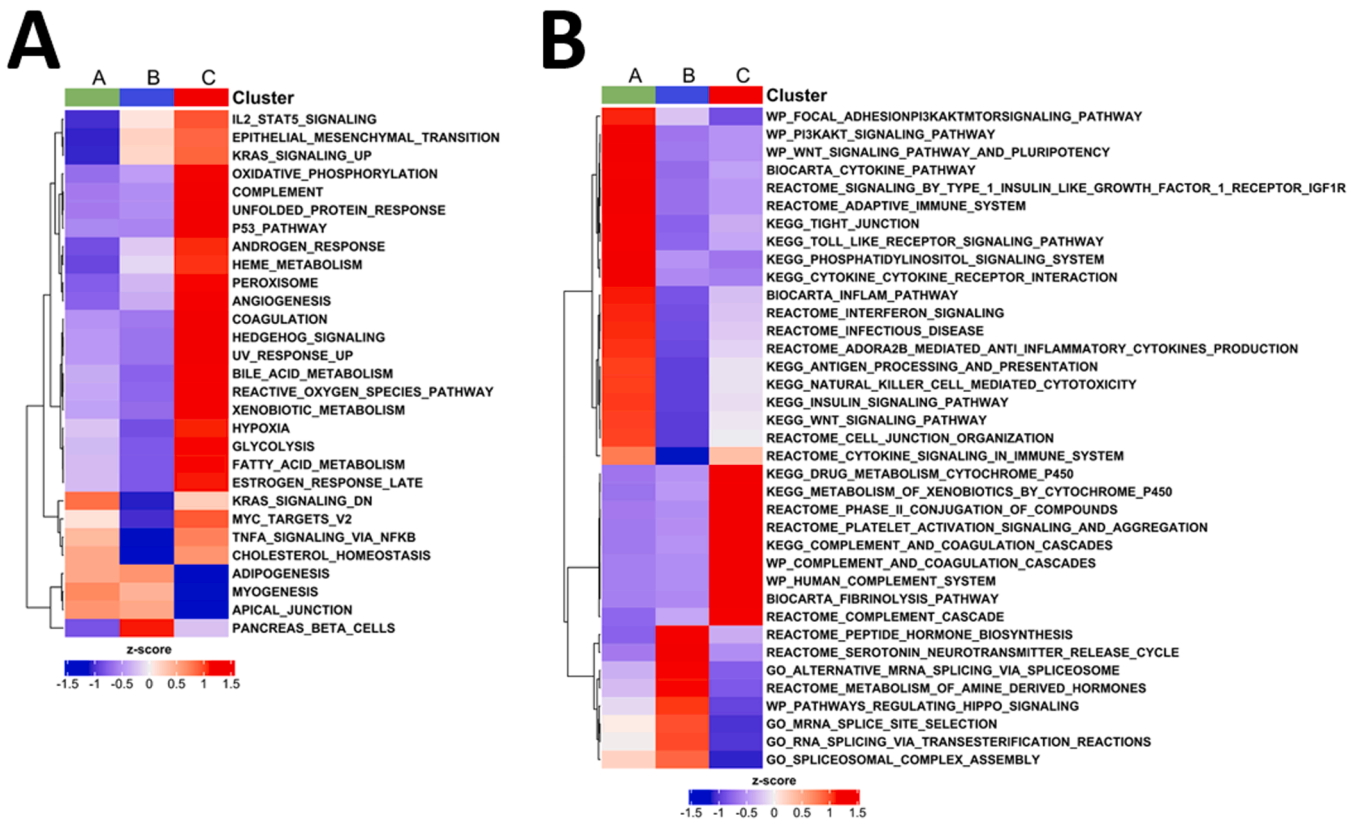

Fig. 2. Gene set enrichment analysis (GSEA) of the different tumour clusters. Heatmaps of: A) hallmark gene sets from MSigDB collections, B) oncogenic signature gene sets (C2).

3.3. Gene set enrichment analysis identified specific biological processes in each cluster

Using GSEA and signatures available on MSigDB, we investigated the most characterizing biological processes for each cluster. This approach was initially based on hallmark signatures, and identified apical cell-junction, genes downregulated by *KRAS* signaling, and myogenesis as the most characterizing signatures associated to CL-A, adipogenesis and pancreatic β-cell signatures to CL-B, and angiogenesis, complement/coagulation, epithelial-to-mesenchymal transition (EMT), and xenobiotic metabolism to CL-C (Fig. 2**A**).

To understand in depth the biological processes previously identified using only hallmark signatures, we also investigated C2 signatures. In particular, we observed that CL-A showed specific positive enrichment z-score for tight junction, cell junction organization, and activation of mTOR and Wnt pathway. At the same time, CL-B showed specific positive enrichment z-score for processes related to neuroendocrine metabolism and splice site machinery-related processes. Of note, this cluster also showed correlation to regulation of Hippo signaling. At last, CL-C confirmed a positive enrichment score for coagulation/complement related pathways and xenobiotic metabolism through cytochrome P450 (Fig. 2**B**).

Furthermore, with the aim of identifying a potential cell of origin for the different clusters, we compared the gene expression profile of each cluster with the available intestinal signatures reported in a recent publication by Gao and colleagues [31], and present in C8 dataset of MSigDB. Based on this comparison, CL-A profile resulted similar to the profile of intestinal goblet cells, CL-B profile showed similarities with large intestinal entero-endocrine cells profile, and CL-C profile was similar to small intestinal enterocyte progenitors profile (Fig. 3**A**).

We also tried to identify potentially specific I-NET genes, by comparing I-NET transcriptome with that of pancreatic neuroendocrine tumors, recently published by our group of research [32]. The DE analysis coupled with volcano plot analysis, applying a cut-off of 0.05 for adjusted p-value, showed *ALB*, *APOA1*, *CHGA*, *DES*, *HP*, *IL6ST*, *SCARNA7*, *TPH1*, and *TPM1* as highly specific genes of I-NETs, and *AK4*, *CA9*, *CHST13*, *CNFN*, *GALNT4*, *HMBS*, *LDHB*, *MAGEB2*, and *PGK1* as highly specific genes for pancreatic neuroendocrine tumors (Fig. 3**B**).

3.4. Characterzation of immune-population of each cluster

Different immune-populations were identified using deconvolution analysis. In particular, XCell algorithm was used to quantify the immune cell sub-populations through the expression of multiple immune-infiltrating cell markers (Fig. 4**)**. CL-A showed a specific enrichment in T CD8 + lymphocytes, cancer associated fibroblasts, and lymphoid/myeloid progenitors. At the same time, CL-B resulted enriched in T CD4 + lymphocytes, and showed the highest immune score among the three different clusters, while CL-C was specifically enriched in M1/2 CD68 + macrophages and dendritic cells.

3.4.1. Validation immunohistochemistry

Based on transcriptome profiles, the following cluster-specific markers for immunohistochemistry (IHC) were selected for transcriptome validation: CD8 (clone: C8/144B; dilution: 1:200; Dako, USA) for cluster A, CD4 (clone: 4B12; dilution: pre-diluted; Novocastra, USA) for cluster B, and CD68 (clone: C14H12; dilution: prediluted; Novocastra, USA) for cluster C (Fig. 4). The results of IHC were as follows: 1) CL-A: mean score of CD8 = 3.8, mean score of CD4 = 1.6, and mean score of CD68 = 1.2; 2) CL-B: mean score of CD8 = 2.0, mean score of CD4 = 3.6, and mean score of CD68 = 1.4; 3) CL-C: mean score of CD8 = 1.8, mean score of CD4 = 1.6, and mean score of CD68 = 4.0). All IHC scores validated the results of transcriptome analysis, showing statistically significant differences among all clusters ($p < 0.05$).

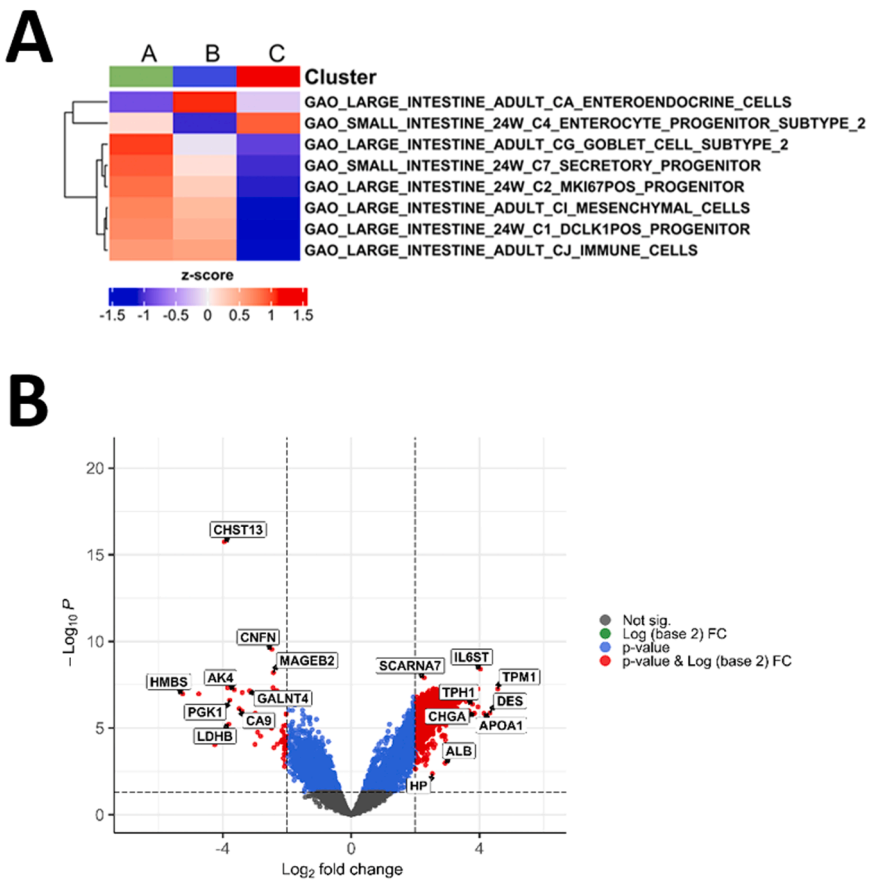

Fig. 3. Origin definition and peculiarities of intestinal neuroendocrine tumors (I-NET). A) Specific cluster-based cell-of-origin investigation of I-NETs based on data of Gao, et al.; B) Volcano plot representation of differential expression analysis. Red dots represents differentially expressed genes reaching statistical significance between intestinal (right-side) and pancreatic (left-side) neuroendocrine tumours.

4. Discussion

In the current study, we performed a transcriptome profiling of 38 surgically resected primary sporadic I-NETs, showing the presence of three distinct biological subgroups in this same entity.

The first subgroup was CL-A, which showed the overexpression of *ARGFX*, *BIRC8*, *NANOS2*, and *SSTR4* genes. Its most characterizing signatures were those related to apical cell-junctions, genes down-regulated by *KRAS* signaling, myogenesis, and activation of mTOR and Wnt pathway. Regarding cell-of-origin profiles, CL-A showed significant similarities to intestinal goblet cells, and about immune cells, this cluster was enriched in T CD8 + lymphocytes, cancer associated fibroblasts, lymphoid/myeloid progenitors, and cells implied in innate-immune response.

The second was CL-B, which showed the overexpression of *PCSK1*, *QPCT*, *ST18*, and *TPH1* genes. Its most characterizing signatures were those related to adipogenesis, pancreatic β-cell signatures, neuroendocrine metabolism, and splice site machinery-related processes. Regarding cell-of-origin profiles, CL-B showed significant similarities to large intestinal entero-endocrine cells, and about immune cells, CL-B was enriched in T CD4 + lymphocytes, also showing the highest immune score.

The third was CL-C, which showed the overexpression of *ALB*, *ANG*, *ARG1*, and *HP* genes. Its most characterizing signatures were complement/coagulation, EMT, and xenobiotic metabolism. Regarding cell-of-origin profiles, CL-C showed significant similarities to small intestinal enterocyte progenitors, and about immune cells, it was enriched in M1/2 macrophages and dendritic cells.

The distinction in different clusters obtained with our analysis may have implications for refining therapeutic approaches to I-NETs patients. Regarding gene expression profiles, indeed, the specific overexpression of *SSTR4* gene in CL-A calls for considering therapeutic approaches based on somatostatin analogs above all for patients belonging to this molecular subgroup [33]. Furthermore, regarding biological process differently activated in each cluster, CL-B demonstrated a specific activation in splice site machinery-related mechanisms. These are highly coordinated processes, regulated and carried out by the spliceosome and its interactions with splicing factors, intronic and exonic sequence elements, and signaling pathways to adequately control gene-expression [34–36]. Of note, emerging therapeutic approaches are exploring actionable vulnerabilities of solid malignancies based on splicing-related targets [37,38]. At last, regarding the findings of immune cell composition with therapeutic implications, CL-C demonstrated a specific enrichment in M1/2 macrophages. Along this line, new therapeutic efforts are now under consideration for targeting gastrointestinal tumors through the perturbation and immunomodulation of macrophages infiltration [39].

Previous investigations on gene-expression profiling of I-NET showed some similarities in terms of biological mechanisms that resulted activated in this tumor entity [14,15]. Moreover, Anderson et al. also showed the presence of an entire cluster totally composed of liver or lymph node metastases. This finding further confirms that context matters in cancer biology [40,41], since also for I-NET the metastatic disease seems to be influenced by the local microenvironment, with an entire biological cluster composed of metastatic tumors. Of note, tissues belonging to that cluster showed a decreased expression of neuroendocrine markers, such as *CHGA*, *NEUROD1* and *SYP* genes. In our series, which was completely composed of primary I-NET, we did not find any

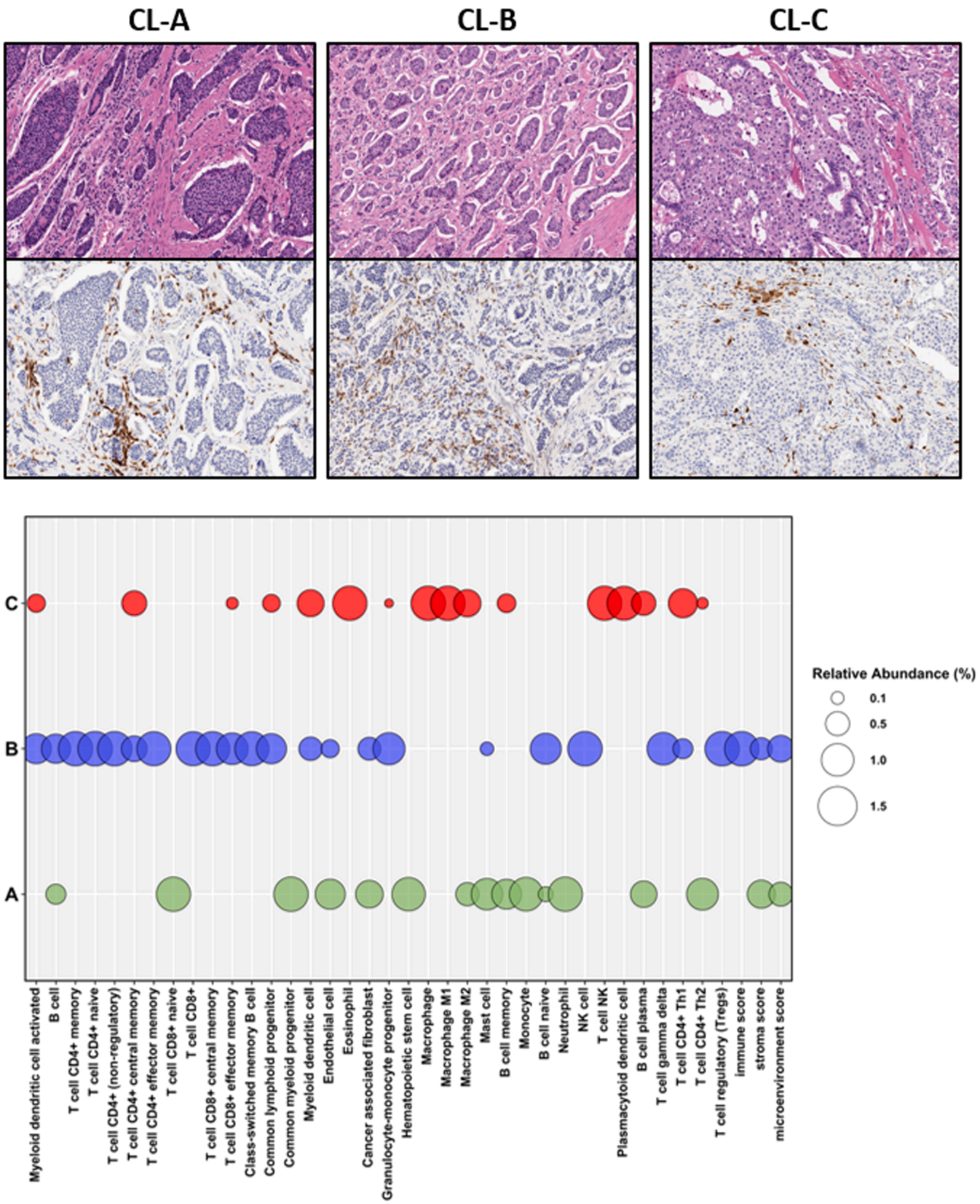

Fig. 4. Immune microenvironment of intestinal neuroendocrine tumors (I-NET), based on clusters. In the upper panel: representative hematoxylin-eosin picture of a case from each cluster (CL-A, CL-B, and CL-C), with the related most specific immunohistochemical marker, as follows: i) CL-A: CD8 (for T CD8 + lymphocytes), ii) CL-B: CD4 (for T CD4 + lymphocytes), and iii) CL-C: CD68 (for macrophages). In the lower panel, bubble plot showing the relative abundance (%) of immune subpopulations inferred by gene expression of immune metagenes significantly enriched in each cluster.

difference between CL-A, CL-B, and CL-C for those genes, suggesting that they may be of importance in the metastatic site and not in primary tumors. Considering together all such findings, as well as the results related to tumor origin, I-NET should be considered as a disease of the entire intestine. Our cohort reflected the differences of site-specific prevalence for I-NET, where the ileum is the most common location. At the same time, our study also showed that duodenum, colon, and jejunum NET displayed gene-expression profiles that can be grouped with NET from ileum without statistical outliers in the clustering process.

With DE analysis coupled with volcano plot, we also showed the presence of statistically significant differences in terms of gene expression profiles between I-NET and pancreatic NET. Indeed, we identified 9 I-NET specific genes, such as *ALB*, *APOA1*, *CHGA*, *DES*, *HP*, *IL6ST*, *SCARNA7*, *TPH1*, and *TPM1*, and 9 pancreatic NET specific genes, such as *AK4*, *CA9*, *CHST13*, *CNFN*, *GALNT4*, *HMBS*, *LDHB*, *MAGEB2*, and *PGK1*. These findings were in line, at least in part, with previous results reported by Wang et al., who described some of our I-NET specific genes as hub genes in I-NET primaries vs liver metastases. Currently, in cases of neuroendocrine metastasis from unknown primary, there are very few tools helping in identifying the exact tumor origin. For example, the loss of DAXX or ATRX proteins for *DAXX* or *ATRX* mutations and the presence of the activation of alternative lengthening of telomeres (so-called ALT) seem to be highly specific for pancreatic origin [42–45], whereas the IHC expression of the marker CDX2 can support the intestinal origin, although it may also be positive in gastric and esophageal NET [46,47]. *DAXX/ATRX* alteration and ALT activation are associated with poor prognosis in pancreatic NETs [42–45], but they are not altered in a significant fraction of pancreatic tumors. Of note, based on our results, also gene expression profiling can be of help in this difficult task, and may be used as a second-level analysis for supporting the distinction of pancreatic vs. intestinal NETs.

Our study does have some limitations. First, the sample size of I-NETs in our study may have been relatively small, but this is mainly due to the relatively rarity of this tumor entity. Furthermore, we did not find any survival difference based on the different cluster, thus further studies should explore in the future the presence of potential prognostic potentials of transcriptome analysis in I-NET. At last, we reported data from transcriptome analysis, but the integration of other –omics analysis could be of interest for further clarifying the complex I-NET biological landscape, ideally with the support of artificial intelligence-based algorithms.

In conclusion, this study provides new insights into I-NET biology. By transcriptome analysis, I-NET clustered into three subtypes that correlate with the activation of different signaling mechanism pathways and tumor microenvironment. Such differences may have potential implications for the clinical management of I-NET patients. We also reported the presence of a gene-set for differentiating I-NET from pancreatic NET. Globally considered, our findings represent an additional step for refining the molecular taxonomy of I-NET and for designing future therapeutic strategies.

Supplementary Files

Supplementary Table 1. List of genes used in unsupervised clustering analysis and differential expression analysis among the different identified clusters.

Funding

This work was supported by Italian Cancer Genome Project (FIRB RBAP10AHJB to A.S.), Italian Ministry of Health (ERP-2017–23671129 “PMTR-pNET” Project to M.M). Giovanni Centonze was supported by a FIRC-AIRC fellowship for Italy. Michele Bevere is supported by a AIRC fellowship for Italy (ID 28054). The funding agencies had no role in the collection, analysis and interpretation of data and in the writing of the manuscript.

CRediT authorship contribution statement

Paola Mattiolo: Conceptualization, Data curation, Investigation, Validation, Writing – original draft, Writing – review & editing, Interpretation. **Anastasios Gkountakos:** Data curation, Validation, Writing – review & editing, Interpretation. **Giovanni Centonze**: Data curation, Validation, Writing – review & editing, Interpretation. **Michele Bevere:** Data curation, Validation, Writing – review & editing, Interpretation. **Paola Piccoli:** Data curation, Validation, Writing – review & editing, Interpretation. **Serena Ammendola**: Data curation, Validation, Writing – review & editing, Interpretation. **Corrado Pedrazzani:** Data curation, Validation, Writing – review & editing, Interpretation. **Luca Landoni:** Data curation, Validation, Writing – review & editing, Interpretation. **Sara Cingarlini:** Data curation, Validation, Writing – review & editing, Interpretation. **Michele Milella:** Data curation, Validation, Writing – review & editing, Interpretation. **Massimo Milione:** Data curation, Funding acquisition, Validation, Writing – review & editing, Interpretation. **Claudio Luchini:** Conceptualization, Data curation, Validation, Writing – review & editing, Interpretation. **Aldo Scarpa**: Conceptualization, Data curation, Funding acquisition, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing, Interpretation. **Michele Simbolo**: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing, Interpretation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data are available in the current manuscript and related supplementary files.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2023.154674.

References

[1] S.Y. Pan, H. Morrison, Epidemiology of cancer of the small intestine, World J. Gastrointest. Oncol. 3 (2011) 33–42, https://doi.org/10.4251/wjgo.v3.i3.33.
[2] I.M. Modlin, K.D. Lye, M. Kidd, A 5-decade analysis of 13,715 carcinoid tumors, Cancer 97 (2003) 934–959, https://doi.org/10.1002/cncr.11105.
[3] I.M. Modlin, K. Oberg, D.C. Chung, R.T. Jensen, W.W. de Herder, R.V. Thakker, M. Caplin, G.D. Fave, G.A. Kaltsas, E.P. Krenning, S.F. Moss, O. Nilsson, G. Rindi, R. Salazar, P. Ruszniewski, A. Sundin, Gastroenteropancreatic neuroendocrine tumours, Lancet Oncol. 9 (2008) 61–72, https://doi.org/10.1016/S1470-2045(07)70410-2.
[4] H. Jann, S. Roll, A. Couvelard, O. Hentic, M. Pavel, J. Müller-Nordhorn, M. Koch, C. Röcken, G. Rindi, P. Ruszniewski, B. Wiedenmann, U.-F. Pape, Neuroendocrine tumors of midgut and hindgut origin: tumor-node-metastasis classification determines clinical outcome, Cancer 117 (2011) 3332–3341, https://doi.org/10.1002/cncr.25855.
[5] M.E. Caplin, J.R. Buscombe, A.J. Hilson, A.L. Jones, A.F. Watkinson, A. K. Burroughs, Carcinoid tumour, Lancet 352 (1998) 799–805, https://doi.org/10.1016/S0140-6736(98)02286-7.
[6] A. Rinke, H.-H. Müller, C. Schade-Brittinger, K.-J. Klose, P. Barth, M. Wied, C. Mayer, B. Aminossadati, U.-F. Pape, M. Bläker, J. Harder, C. Arnold, T. Gress, R. Arnold, Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the PROMID study group, JCO 27 (2009) 4656–4663, https://doi.org/10.1200/JCO.2009.22.8510.
[7] A. Rinke, M. Wittenberg, C. Schade-Brittinger, B. Aminossadati, E. Ronicke, T. M. Gress, H.-H. Müller, R. Arnold, for the PROMID Study Group, Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine

midgut tumors (PROMID): results of long-term survival, Neuroendocrinology 104 (2016) 26–32, https://doi.org/10.1159/000443612.
[8] L. Bottarelli, C. Azzoni, S. Pizzi, T. D'Adda, E.M. Silini, C. Bordi, G. Rindi, Adenomatous polyposis coli gene involvement in ileal enterochromaffin cell neuroendocrine neoplasms, Hum. Pathol. 44 (2013) 2736–2742, https://doi.org/10.1016/j.humpath.2013.06.019.
[9] A. Karpathakis, H. Dibra, C. Pipinikas, A. Feber, T. Morris, J. Francis, D. Oukrif, D. Mandair, M. Pericleous, M. Mohmaduvesh, S. Serra, O. Ogunbiyi, M. Novelli, T. Luong, S.L. Asa, M. Kulke, C. Toumpanakis, T. Meyer, M. Caplin, M. Meyerson, S. Beck, C. Thirlwell, Prognostic impact of novel molecular subtypes of small intestinal neuroendocrine tumor, Clin. Cancer Res. 22 (2016) 250–258, https://doi.org/10.1158/1078-0432.CCR-15-0373.
[10] J.M. Francis, A. Kiezun, A.H. Ramos, S. Serra, C.S. Pedamallu, Z.R. Qian, M. S. Banck, R. Kanwar, A.A. Kulkarni, A. Karpathakis, V. Manzo, T. Contractor, J. Philips, E. Nickerson, N. Pho, S.M. Hooshmand, L.K. Brais, M.S. Lawrence, T. Pugh, A. McKenna, A. Sivachenko, K. Cibulskis, S.L. Carter, A.I. Ojesina, S. Freeman, R.T. Jones, D. Voet, G. Saksena, D. Auclair, R. Onofrio, E. Shefler, C. Sougnez, J. Grimsby, L. Green, N. Lennon, T. Meyer, M. Caplin, D.C. Chung, A. S. Beutler, S. Ogino, C. Thirlwell, R. Shivdasani, S.L. Asa, C.R. Harris, G. Getz, M. Kulke, M. Meyerson, Somatic mutation of CDKN1B in small intestine neuroendocrine tumors, Nat. Genet. 45 (2013) 1483–1486, https://doi.org/10.1038/ng.2821.
[11] C. Park, S.Y. Ha, S.T. Kim, H.C. Kim, J.S. Heo, Y.S. Park, G. Lauwers, J. Lee, K.-M. Kim, Identification of the BRAF V600E mutation in gastroenteropancreatic neuroendocrine tumors, Oncotarget 7 (2015) 4024–4035, https://doi.org/10.18632/oncotarget.6602.
[12] M. Simbolo, C. Vicentini, A. Mafficini, M. Fassan, S. Pedron, V. Corbo, L. Mastracci, B. Rusev, C. Pedrazzani, L. Landoni, F. Grillo, S. Cingarlini, G. Rindi, C. Luchini, A. Scarpa, R.T. Lawlor, Mutational and copy number asset of primary sporadic neuroendocrine tumors of the small intestine, Virchows Arch. 473 (2018) 709–717, https://doi.org/10.1007/s00428-018-2450-x.
[13] M.S. Banck, R. Kanwar, A.A. Kulkarni, G.K. Boora, F. Metge, B.R. Kipp, L. Zhang, E. C. Thorland, K.T. Minn, R. Tentu, B.W. Eckloff, E.D. Wieben, Y. Wu, J. M. Cunningham, D.M. Nagorney, J.A. Gilbert, M.M. Ames, A.S. Beutler, The genomic landscape of small intestine neuroendocrine tumors, J. Clin. Invest 123 (2013) 2502–2508, https://doi.org/10.1172/JCI67963.
[14] E. Andersson, Y. Arvidsson, C. Swärd, T. Hofving, B. Wängberg, E. Kristiansson, O. Nilsson, Expression profiling of small intestinal neuroendocrine tumors identifies subgroups with clinical relevance, prognostic markers and therapeutic targets, Mod. Pathol. 29 (2016) 616–629, https://doi.org/10.1038/modpathol.2016.48.
[15] Q. Wang, C. Yu, Expression profiling of small intestinal neuroendocrine tumors identified pathways and gene networks linked to tumorigenesis and metastasis, Biosci. Rep. 40 (2020), https://doi.org/10.1042/BSR20193860.
[16] W.H.O. Classification of Tumours Editorial Board. Digestive system tumours., 5th ed., International Agency for Research on Cancer, Lyon, 2019. http://publication.iarc.fr/579.
[17] M.B. Amin, S. Edge, F. Greene, D.R. Byrd, R.K. Brookland, M.K. Washington, J. E. Gershenwald, C.C. Compton, K.R. Hess, D.C. Sullivan, J.M. Jessup, J.D. Brierley, L.E. Gaspar, R.L. Schilsky, C.M. Balch, D.P. Winchester, E.A. Asare, M. Madera, D. M. Gress, L.R. Meyer (Eds.), AJCC Cancer Staging Manual, eighth ed., Springer International Publishing, 2017 accessed October 31, 2020, ⟨https://www.springer.com/gp/book/9783319406176⟩.
[18] M. Simbolo, M. Gottardi, V. Corbo, M. Fassan, A. Mafficini, G. Malpeli, R.T. Lawlor, A. Scarpa, DNA qualification workflow for next generation sequencing of histopathological samples, PLoS One 8 (2013), e62692, https://doi.org/10.1371/journal.pone.0062692.
[19] M.I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, Genome Biol. 15 (2014) 550, https://doi.org/10.1186/s13059-014-0550-8.
[20] C.W. Law, M. Alhamdoosh, S. Su, X. Dong, L. Tian, G.K. Smyth, M.E. Ritchie, RNA-seq analysis is easy as 1–2-3 with limma, Glimma and edgeR, (2018). https://doi.org/10.12688/f1000research.9005.3.
[21] W.E. Johnson, C. Li, A. Rabinovic, Adjusting batch effects in microarray expression data using empirical Bayes methods, Biostatistics 8 (2007) 118–127, https://doi.org/10.1093/biostatistics/kxj037.
[22] Z. Gu, R. Eils, M. Schlesner, Complex heatmaps reveal patterns and correlations in multidimensional genomic data, Bioinformatics 32 (2016) 2847–2849, https://doi.org/10.1093/bioinformatics/btw313.
[23] A. Subramanian, P. Tamayo, V.K. Mootha, S. Mukherjee, B.L. Ebert, M.A. Gillette, A. Paulovich, S.L. Pomeroy, T.R. Golub, E.S. Lander, J.P. Mesirov, Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles, Proc. Natl. Acad. Sci. 102 (2005) 15545–15550, https://doi.org/10.1073/pnas.0506580102.
[24] A. Liberzon, A. Subramanian, R. Pinchback, H. Thorvaldsdóttir, P. Tamayo, J. P. Mesirov, Molecular signatures database (MSigDB) 3.0, Bioinformatics 27 (2011) 1739–1740, https://doi.org/10.1093/bioinformatics/btr260.
[25] T. Li, J. Fu, Z. Zeng, D. Cohen, J. Li, Q. Chen, B. Li, X.S. Liu, TIMER2.0 for analysis of tumor-infiltrating immune cells, Nucleic Acids Res. 48 (2020) W509–W514, https://doi.org/10.1093/nar/gkaa407.
[26] D. Aran, Z. Hu, A.J. Butte, xCell: digitally portraying the tissue cellular heterogeneity landscape, Genome Biol. 18 (2017) 220, https://doi.org/10.1186/s13059-017-1349-1.
[27] W. Luo, M.S. Friedman, K. Shedden, K.D. Hankenson, P.J. Woolf, GAGE: generally applicable gene set enrichment for pathway analysis, BMC Bioinforma. 10 (2009) 161, https://doi.org/10.1186/1471-2105-10-161.
[28] F. Sabbatino, V. Villani, J.H. Yearley, V. Deshpande, L. Cai, I.T. Konstantinidis, C. Moon, S. Nota, Y. Wang, A. Al-Sukaini, A.X. Zhu, L. Goyal, D.T. Ting, N. Bardeesy, T.S. Hong, C. Fernandez-del Castillo, K.K. Tanabe, K.D. Lillemoe, S. Ferrone, C.R. Ferrone, PD-L1 and HLA Class I antigen expression and clinical course of the disease in intrahepatic cholangiocarcinoma, Clin. Cancer Res. 22 (2016) 470–478, https://doi.org/10.1158/1078-0432.CCR-15-0715.
[29] C. Luchini, J. Cros, A. Pea, C. Pilati, N. Veronese, B. Rusev, P. Capelli, A. Mafficini, A. Nottegar, L.A.A. Brosens, M. Noë, G.J.A. Offerhaus, P. Chianchiano, G. Riva, P. Piccoli, C. Parolini, G. Malleo, R.T. Lawlor, V. Corbo, N. Sperandio, M. Barbareschi, M. Fassan, L. Cheng, L.D. Wood, A. Scarpa, PD-1, PD-L1, and CD163 in pancreatic undifferentiated carcinoma with osteoclast-like giant cells: expression patterns and clinical implications, Hum. Pathol. 81 (2018) 157–165, https://doi.org/10.1016/j.humpath.2018.07.006.
[30] M. Simbolo, S. Bersani, C. Vicentini, S.V. Taormina, C. Ciaparrone, F. Bagante, B. Rusev, G. Centonze, M. Montresor, M. Brunelli, S. Pedron, A. Mafficini, G. Paolino, P. Mattiolo, S. Conci, M. Milione, A. Guglielmi, A. Ruzzenente, A. Scarpa, C. Luchini, Molecular characterization of extrahepatic cholangiocarcinoma: perihilar and distal tumors display divergent genomic and transcriptomic profiles, Expert Opin. Ther. Targets 25 (2021) 1095–1105, https://doi.org/10.1080/14728222.2021.2013801.
[31] S. Gao, L. Yan, R. Wang, J. Li, J. Yong, X. Zhou, Y. Wei, X. Wu, X. Wang, X. Fan, J. Yan, X. Zhi, Y. Gao, H. Guo, X. Jin, W. Wang, Y. Mao, F. Wang, L. Wen, W. Fu, H. Ge, J. Qiao, F. Tang, Tracing the temporal-spatial transcriptome landscapes of the human fetal digestive tract using single-cell RNA-sequencing, Nat. Cell Biol. 20 (2018) 721–734, https://doi.org/10.1038/s41556-018-0105-4.
[32] M. Simbolo, M. Bilotta, A. Mafficini, C. Luchini, D. Furlan, F. Inzani, G. Petrone, D. Bonvissuto, S. La Rosa, G. Schinzari, A. Bianchi, E. Rossi, R. Menghi, F. Giuliante, S. Boccia, A. Scarpa, G. Rindi, Gene expression profiling of pancreas neuroendocrine tumors with different Ki67-based grades, Cancers 13 (2021) 2054, https://doi.org/10.3390/cancers13092054.
[33] R. Baldelli, Somatostatin analogs therapy in gastroenteropancreatic neuroendocrine tumors: current aspects and new perspectives, Front. Endocrinol. 5 (2014). ⟨https://www.frontiersin.org/articles/10.3389/fendo.2014.00007⟩. accessed June 21, 2023.
[34] M. Chen, J.L. Manley, Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches, Nat. Rev. Mol. Cell Biol. 10 (2009) 741–754, https://doi.org/10.1038/nrm2777.
[35] V. Pelechano, W. Wei, L.M. Steinmetz, Extensive transcriptional heterogeneity revealed by isoform profiling, Nature 497 (2013) 127–131, https://doi.org/10.1038/nature12121.
[36] I. Kozlovski, Z. Siegfried, A. Amar-Schwartz, R. Karni, The role of RNA alternative splicing in regulating cancer metabolism, Hum. Genet 136 (2017) 1113–1127, https://doi.org/10.1007/s00439-017-1803-x.
[37] W. Wojtyś, M. Oroń, How driver oncogenes shape and are shaped by alternative splicing mechanisms in tumors, Cancers 15 (2023) 2918, https://doi.org/10.3390/cancers15112918.
[38] J.D. Fackenthal, Alternative mRNA splicing and promising therapies in cancer, Biomolecules 13 (2023) 561, https://doi.org/10.3390/biom13030561.
[39] Y. Shen, J.-X. Chen, M. Li, Z. Xiang, J. Wu, Y.-J. Wang, Role of tumor-associated macrophages in common digestive system malignant tumors, World J. Gastrointest. Oncol. 15 (2023) 596–616, https://doi.org/10.4251/wjgo.v15.i4.596.
[40] G. Schneider, M. Schmidt-Supprian, R. Rad, D. Saur, Tissue-specific tumorigenesis: context matters, Nat. Rev. Cancer 17 (2017) 239–253, https://doi.org/10.1038/nrc.2017.5.
[41] R.T. Lawlor, A. Mafficini, C. Sciammarella, C. Cantù, B.C. Rusev, M.L. Piredda, D. Antonello, S. Grimaldi, G. Bonizzato, N. Sperandio, G. Marchegiani, G. Malleo, A. Pea, R. Salvia, A. Mombello, G. Mazzoleni, A. Nottegar, E. Hanspeter, G. Riva, A. Tomezzoli, M. Bencivenga, G. de Manzoni, S. Pedron, G. Paolino, P. Mattiolo, L. A. Brosens, N. Silvestris, M. Fassan, S.L. Cooke, P.A. Beer, M. Milella, V.N. Adsay, L. Cheng, A. Scarpa, C. Luchini, Genomic characterization of hepatoid tumors: context matters, Hum. Pathol. 118 (2021) 30–41, https://doi.org/10.1016/j.humpath.2021.09.006.
[42] W.M. Hackeng, L.A.A. Brosens, J.Y. Kim, R. O'Sullivan, Y.-N. Sung, T.-C. Liu, D. Cao, M. Heayn, J. Brosnan-Cashman, S. An, F.H.M. Morsink, C.M. Heidsma, G. D. Valk, M.R. Vriens, E. Nieveen van Dijkum, G.J.A. Offerhaus, K.M.A. Dreijerink, H. Zeh, A.H. Zureikat, M. Hogg, K. Lee, D. Geller, J.W. Marsh, A. Paniccia, M. Ongchin, J.F. Pingpank, N. Bahary, M. Aijazi, R. Brand, J. Chennat, R. Das, K. E. Fasanella, A. Khalid, K. McGrath, S. Sarkaria, H. Singh, A. Slivka, M. Nalesnik, X. Han, M.N. Nikiforova, R.T. Lawlor, A. Mafficini, B. Rusev, V. Corbo, C. Luchini, S. Bersani, A. Pea, S. Cingarlini, L. Landoni, R. Salvia, M. Milione, M. Milella, A. Scarpa, S.-M. Hong, C.M. Heaphy, A.D. Singhi, Non-functional pancreatic neuroendocrine tumours: ATRX/DAXX and alternative lengthening of telomeres (ALT) are prognostically independent from ARX/PDX1 expression and tumour size, Gut 71 (2022) 961–973, https://doi.org/10.1136/gutjnl-2020-322595.
[43] C. Luchini, R.T. Lawlor, S. Bersani, C. Vicentini, G. Paolino, P. Mattiolo, A. Pea, S. Cingarlini, M. Milella, A. Scarpa, Alternative lengthening of telomeres (ALT) in pancreatic neuroendocrine tumors: ready for prime-time in clinical practice? Curr. Oncol. Rep. 23 (2021) 106, https://doi.org/10.1007/s11912-021-01096-w.
[44] A. Pea, J. Yu, L. Marchionni, M. Noe, C. Luchini, A. Pulvirenti, R.F. de Wilde, L. A. Brosens, N. Rezaee, A. Javed, P. Chianchiano, S. Gobbo, P. Regi, R. Salvia, C. Bassi, J. He, M.J. Weiss, J.L. Cameron, G.J.A. Offerhaus, R.H. Hruban, R. T. Lawlor, A. Scarpa, C.M. Heaphy, L.D. Wood, C.L. Wolfgang, Genetic analysis of small well-differentiated pancreatic neuroendocrine tumors identifies subgroups with differing risks of liver metastases, Ann. Surg. 271 (2020) 566–573, https://doi.org/10.1097/SLA.0000000000003022.

[45] R.T. Lawlor, N. Veronese, A. Pea, A. Nottegar, L. Smith, C. Pilati, J. Demurtas, M. Fassan, L. Cheng, C. Luchini, Alternative lengthening of telomeres (ALT) influences survival in soft tissue sarcomas: a systematic review with meta-analysis, BMC Cancer 19 (2019) 232, https://doi.org/10.1186/s12885-019-5424-8.

[46] R.S. Saad, Z. Ghorab, M.A. Khalifa, M. Xu, CDX2 as a marker for intestinal differentiation: Its utility and limitations, World J. Gastrointest. Surg. 3 (2011) 159–166, https://doi.org/10.4240/wjgs.v3.i11.159.

[47] M.K. Li, A.L. Folpe, CDX-2, a new marker for adenocarcinoma of gastrointestinal origin, Adv. Anat. Pathol. 11 (2004) 101–105, https://doi.org/10.1097/00125480-200403000-00004.