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Genetic insight into lung neuroendocrine tumors: Notch and Wnt signaling pathways as potential targets

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Abstract

Background The molecular landscape of lung neuroendocrine neoplasms is still poorly characterized, making it difficult to develop a molecular classification and personalized therapeutic approaches. Significant clinical heterogeneity of these malignancies has been highlighted among poorly differentiated histotypes and within the subgroup of well-differentiated neuroendocrine tumors (NET). Currently, the main prognostic factors of lung NET include stage, histotype, grade, peripheral location, and demographic parameters. To gain deeper insights into the genomic underpinnings of lung NETs, we conducted a pilot investigation to uncover potential genetic mutations and copy number variations (CNVs) implicated in their pathogenesis.

Methods Formalin-fixed, paraffin-embedded intraoperative tumor biopsies and matched peripheral blood mononuclear cell samples were collected from six consecutive patients with lung NETs. The whole exome sequencing (WES) was performed to profile germline and somatic mutations, identify novel genetic alterations, and detect CNVs. Clinical and pathological data were systematically documented at diagnosis and during follow-up.

Results The WES analysis identified a subset of mutations shared between germline and somatic; some were of particular clinical interest as they were associated with tumor proliferation and potential therapeutic targets such as the genes *KDM5C, ATR, COL7A1, NOTCH4, PTPRS, SMO, SPEN, SPTA1, TAF1*. These mutations were predominantly linked to chromatin remodeling and were involved in critical oncogenic pathways such as Notch and Wnt signaling.

Conclusions This pilot study highlights the potential role of NGS analysis on solid biopsy in the assessment of the mutational profile of lung NET. A comparison of germline and somatic mutations is critical to identifying putative tumor driver mutations. In perspective, the enrichment of a subpopulation of cancer cells in the blood, with one or more specific mutations, is information of enormous clinical relevance, either for prognosis or therapeutic decisions. Translational studies on large prospective series are required to establish the role of liquid biopsy in lung NET.

Keywords Lung neuroendocrine tumors, Carcinoids, Next generation sequencing, Somatic mutation, Germline mutation, Whole exome sequencing, NET epigenetics, Notch pathway, Wnt pathway, Chromatin remodeling

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Background

Neuroendocrine Tumors (NET) are a miscellaneous group of malignancies originating from neuroendocrine cells distributed throughout the body and arising in various organs, including the gastrointestinal tract, lungs, pancreas, and thymus [1, 2]. For this reason are characterized by elevated heterogeneity, which makes difficult to achieve an uniform diagnostic definition and prognostic stratification. Lung NET are classified depending on morphology, mitotic count, necrosis, and cytological features. High-grade neuroendocrine carcinomas (NEC) include poorly differentiated histotypes. Low-grade NET includes the well-differentiated forms, the so-called typical (TC) and atypical carcinoids (AC) [3]. NET commonly overexpress somatostatin receptors (SSTR), which are becoming used as diagnostic and therapeutic targets [4]. Most carcinoids can be cured by surgery [5].

However, in the setting of advanced disease [6], somatostatin analogs (SSAs) are commonly used in non-rapidly progressive SST-positive L-NETs, although there is not a formal approval for this indication [7, 8], octreotide and lanreotide [9, 10], chemotherapy [11], and everolimus [12, 13], with variable tumor response. There are few validated molecular biomarkers in lung NET [14–17] and, therefore, no personalized strategies for clinical practice. However, in recent years, several works have highlighted the central role of genes involved in a few biological mechanisms such as chromatin remodeling, DNA repair and splicing [16–18]. This data alone makes urgent the need for accurate biomarkers for early diagnosis of NET patients, as well as their prognostic and therapeutic assessment to improve survival and clinical management [19, 20].

In this context we have conducted a pilot study on a cohort of six well differentiated lung NET, by high-throughput whole exome sequencing (WES) analysis, at germline and somatic level, to uncover any genetic mutation and copy number variations (CNVs) that might contribute to lung NET carcinogenesis. Our results could add new knowledge about the mutational landscape of NET to identify novel prognostic biomarkers and therapeutic targets that shall contribute to incorporating precision medicine in clinical practice and ameliorating lung NET outcomes.

Material and methods

Patients

Blood and tumor samples were obtained from 6 consecutive surgically treated lung NET patients collected at the AOU Sant'Andrea NET Unit. Inclusion criteria were a histologically confirmed diagnosis of lung NET (TC or AC, according to 2021 World Health Organization classification). Patients with poorly differentiated neuroendocrine carcinomas (LCNEC and SCLC), high-grade NET G3, mixed neuroendocrine-epithelial histology, and no-neuroendocrine histology were excluded. Two patients were siblings, achieving the diagnosis of NET the same year. Expert NEN-dedicated pathologist (M. M) reviewed all histological samples in this study. For each patient, we collected formalin-fixed, paraffin-embedded (FFPE) tissue samples from the primary tumor and obtained corresponding blood samples at diagnosis (surgical or bioptical tissue sample). Clinical, biochemical and radiological data were collected at the diagnosis and during the follow-up. Written informed consent was obtained from all patients. This study was performed by the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Institutional Ethical Committee (n. 7269 protocol 0730/2023).

Clinical characteristics of the lung NET cohort

Patients with lung NET included were three women and three men, with an average age of 57.5 years (range 38–77). According to the pathological classification, five out of six lung NET were classified as TC, the remaining one was classified as AC. Tumor staging revealed that two patients had stage I NET, three had stage II NET, and one had stage III NET. The Ki-67 index was 0.5% in one patient, 1% in four patients, 3% in one patient. Mitotic counts ranged from 0.5 to 3.5 per 10 high-power fields. All patients with typical carcinoid were low grade NET G1, the patient with atypical carcinoid was intermediate grade NET G2. The mean diameter of the lesions was 3.5 cm, and all patients had positive immunostaining for neuroendocrine markers such as Cytokeratin AE1/AE3 (CK AE1/ AE3), Chromogranin A (CGA), Synaptophysin (SYN), and Insulinoma- associated protein 1 (INSM1). As regards regional nodal status, four patients had no regional lymph node involvement (N0), one patient had not evaluable regional nodal status (Nx) and one patient was N2. Tissue and blood samples were collected from all six patients before surgery. All enrolled patients displayed stable disease along the follow-up (7–17 months). The cohort's clinical characteristics and IHC neuroendocrine markers were summarized in Table 1.

PBMC and tissue sample processing

Peripheral blood mononuclear cells (PBMC) were isolated by a density gradient, using Lympholyte (Cedarlane), following the manufacturer's instructions [21]. The tumor tissue analysis was based on the FFPE samples. To minimize the generation of artefacts, in particular cytosine deamination, all samples used were

Table 1 Clinical pathological characteristics and IHC markers of lung NET cohort

ID	SANET_002	SANET_004	SANET_005	SANET_006	SANET_011	SANET_016
Sex	F	F	M	F	M	M
Age	77	43	66	64	38	57
Histotype	TC	TC	TC	TC	TC	AC
Localization	cr	cr	Cl	pr	cr	cl
Ki-67(%)	1	1	0.5	1	1	3
MI (mm^2)	1.5	1	1	0.5	0.5	3.5
Grade	G1	G1	G1	G1	G1	G2
Diameter (cm)	1.8	4.5	2.8	5	4.2	2.5
Stage	IA2	IIA	IIIB	IIB	IIA	IA3
CK AE1/AE3	+	+	+	+	+	+
CGA	+	+	+	+	+	−/+
SYN	+	+	+	+	+	+
INSM	+	+	+	+	+	+
TNM	pT1b pN0 LVI0	pT2b pN0 LVI0	pT4 pN2 LVI1	pT3 pNx LVI1	pT2b pN0 LVI1	pT1c pN0 LVI0
staging	PL0 R0	PL2 R1	PL2 R0	PL0 R0	PL0 R0	PL0 R0

TC = typical carcinoids; AC = atypical carcinoids; cr = central right; cl = central left; pr = peripheral right; MI = mitotic index; CK = Cytokeratin; CGA = Chromogranin A; SYN = Synaptophysin; INSM1 = Insulinoma-associated protein 1; TNM = Tumor– Nodes– Metastasis for IHC staining + = 100%; ± = 50–75%; −/+ = 25–50%; − = 0%

fixed at 4 °C. Afterwards the paraffin blocks were cut, and DNA was extracted using the GeneRead DNA FFPE Kit (Qiagen).

Bulk exome sequencing

DNA was extracted from PBMC and FFPE tissue using the DNeasy Blood & Tissue Kit (Qiagen) and GeneRead DNA FFPE Kit (Qiagen), respectively. Genomic DNA was quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). In contrast, DNA quality (DIN range from 1 to 10) was assessed using the 4200 TapeStation and the corresponding Genomic DNA ScreenTape assay (Agilent Technologies, CA, USA). 100 ng of genomic DNA was enzymatically fragmented using the SureSelect Enzymatic Fragmentation Kit (Agilent Technologies, CA). WES was carried out using the SureSelect XT HS2 DNA Reagent Kit for library preparation, and the coding regions were enriched using the all-exon probes V7 according to the manufacturer's instructions (Agilent Technologies, CA). The quality and quantity of the intermediate whole genome library was controlled on the 4200 TapeStation with the D1000 ScreenTape Analysis (Agilent Technologies, CA). After the exome enrichment, the quality of the final library was assessed using the 4200 TapeStation (High Sensitivity D1000 ScreenTape assay), and the quantity via RT-qPCR. The libraries were sequenced on an Illumina NovaSeq6000 platform (Illumina, San Diego, CA, USA) to generate 100 bp paired-end reads.

Bioinformatic analysis

Whole exome sequencing data was analyzed with Sarek version 3.1.1, a nf-core workflow designed to detect germline and somatic variants [22]. A comparison analysis was performed between germline and somatic, and then, for each patient, a head-to-head comparison was performed. Gene and variant annotations were performed with VEP (Variant Effect Predictor). Detection of oncogenic and clinically actionable mutations was performed with PCGR (Personal Cancer Genome Reporter) software [23]. Sites of mutation were chosen based on the following criteria: a minimum of 50 sequencing reads and minor allele frequency (MAF) ≥ 0.5. Oncoprint and Transition/Transversion plots and statistical tables on mutations were generated with Maftools R package [23]. The jaccard distance and hierarchical clustering were generated with the 'dendextend' library of R software v. 4.2. Gene ontology and pathway enrichment analyses were performed using ShinyGO, considering only terms with a false discovery rate (FDR) below 0.05. The protein–protein interaction network was constructed using STRINGdb.

Statistical analysis

Categorical data were summarized using frequencies and percentages and were compared using Fisher's exact test. Continuous variables were reported as median and were compared using the Mann–Whitney U test. A p-value < 0.05 was considered significant. Hierarchical clustering explored the relationship between PBMC and FFPE samples across patients. The Jaccard index distance

was employed to capture mutation similarity. Clustering results were compared to assess consistency and highlight systematic differences between sample types. The distance matrix was constructed based on the number of exonic mutations per sample.

Results

Comprehensive mutational profiling

To thoroughly characterize lung NET's genomic landscape, all samples underwent detailed mutational profiling. This included the assessment of CNVs, single nucleotide variant (SNV) classifications, mutation burden, and variant annotations through WES analysis. Notably, no significant focal CNVs were identified across the cohort. Our data highlighted a subversion of the type of mutation from germline to somatic. In particular, C > T transitions doubled from germline to somatic, becoming the most prevalent mutation in lung NET independently from patients (Fig. 1a, b). The total number of germline mutations was quintupled compared to the somatic (Fig. 1 and Table 2). In detail, we revealed 11.889 germline mutations and 2.248 somatic mutations, with an average number of variants/sample of 1981.5 and 374.6, respectively. Notably, the patient with the most advanced tumor stage shows a more significant number of somatic mutations with p-value = 0.05 (Fig. 1). Concerning the tumor mutational burden (TMB), carcinoids showed low TMB (0.18–2.79 mut/Mb), confirming existing data in the literature [15, 24] (Table 2). Different types of genomic alterations were found, such as missense variants, stop gained, stop lost, frameshift variants, inframe deletions, inframe insertion, splice variants, multihit, and others (Fig. 1). Germline and somatic mutations were mainly determined by single nucleotide variants (SNVs), such as frameshift deletions (44% vs. 35%) and missense mutations (31% vs. 24%), respectively. Furthermore, mutations affecting splicing sites increased from germline to somatic (5% vs. 14%) (Table 2). In summary, our analysis revealed a notable shift in the mutation profile from germline to somatic, characterized by an increased prevalence of mutations at splicing sites. Additionally, we found a correlation between TMB and the stage of the disease.

Key genetic mutations and epigenetic alterations

Germline and somatic genes mutations identified in the study cohort are summarized in Fig. 2. Among the most frequent mutations, we found 50 genes known to be related to carcinogenesis. The genes displayed in the Oncoprint (Fig. 2) were selected from the lung NET cohort included in The AACR Project GENIE Consortium, using their online platform [25–27]. The recurrence rate of somatic mutations in this study is lower than that found in gastroenteropancreatic NET (GEP-NET), confirming the greater clinical and molecular heterogeneity of the former compared to the latter [28]. Lysine (K)-specific demethylase 5C (*KDM5C*) was mutated in 50% of cases. In contrast, another subset of genes such as Ataxia telangiectasia and Rad3-related (*ATR*), Collagen Type VII Alpha 1 Chain (*COL7A1*), Notch Receptor 4 (*NOTCH4*), Receptor-type tyrosine-protein phosphatase S (*PTPRS*), Smoothened (*SMO*), Spen Family Transcriptional Repressor (*SPEN*), Spectrin Alpha, Erythrocytic 1 (*SPTA1*) and TATA-box binding protein- associated factor 1 (*TAF1*) was mutated in 33% of cases. Other genes were mutated in 17% of cases. Interestingly, all the samples analyzed had at least one gene mutation involved in chromatin remodeling. These genes encoded covalent histone modifiers and subunits of the SWI–SNF complexes such as *KDM5C*, AT-rich interaction domain 1A (*ARID1A*), *PTPRS*, *TAF1*, Axis inhibition protein 2 (*AXIN2*), Spen Family Transcriptional Repressor (*SPEN*), Lysine Methyltransferase 2A (*KMT2A*), Lysine Methyltransferase 2B (*KMT2B*) and DNA methyltransferase 3 beta (*DNMT3B*). Interestingly, patients sharing somatic mutations in the *KDM5C* (c.2623-51_2729del), *NOTCH4* (c.1625-86_1729del), *SMO* (c.1779_1801 + 83del), *TAF1* (c.4315_444 + 96del) genes carried the same specific mutations (see Additional file 1). Furthermore, consistent with data shown in Fig. 1b, the patient with the highest number of somatic mutations and the most advanced stage of the disease also had the most mutated genes. Regarding the prognostic and predictive mutation potential, all mutations were classified as TIER 3 or TIER 4 (data not shown), according to the ESMO Scale for Clinical Actionability of Molecular Targets (ESCAT) [29]. Germline genes such as *FAT1* (100%), *BRCA2* (83%), *LRP1B* (83%), *NSD1* (83%), *APC* (67%), *ARID1A* (67%), *COL7A1*(67%), *PRKDC* (67%) and *ZFHX3* (67%) were consistently mutated. Overall, the oncogenic mutations shared between germline and somatic involved *ARID1A*, *COL7A1*, *SPTA1*, *FAT1*, *APC*, *CUX1*, *BCR*, *KMT2A*, *KMT2B*, *KMT2D*, *BCOR*, *IGF2R*, *MED12* and *ERBB4* genes (see Additional file 2). In addition, other highly shared somatic mutations affected genes *REG3A* (100%), *FMNL1* (83%), *TCF15* (83%), *CC2D2A* (67%), *KRTAP9-9* (67%), *SOHLH1* (67%), *TLE4* (67%) (see Additional file 3). In summary, lung NET showed a marked molecular heterogeneity with the *KDM5C* gene somatically mutated in half of the patients. In contrast, *ATR*, *COL7A1*, *NOTCH4*, *PTPRS*, *SMO*, *SPEN*, *SPTA1*, and *TAF1* genes were mutated in a third of cases. Notably, all the samples analyzed had at least one gene mutation involved in epigenetic mechanisms.

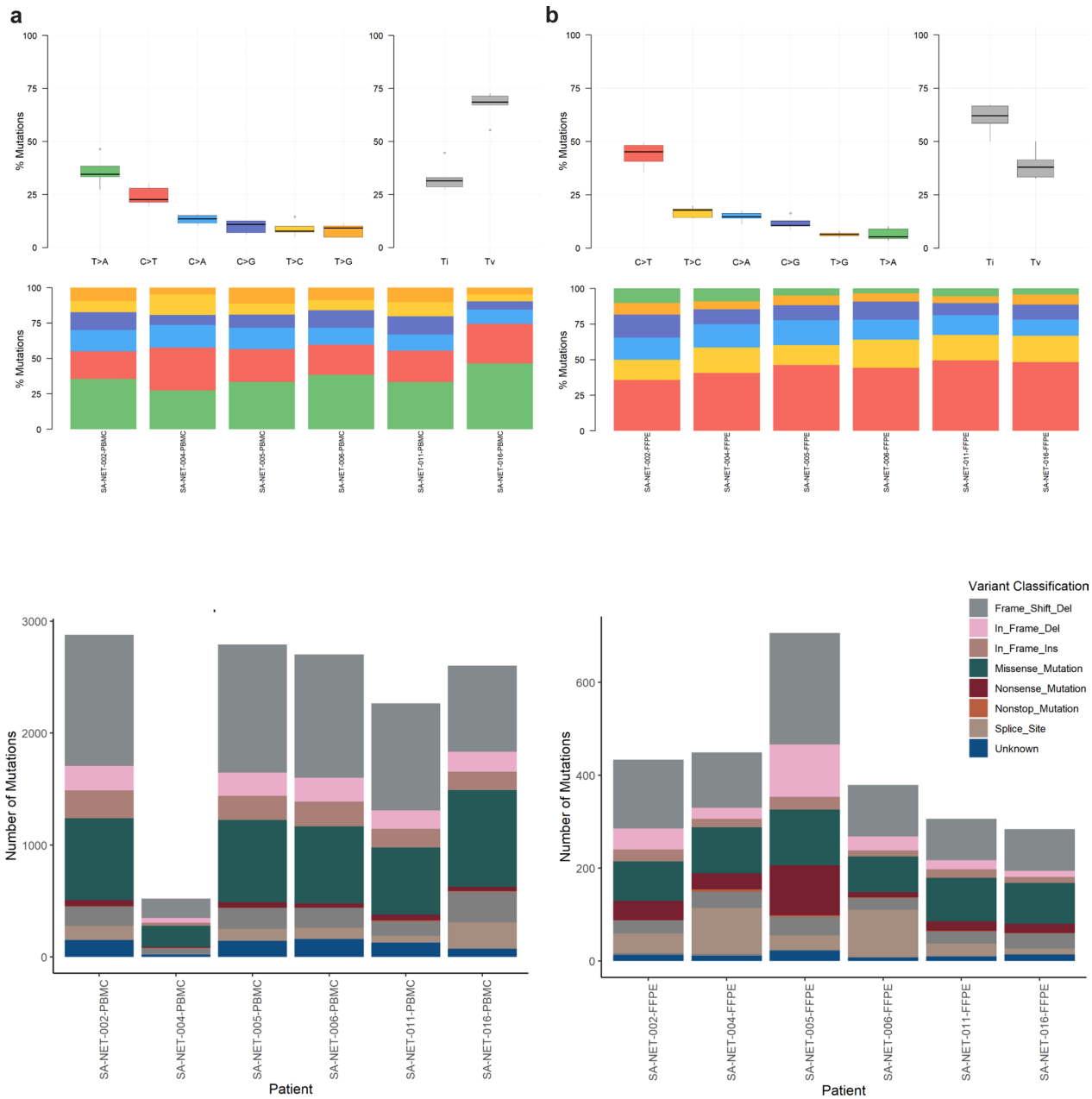

Fig. 1 Mutational profiling of samples derived from PBMC and FFPE. **a** PBMC-derived samples. The upper panel shows the transitions (Ti) and transversions (Tv) ratio, along with the distribution of single nucleotide variants (SNVs) for each sample. The lower panel displays the total number of mutations identified, categorized by variant classification, for each patient. **b** FFPE-derived samples. The upper panel shows the Ti/Tv ratio and SNV distribution for each sample. The lower panel displays the total number of mutations identified, categorized by variant classification, for each patient. FFPE-derived samples show a significantly higher number of mutations compared to PBMC-derived samples, with a predominance of variants classified as Missense Mutation and Frame Shift Del

Hierarchical clustering dendrogram

We performed hierarchical clustering analysis using exonic non-silent genetic mutations from each sample (Fig. 3). The cluster dendrogram supported genetic similarity, in terms of their mutational profile, between FFPE SA-002 and FFPE SA-011 samples and between FFPE SA-005 and FFPE SA- 006 samples. The genetic similarity between the SA-005 and SA-006 samples and germinal and somatic samples was justified because the two patients were siblings. Interestingly, despite the genetic similarity, the two siblings showed different clinical pathological features (Table 1). Furthermore,

Table 2 Number and type of germline and somatic mutations and TMB for each patient

ID	Deletion		Insertion	MSS	NSS	NST	Splice Site	Translation Start Site	TMB (mut/Mb)	Total
	Frame Shift	In Frame	In Frame							
SA-NET-002-PBMC	1156	213	248	717	53	7	128	1		2523
SA-NET-002-FFPE	142	44	26	81	41	1	42	4	0.44	381
SA-NET-004-PBMC	169	42	25	190	8	1	3	0		438
SA-NET-004-FFPE	116	24	18	98	34	5	98	4	1.53	397
SA-NET-005-PBMC	1118	207	214	714	47	5	104	3		2412
SA-NET-005-FFPE	236	110	26	119	105	3	30	2	2.79	631
SA-NET-006-PBMC	1082	209	215	679	36	4	98	2		2325
SA-NET-006-FFPE	111	28	13	74	11	2	101	2	0.68	342
SA-NET-011-PBMC	940	160	163	589	48	9	57	3		1969
SA-NET-011-FFPE	88	20	18	91	20	2	27	0	0.35	266
SA-NET-016-PBMC	760	176	164	846	36	10	227	3		2222
SA-NET-016-FFPE	89	13	13	83	19	1	12	1	0.18	231

Missense (MSS), Nonsense (NSS), Nonstop (NST), Tumor Mutational Burden (TMB)

patient 004 demonstrated a high degree of genetic overlap between germline and somatic components.

Pathway enrichment reveals critical roles of Notch and Wnt signaling

Afterward, we conducted a Gene Ontology (GO), such as Pathway enrichment analysis, to understand whether the mutated genes were part of any significant signaling pathway for lung NET, by Biological Process Enrichment and Elsevier Pathway Collection. Our analyses uncovered that six genes (*KDM5C*, *NOTCH4*, *TAF1*, *ARID1A*, *SPEN*, *FAT1*) were involved in the "*NOTCH signaling pathway*" and "*Positive regulation of transcription of NOTCH receptor target*" in the GO Biological Process Enrichment (Fig. 4a and Additional file 4). Furthermore, two genes (*SPEN*, *NOTCH4*) were also involved in the "*NOTCH receptor signaling*" in the GO Elsevier Pathway Collection (Fig. 4b and Additional file 5). Three genes (*AXIN2, APC, SMO*) were involved in the "*Activation of the Wnt pathway by blocking tumor suppressor genes*" and in "*Wnt canonical signaling activation in cancer*" in the Elsevier Pathway Collection (Fig. 4b). In conclusion, we highlighted that most mutated genes had a role in chromatin remodeling mechanism, contributing to shared functions that mainly involved the Notch and Wnt pathways (Fig. 4c).

Discussion

WHO classification defines clinically relevant subgroups of lung NET, but there is still a need for better diagnostic definition and prognostic stratification within histological subtypes. Lung NET are malignant tumors with variable clinical aggressiveness only in part predictable. The most relevant prognostic factors include age, gender, performance status, peripheral location, tumor stage, and histotype [1]. In particular, the well-differentiated forms TC and AC show low proliferative activity but increased from TC to AC [7]. Unfortunately, to date, only a few biomarkers have been established as clinically useful and reliable tools for the prediction of prognosis or response to treatment [14]. The considerable heterogeneity in their clinical presentation and histological and biological features could improve their clinical management and prompt diagnosis [20]. NET commonly overexpress somatostatin receptors (SSTR), which are becoming used as diagnostic and therapeutic targets [4]. The gold standard of care for early-stage patients is surgery [5]. However, a variable range of postoperative recurrence has been reported [6, 30]. Metastatic diseases at first diagnosis range from 20 to 70%, which hinders complete tumor debulking [31]. In the setting of advanced disease, few options are available. SSAs are commonly used in non-rapidly progressive SST-positive L-NETs, although there is not a formal approval for this indication [7, 8], octreotide and lanreotide [9, 10], chemotherapy [11], and everolimus [12, 13], with variable tumor response. In addition, a promising therapeutic option for the future is peptide receptor radionuclide therapy (PRRT) with 177Lu-DOTATATE (a somatostatin analog linked to a radioisotope that mainly targets SSTR2 and SSTR5). PRRT, indeed, has shown a certain degree of efficacy in controlling the progression of disease also in lung NET [32]. Tumor relapse in surgically treated disease and tumor progression in the advanced disease under systemic therapy are for the most unpredictable, required validation of reliable prognostic and predictive markers.

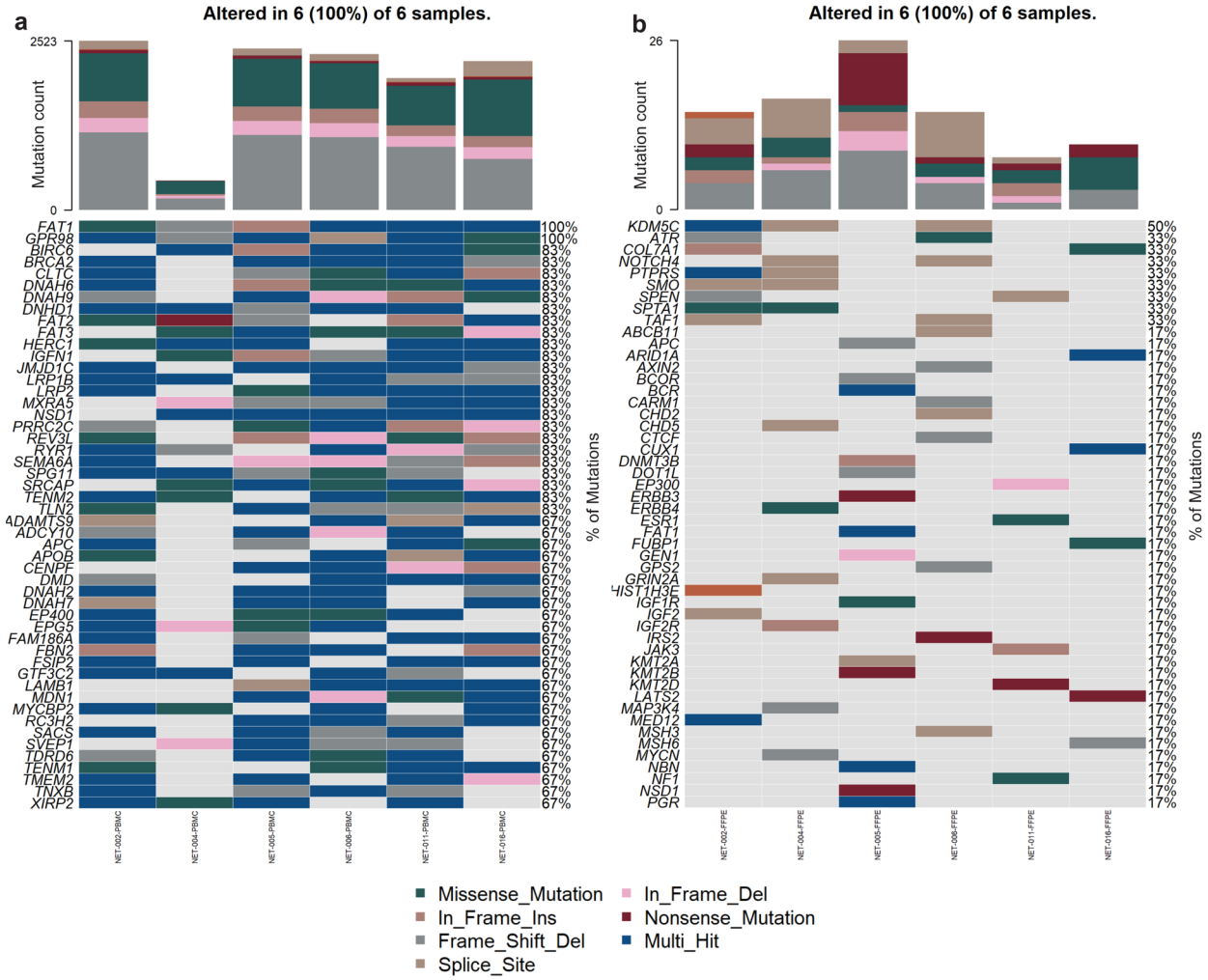

Fig. 2 Oncoprint representation of mutational frequencies in genes across PBMC and FFPE samples. **a** PBMC-derived samples. The Oncoprint shows the mutation frequency of selected genes across all samples (n = 6), with mutation counts represented in the top bar plot. The heatmap displays the percentage of mutations identified per gene for each sample, categorized by variant classification (e.g., Missense Mutation, Frame Shift Del, Nonsense Mutation, etc.). **b** FFPE-derived samples. The Oncoprint illustrates the mutation frequency of the same genes across FFPE samples (n = 6). The mutation counts are represented in the top bar plot, while the heatmap shows the percentage of mutations for each gene, categorized similarly to the PBMC samples

Nowadays, there are no validated molecular biomarkers in lung NET and no personalized strategies or clinical practice. In this context, the NETest, a NET-specific liquid biopsy, evaluates the expression of 51 NET genes by RT-qPCR. Its diagnostic utility has been widely demonstrated, while its prognostic and predictive role is still debated [33]. To date, the most comprehensive and robust genomic analysis by whole-genome sequencing was conducted in pancreatic NET (PanNET) [34].

On the other hand, lung NET's genetic profile could represent a valid tool for better characterizing tumor behavior and outcomes, and recent improvements in Next-Generation Sequencing (NGS) technologies have enhanced the exploration of lung NET's genetic background. Many efforts have been made and are ongoing to decipher the molecular landscape of lung NET, such as the lungNENomics project and the Rare Cancers Genomics initiative [35]. Lung NET rarely harbors driver mutations commonly found in non-small cell lung cancer or *TP53/RB1* mutations found universally in small cell lung cancer (SCLC) [14, 36]. A genome/exome sequencing analysis, collecting specimens from different biobanks, mostly TC, has reported that chromatin-remodeling is the most frequently altered molecular pathway in lung NET [16]. In this regard, some critical studies have highlighted some recurrent mutations that

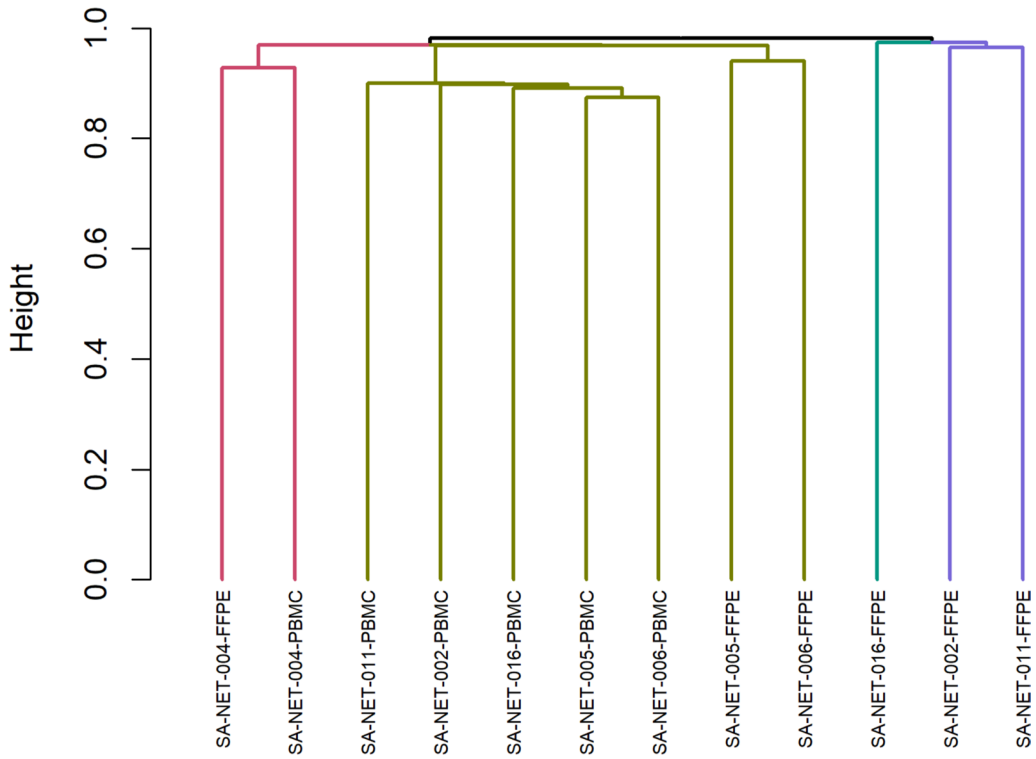

Fig. 3 Clustering of PBMC and FFPE samples based on Jaccard Index. Hierarchical clustering dendrogram showing the similarity between PBMC and FFPE samples based on their mutational profiles, as measured by the Jaccard Index. The clustering reveals distinct separation between PBMC-derived and FFPE-derived samples, suggesting systematic differences in the mutational landscape captured by the two sample types

mainly affect the genes that regulate chromatin remodeling, such as *MEN1, ARID1A, KMT2D, KTMD2C, NOTCH2, EIF1AX, TERT1*, and *PCLO* [14–16]. In addition, the dysregulation of the splicing machinery in lung NET has been demonstrated, suggesting the therapeutic druggability of *NOVA1, PRPF8*, and *SRSF10* [18]. Nevertheless, the mutations across samples were often non-overlapping, posing potential difficulty for the design of targeted therapeutic strategies. Furthermore, no recurrent genomic alterations were found in the PI3K/AKT/mTOR pathway [16], reported only in 2% of these tumors in another study [17]. A low expression of the pro-apoptotic tumor suppressor gene *CD44* and the transcription factor *OTP* expression were indicators of poor outcomes in lung NET [37]. Moreover, other studies demonstrated that the *OTP* expression was associated with the prognosis [38] and most likely due to changes in DNA methylation levels [39].

The results of another study demonstrated that high *TERT* expression defines clinically aggressive lung NET with fatal outcomes, similar to neuroblastoma [40]. Leunissen's work enabled the identification of molecular-defined lung NET subgroups (A1, A2, B), using an IHC marker panel (OTP, ASCL1, and HNF1A) [41].

Our study was performed to identify potential therapeutic targets within lung NET, similar to how everolimus

(See figure on next page.)

Fig. 4 Enrichment analysis and network visualization of mutated genes. **a** Biological process enrichment analysis of mutated genes. The bar plot displays the significantly enriched biological processes based on gene ontology (GO) terms, ranked by fold enrichment. The size of the dots represents the number of genes involved, while the color indicates statistical significance (-log10(FDR)). Notch signaling pathway appears prominently enriched. **b** Pathway enrichment analysis using the Elsevier Pathway Collection. The plot shows the significantly enriched pathways among the mutated genes, with the most relevant pathways being related to WNT signaling activation, NOTCH receptor signaling, and DNA damage checkpoint regulation. The size of the dots indicates the number of genes involved, and the color scale represents the significance level. **c** Protein–protein interaction network of mutated genes. The network highlights interactions between genes, with nodes related to Notch signaling shown in green and nodes related to WNT signaling shown in red. The network suggests potential cross-talk between these pathways, which may contribute to tumor progression and resistance to therapy

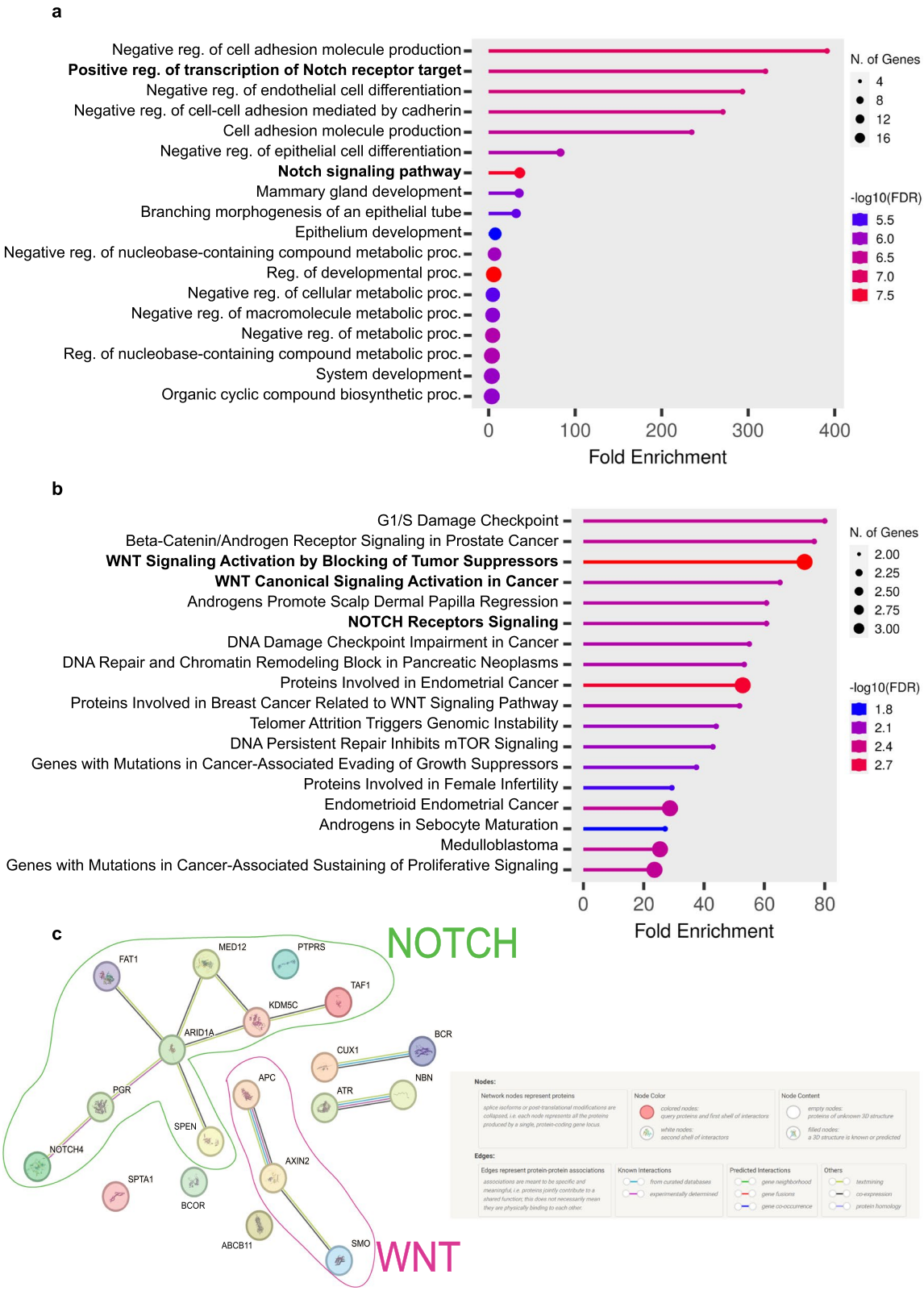

Fig. 4 (See legend on previous page.)

targets the mTOR pathway [42]. We directly compared germline and somatic genetic alterations on a cohort of 6 lung NET, which is scarcely described to date conducted by WES analysis. As for the total number of mutations in the germline it was five times more than in the somatic and C > T transitions were double in the somatic compared to the germline, in which mostly transversions were detected. As observed in our data (11.889 germline vs. 2.248 somatic mutations), this disproportion may reflect several factors, including the intrinsic biological stability of well- differentiated lung NETs, which tend to have a low somatic mutation burden. This observation is consistent with previously published studies [16, 18], reporting low tumor mutational burden and highlighting chromatin remodeling genes as major contributor in lung carcinoids. In addition, our results highlighted a correlation between disease progression and genomic instability. Gagliardi's recent study also highlighted the highest percentage of variants found consisting of a C > T transition [28].

Furthermore, the increased mutation rate in the splicing site, at somatic level, corroborates the results of the Blázquez-Encinas, which demonstrated the alteration of splicing machinery in lung carcinoids, also by in vitro functional studies [18]. However, we identified some common somatic mutations, involving *KDM5C, ATR, COL7A1, NOTCH4, PTPRS, SMO, SPEN, SPTA1, TAF1* genes, that had only been partially described in lung NET.

The comparison of germline and somatic mutations, critical to identifying putative tumor driver mutations, identified four specific recurrent mutations that were present at both germline and somatic levels which included genes as *KDM5C, NOTCH4, SMO* and *TAF1*.

Furthermore, as already argued by F. Cuesta and coworkers, our data also suggest that inactivation of chromatin-remodelling genes is sufficient to drive transformation in lung NET [16]. Infact, we detected mutations in chromatin remodeling genes in all the samples analyzed. These genes encoded covalent histone modifiers and subunits of the SWI–SNF complex such as *KDM5C, ARID1A, PTPRS, TAF1, AXIN2, SPEN, KMT2A, KMT2B* and *DNMT3B*; confirming that chromatin modifiers are fundamental players in the pathogenesis of lung NET [16].

The genetic cluster dendrogram highlighted genetic similarity between the SA-005 and SA-006 samples that were siblings. Interestingly, despite the genetic similarity, they showed different clinical pathological features (Table 1). This concept is probably related to the fact that somatic oncogenic mutations differed in the number and type of involved genes. This aspect is a valuable starting point for future investigations. Furthermore, patient 004 demonstrated a high degree of genetic overlap between germline and somatic components, raising the possibility of an underlying hereditary predisposition.

Furthermore, the pathway enrichment analysis highlighted that mostly genes are involved in Notch signaling (*KDM5C, NOTCH4, TAF1, ARID1A, SPEN, FAT1*) and in the activation of the Wnt pathway (*AXIN2, APC, SMO*).

The histone demethylase *KDM5C* alterations were common in various cancers, regulating cancer cell proliferation invasion, drug resistance [43]. Abnormality of *NOTCH4* expression affects several tumor-cell behaviors, including stemness, the epithelial–mesenchymal transition (EMT), radio/chemoresistance, and angiogenesis [44]. *TAF1* aberrant activity has been implicated in cancer progression through its involvement in chromatin remodeling, its interaction with the androgen receptor [45] and the inactivation of tumor suppressor mechanisms, such as p53 [46]. The *ARID1A* protein is known to comprise the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complexes1. Gene alterations, leading to loss of function of *ARID1A*, occur in ~ 6% of cancers [47]. *SPEN* has been suggested to play a role in gene regulation in cell signaling, including the Notch signaling pathways. It has been also identified as a major regulator of the X Chromatin inactivation (XCI) in mammals and its alterations have been identified in several cancers [48]. In numerous cancers, disruption in *FAT1* activity facilitates EMT and contributes to cancer initiation/stem-like cell development [49]. *APC* is primarily recognized for its role as a negative regulator of the Wnt/b-catenin pathway and it is frequently mutated in colorectal and other epithelial cancers especially in the early stages of cancer development, establishing *APC* as a critical gatekeeper of tumor progression and a promising therapeutic target [50]. *AXIN2* is a key regulator of the Wnt/b- catenin signaling pathway, influencing cell proliferation, migration, and apoptosis, and acting as a tumor suppressor gene and epigenetic regulator in tumorigenesis [51]. *SMO* gene, essential in the Hedgehog (Hh) signaling pathway, is mutated in basal cell carcinoma and medulloblastoma [52]. It has a role in tumor cell growth, differentiation and migration, as well as therapeutic resistance [53, 54]. These results confirm current knowledge but further emphasize a small set of cellular pathways in lung NET, defining these as the key pathways in this tumor type [41, 51, 55, 56]. The fact that most mutated genes participate in shared functions, mainly involving Notch and Wnt signaling, could be explained by their potential interaction, already known in other types of tumor, through various mechanisms such as that orchestrated by *Jagged 1* [57, 58]. So, our findings highlighted the Notch and Wnt signaling pathways as promising areas for therapeutic intervention. Notch signaling is a critical pathway involved in lung cancer progression,

dysregulation of NOTCH receptors, such as NOTCH4, affects tumor-cell behaviors, including stemness and chemoresistance [59]. Targeting Notch signaling represents a promising therapeutic strategy. Various approaches, including the use of γ-secretase inhibitors, have been explored to modulate NOTCH activity in other cancer treatments [60]. The Wnt/b-catenin signaling pathway is known to play a significant role in various cancers, including lung cancer. Inhibitors targeting this pathway have demonstrated antitumor properties. For instance, the PORCN inhibitor WNT974 has shown efficacy in NET cell lines by inhibiting Wnt signaling, leading to reduced tumor cell viability. Similarly, the b-catenin inhibitor PRI-724 has exhibited growth-inhibitory effects in NET cells [61]. Therefore, these studies should be extended to a larger population to uncover potential molecular targets that could lead to the development of targeted therapies even for lung NET.

The major limitation of our study is represented by the low sample size and by the intrinsic heterogeneity of these tumors. In fact, among patients there was a lower percentage of shared comparing to our previous work on GEP-NET [28]. On the other hand, the strengths are the in-depth genomic analysis and the correlation with the patients' clinicopathological data.

Conclusions

This research on a hand confirms previous knowledge but on the other hand to focuses attention on unknown genes mutations involved in two essential signaling pathways, such as Notch and Wnt, only partially investigated in lung NET. Nevertheless, NGS data, even if highly informative, need to be validated with transcriptomics and proteomics data and within vivo/in vitro functional studies. In prospective, if confirmed, the enrichment of a subpopulation of cancer cells in the blood, with one or more specific mutations, will be an information of enormous clinical significance because this would allow the progress of the disease to be monitored with an alternative less invasive procedure as ctDNA sequencing from liquid biopsy. The next objective will therefore be to compare the tissue mutational profile from solid biopsy with that resulting from liquid biopsy in lung NET.

Abbreviations

NET	Neuroendocrine tumors
CNVs	Copy number variations
FFPE	Formalin-fixed Paraffin-embedded
PBMC	Peripheral blood mononuclear cells
TC	Typical carcinoid
AC	Atypical carcinoid
NEC	Neuroendocrine carcinomas
SSTR	Somatostatin receptor
GEP	Gastroenteropancreatic
LCNEC	Large cell neuroendocrine carcinoma
SCLC	Small cell lung cancer
NEN	Neuroendocrine neoplasm
CK	Cytokeratin
CGA	Chromogranin A
SYN	Synaptophysin
INSM1	Insulinoma-associated protein 1
RT-qPCR	Real-time quantitative PCR
NGS	Next generation sequencing
WES	Whole exome sequencing
VEP	Variant effect predictor
PCGR	Personal cancer genome reporter
MAF	Minor allele frequency
SNV	Single nucleotide variants
Ts	Transitions
Tv	Transversion
TMB	Tumor mutational burden
MSS	Missense
NSS	Nonsense
NST	Nonstop
ESCAT	ESMO Scale for Clinical Actionability of Molecular Targets
AACR	American association for cancer research
XCI	X Chromatin inactivation
GO	Gene ontology
PanNET	Pancreatic NET
EMT	Epithelial–Mesenchymal transition
WHO	World Health Organization
SSAs	Somatostatin analogs
PRRT	Peptide receptor radionuclide therapy
ctDNA	Circulating tumoral DNA

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06442-1.

Additional file 1. Somatic mutation dataset.

Additional file 2. Germline and somatic common mutations. Mutated samples per oncogenic mutation shared between germline and somatic.

Additional file 3. Top fifty somatic mutations panel. The fifty most frequent somatic mutations.

Additional file 4. Biological process enrichment analysis data. Biological process enrichment analysis raw data.

Additional file 5. Elsevier Pathway Collection analysis data. Elsevier Pathway Collection enrichment analysis raw data.

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Author contributions

AF, AMI, CDV, MF, RM: conceptualization and design; CDV, GP, SS: methodology; AS, CM, FDN, GP, LC, MM, RM, SS, ST, VZ: investigation; ALS, CDV, RM, VZ: formal analysis; CDV, CM, GP: writing—original draft preparation; AF, ALS, CDV, DB: writing—review and editing; AF, AMI, AV, CDV, MF, MI, MMS, RM: supervision. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article will be available on the European Genome-phenome Archive (EGA), https://ega-archive.org. In addition, all data from this study can be obtained from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Written informed consent was obtained from all patients. This study was performed by the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Institutional Ethical Committe (n. 7269 protocol 0730/2023). All human samples, encompassing sequencing samples and IHC staining specimens, were performed on existing samples collected during standard diagnostic tests, posing no extra burden to patients.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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