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Genetic Alterations and Tumor Mutation Burden of Poorly Differentiated Small Cell Euro-endocrine Carcinomas are Similar in Lung Lesions and Distant Metastatic Foci

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Abstract

Objective: Studying the genetic alterations of poorly differentiated small cell neuroendocrine carcinomas to improve the understanding of the biology of these aggressive cancers.

Methods: Next generation sequencing was performed on the DNA extracted samples, using the Illumina HiSeq2000/4000 on 315 cancer related genes and tumor mutation burden was reported.

Results: In 914 small cell lung cancer (SCLC) and 115 small cell of undefined primary (SCUP), there were similar and close rates of genetic alterations in lung lesions and distant metastatic foci in SCLC and SCUP. Also, the majority of tumors, both lung lesions and distant metastatic foci, did not carry a high tumor mutation burden. Multiple potentially targetable driver genes were identified. Despite common involvement of transmembrane signaling pathways and transcription machinery, other than *TP53* and *RB1*, there was no considerable concurrent gene alteration.

Conclusion: This study showed similar genetic alteration and tumor mutation burden in the lung lesions and in distant metastatic foci. TP53 and *RB1* were the frequently altered concurrently.

Keywords: Small cell lung cancer; Oat cell; Neuroendocrine; Tumor mutation burden; Metastases; Mutation

Introduction

Neuroendocrine tumors are a wide array of different neoplasms arising from endocrine and nervous system origin. They commonly express chromogranin A, synaptophysin (p38), neural adhesion molecule (CD56), neuron-specific enolase, or neurofilament. They are generally categorized based on their degree of differentiation, grade and mitotic rate. Small cell poorly differentiated neuroendocrine carcinomas are small round blue cells with rapid proliferation rate, lack of structural formation, and poor differentiation. These cancers are aggressive, invade and metastasize early, respond to chemotherapy and radiation, and relapse almost universally. The most common type is the small cell lung cancer (SCLC), counting for 15% of lung cancers with higher incidence among older males with smoking history [1-6]. However, they can arise in many different organs and can be present in other cancers as well. Presence of a component of small cell neuroendocrine neoplasm in other tumors is associated with poor prognosis [7-9].

Multiple prior studies on small carcinoma cell lines, xenografts and primary human tumors have reported a variety of mutations, most commonly *TP53* and *RB1* [10-12]. These studies have shown variable genetic mutations in tumors arising from different organs. As an example, some have showed similar genetic alterations in small cell cancers arising in the pancreas and esophagus but showed different findings in those rare cancers originating in the bladder [13-15]. Similar mutational analysis leads to re-classification of small cell carcinoma of the ovaries as ovarian rhabdoid tumor [16]. Similarly, other studies have helped identify Merkel cell associate papilloma virus as a cause of this small cell poorly differentiated cancer of skin [17,18]. Understanding the altered genetic pathways in the tumors may help understand their biology, mechanisms involved in their development, their proper classification and potentially help with identifying therapeutic strategies. Previous studies with smaller sample size had shown limited co-occurrence of the genetic alterations and comparing genetic alterations in metastasis lesions in different organs [10,19-21]. In order to improve our understanding of the genes and pathways involved in these tumors, the current study compares mutations in a larger set of lung lesions, distant metastatic foci and small cell carcinoma of undefined primary (SCUP), and also evaluates co-occurrence of those mutations.

Methods

Genetic sequencing methods are fully explained in a previous publication [22]. Briefly, samples were diagnosed by local pathologist and then slides were submitted for hybrid capture based comprehensive genomic profiling (CGP) to Foundation Medicine (Cambridge, MA). Tumor samples analyzed between January 2010 to

December 2016 was included. Origins of the primary tumor were based on the documented report by the requesting local institutions. Tumors without documented origin were classified as small cell carcinoma of undefined primary (SCUP). DNA was extracted from formaldehyde fixed paraffin embedded biopsy or surgical specimens. CGP was performed using the Illumina HiSeq2000/4000 on indexed, adaptor ligated, hybridization-captures libraries for exons of 315 cancer related genes and 47 introns of 19 genes frequently involved in rearrangements.

Genetic alterations included base substitutions, short insertions and deletions, amplifications, homologous deletions and chromosomal rearrangements. Alterations likely or known to be bona-fide oncogenic drivers and germ-line polymorphisms were included. Alterations were reported as short variants, copy number for genes (amplifications and losses), or rearrangements. Short variants include single-base nucleotide substitutions, small-scale multi-base deletions or insertions, and microsatellite repeats. Publicly available and validated analysis tools were used to analyze the data. Median exon unique coverage was 647X. For tumor mutation burden (TMB), the number of somatic mutations detected on NGS (interrogating 1.2 Mb of the genome) were quantified and that value extrapolated to the whole exome using a validated algorithm [23,24]. TMB was measured in mutations per mega base (Mb) and was divided into three groups: low (1-5 mutations/Mb), intermediate (6-19 mutations/Mb), and high (≥ 20 mutations/Mb). One hundred non-synonymous mutations per exome were used as a threshold. The threshold of 20 coding mutations per Mb was used as equivalent to 400 non-synonymous mutations per exome. In a large cohort of patients this approximately divided patients to 50% as low TMB, about 40% as intermediate TMB, and about 10% as high TMB [25]. Gene rearrangements were detected by identifying clusters of chimeric read pairs from both DNA (pairs mapping 0.10 kilo bases (kb) apart or on different chromosomes) and RNA (pairs mapping to RefSeq sequences corresponding to different genes or to genomic loci 0.10 kb apart). Chimera clusters were filtered for repetitive sequence and by distribution of mapped positions. Identified rearrangements were then annotated according to the genomic loci of both clusters and categorized as gene fusions, gene rearrangements, or truncating events. Throughout the study, cumulative de-identified data were extracted from data-bank, without access to the patients' data, was used. In agreement with Declaration of Helsinki, In addition to Independent Review Board (IRB) of the institutions sending the samples, Western IRB was used to obtain approval for our publication, including a waiver of informed consent and HIPAA waiver of authorization.

Statistical methods

Simple statistical methods were used to compare the frequency of mutations in different groups, to calculate odds ratios and p-values by publicly available online calculator (https://www.medcalc.org/calc/odds_ratio.php). The difference of TMB between the lung lesions and distant metastatic foci was calculated with 2 × 3 chi-square contingency test. Gene enrichment scores (ES) were calculated by a one tailed hyper-geometric test using publicly available online programs (http://systems.crump.ucla.edu/hypergeometric/index.php).

Results

Among all reported gene alterations, short variants were the most common alterations and rearrangements were the least common ones (Tables 1 and 2).

Tissue of origin

In total, 914 SCLC were identified, 406 as lung lesions, and 310 as SCLC distant metastatic foci, with 198 SCLC as ambiguous state (unknown site of biopsy). The most common site of biopsy of metastatic SCLC was the liver (205/310; 58.9%), followed by the brain (33/310; 9.5%), bone (26/310; 7.5%), soft tissue (24/310; 6.9%), adrenal glands (21/310; 6%) and other organs (40/310; 11%). One hundred fifteen (115) samples were categorized as SCUP.

Gene alterations in SCLC

The most common alterations (>4.5%) included: *TP53* (90.4%) followed by *RB1* (69.1%), *KMT2D* (11.9%), *LRP1B* (11.4%), *PTEN* (8.3%), *MYCL* (7.7%), *RICTOR* (6.2%), *MYC* (6%), *CREBBP* (5.7%), *SPTA1* (5.5%), *FAT1* (5.3%), *PIK3CA* (4.9%). Findings are summarized in Table 1. Among the targetable genes in *SCLC*, *PTEN* alteration was seen in 8.3% and *RICTOR* amplification was observed in 6.1% of cases. *EGFR* alterations was seen in 3.5%, *KIT* 3%, *BRCA2* 1.3%, *JAK2* 1.2%, and *BRAF* in 1% of cases. *ERBB2* alterations were reported in 4.3% (2.6% short variant and 1.7% amplifications) were reported in *SCLC*. *TERC* mutation was reported in 1% of cases. There was also alteration detected in *NOTCH*1 4.7%, *NOTCH2* 2%, *NOTCH3* 2%, *NOTCH4* 1.6%.

Gene	All alterations	Short variant	Copy number	Rearrangement
TP53	90.40%	89.20%	1.10%	0.00%
RB1	69.10%	59.40%	8.60%	0.70%
MLL2	11.90%	11.40%	0.00%	0.40%
LRP1B	11.40%	10.50%	0.50%	0.30%
PTEN	8.30%	4.00%	4.00%	0.10%
MYCL1	7.70%	0.00%	7.50%	0.10%
RICTOR	6.20%	0.10%	6.10%	0.00%
MYC	6.00%	0.00%	6.00%	0.00%
CREBBP	5.70%	4.90%	0.60%	0.20%
SPTA1	5.50%	5.30%	0.00%	0.10%
FAT1	5.30%	5.10%	0.30%	0.00%
PIK3CA	4.90%	3.40%	1.30%	0.00%

Table 1: Common gene alterations in Small Cell Lung Cancer (SCLC).

Gene alterations in SCUP

Most common *SCUP* alterations (>4.5 %) included: *TP53* (78.3%) followed by *RB1* (62.6%), *PTEN* (13.9%), *KRAS* (9.6%), *CREBBP* (7.8%), *APC* (7.8%), *KMT2D* (7%), *MYC* (7%), *PIK3CA* (6.1%), *MUTYH* (5.3%), *LRP1B* (4.3%). Findings are summarized in Table 2. Among the targetable genes in *SCUP*, *PTEN* alteration was seen in 13.9%, *BRCA2* 4.3%, *ATM* 2.6%, *FLT3* 1.7%, *MET* 1.7%, and *VEGFA* in 1% of cases. Among DNA repair genes, there was 5.2% mutation reported in *MUTYH*, and 1.7% in *MLH1* and *MSH6*. Also, *NOTCH1* alteration was found in 2.6% of cases. The alteration in *hTERT* promoter was seen in 1% and *TERC* alteration occurred in 2% of cases.

Gene	All alterations	Short variant	Copy number	Rearrangement
TP53	78.30%	75.70%	1.70%	0.00%
RB1	62.60%	51.30%	9.60%	1.70%
PTEN	13.90%	7.80%	6.10%	0.00%
KRAS	9.60%	8.70%	0.00%	0.00%
CREBBP	7.80%	7.00%	0.00%	0.90%
APC	7.80%	6.10%	1.70%	0.00%
MLL2	7.00%	7.00%	0.00%	0.00%
MYC	7.00%	0.00%	7.00%	0.00%
PIK3CA	6.10%	5.20%	0.90%	0.00%
MUTYH	5.20%	5.20%	0.00%	0.00%
LRP1B	4.30%	3.50%	0.90%	0.00%

Table 2: Common gene alterations in Small Cell Cancer of Unknown Primary (SCUP).

Comparing gene alterations in SCLC and SCUP

Difference between the frequency of more common mutations (those with frequency >5%) were compared between *SCLC* with *SCUP*. Significant differences were found in *TP53*, (12.1% difference; p<0.001), *RB1* (6.5% difference; p=0.03), *LRP1B* (7.1% difference; p=0.02), *PTEN* (5.6% difference; p=0.03) and *SPTA1* (3.5% difference; p=0.03). Findings are summarized in Table 3.

Gene	SCLC	SCUP	p value	Odds ratio	CI 95%	Z
TP53	90.40%	78.30%	0.01%	38.00%	0.23-0.63	3.8
RB1	69.10%	62.60%	3.00%	64.00%	0.42-0.95	2.2
MLL2	11.90%	7.00%	12.00%	55.00%	0.26-1.2	1.5
LRP1B	11.40%	4.30%	2.00%	33.00%	0.13-0.84	2.3
PTEN	8.30%	13.90%	3.00%	186.00%	1.04-3.32	2.1
MYCL1	7.70%	4.30%	20.00%	54.00%	0.21-1.38	1.3
RICTOR	6.20%	2.60%	13.00%	40.00%	0.12-1.3	1.5
MYC	6.00%	7.00%	65.00%	119.00%	0.55-2.5	0.4
CREBBP	5.70%	7.80%	36.00%	140.00%	0.67-2.93	1.4
SPTA1	5.50%	2.00%	3.00%	0.29	0.07-1.22	1.68
FAT1	5.30%	1.00%	6.00%	0.14	0.02-1.06	1.9

Table 3: Common Mutations (Incidence >5%) Small Cell Lung Cancer (SCLC) *vs.* Small Cell Cancer of Undetermined Primary (SCUP).

Comparing gene alterations in SCLC lung lesion and distant metastatic foci

Difference between frequency of the more common mutations (those with frequency >5%) were compared between most common mutations of the lung tumor *vs.* distant metastatic foci in *SCLC*. Significant differences were found only in *TP53* alterations (4.2% difference; p<0.04). Findings are summarized in Table 4.

Gene	Primary	Metastatic	p-value	Odds ratio
TP53	93.3%	89.1%	0.04	0.58
RB1	66.0%	70.4%	0.19	1.23
KMT2D	12.4%	12.1%	0.91	0.97
LRP1B	9.2%	10.1%	0.71	1.10
PTEN	9.0%	6.9%	0.35	0.75
MYCL	7.1%	8.3%	0.59	1.18

CREBBP	6.9%	5.2%	0.37	0.74
MYC	6.4%	6.0%	0.88	0.93
PIK3CA	5.7%	5.2%	0.75	0.89
FGFR1	5.5%	5.7%	1.00	1.04
RICTOR	5.1%	6.6%	0.36	1.33
SPTA1	5.1%	4.3%	0.74	0.85

Table 4: Primary *vs.* Metastatic Small Cell Lung Cancer (SCLC): comparing frequency of common mutations (frequency >5%).

Tumor mutation burden (TMB) was also compared between SCLC lung lesion and distant metastatic foci. Most common group was TMB-intermediate (64.8% in lung lesions *vs.* 60.6% in distant metastatic foci), followed by TMB-low (26.9% in lung lesions *vs.* 31.3% in distant metastatic foci). The least common group was TMB-high (8.3% in lung lesions *vs.* 8.0% in distant metastatic foci); There was no significant difference between TMB groups in SCLC lung lesions with distant metastatic foci (p=1).

Concurrent mutations in SCLC

Notably, there was significant enrichment score (ES) between *TP53* and *RB1* (ES=1.06; p<0.0001), *TP53* and *MYC* (ES=1.09; p=0.02) and *MYCL* and *RICTOR* (ES=3.33; p=0.05). Other events had lower incidence and were not statistically significant. Findings are summarized in Table 5.

Gene 1	Gene 2	gene1	gene2	Concurrence	Expected	RR	95% CI	p value
TP53	*RB1*	794	613	594	560	1.06	1.03-1.09	<0.0001
TP53	*PTEN*	794	77	74	70	1.05	0.97-1.14	0.19
TP53	*MYCL1*	794	66	55	60	0.91	0.80-1.04	0.19
TP53	*RICTOR*	794	50	49	45	1.08	0.98-1.2	0.09
TP53	*MYC*	794	57	57	52	1.09	1.01-1.18	0.02
TP53	*CREBBP*	794	49	48	44	1.09	0.98-1.2	0.09
TP53	*PIK3CA*	794	44	41	40	1.02	0.90-1.15	0.69
RB1	*PTEN*	613	77	56	54	1.03	0.84-1.26	0.72
RB1	*MYCL1*	613	66	51	46	1.11	0.9-1.36	0.32
RB1	*RICTOR*	613	50	36	35	0.98	0.77-1.26	0.91
RB1	*MYC*	613	57	38	40	0.95	0.74-1.21	0.68
RB1	*CREBBP*	613	49	39	34	1.14	0.90-1.44	0.25
RB1	*PIK3CA*	613	44	27	31	0.87	0.64-1.79	0.37
PTEN	*MYCL1*	77	66	7	5	1.4	0.46-4.19	0.54
PTEN	*RICTOR*	77	50	8	4	2	0.64-6.21	0.23
PTEN	*MYC*	77	57	10	5	2	0.73-5.4	0.18
PTEN	*CREBBP*	77	49	4	4	1	0.27-3.77	1
PTEN	*PIK3CA*	77	44	8	3	2.67	0.74-9.5	0.13
MYCL1	*RICTOR*	66	50	10	3	3.33	0.97-11.4	0.05
MYCL1	*MYC*	66	57	3	4	0.75	1.18-3.2	0.69
MYCL1	*CREBBP*	66	49	1	3	0.33	0.04-3.09	0.33
MYCL1	*PIK3CA*	66	44	2	3	0.67	0.12-3.78	0.64
RICTOR	*MYC*	50	57	8	3	2.67	0.74-9.5	0.13
RICTOR	*CREBBP*	50	49	0	2	0.2	0.01-4	0.29
RICTOR	*PIK3CA*	50	44	3	2	1.27	0.22-7.26	0.79
MYC	*CREBBP*	57	49	2	3	0.67	0.12-3.82	0.65
MYC	*PIK3CA*	57	44	6	2	3	0.64-14.06	0.16
CREBBP	*PIK3CA*	49	44	1	2	0.5	0.05-5.32	0.56

Table 5: Incidence of Concurrent Common Mutations in Small Cell Lung Cancer (SCLC).

Discussion

Poorly differentiated small cell neuroendocrine cancers are a major problem in oncology. The pathways and mechanisms involved in the development of these cancers have remained unclear. Multiple studies have reported various mutations in cell lines, xeno-grafts and primary human tumors with limited or whole genome sequencing [10,11]. Besides the high prevalence of concurrent loss-of-function of *TP53* and *RB1*, there were frequent alterations of genes involved in trans-membrane signaling and transcription machinery, including chromosome remodeling, histone modifications, and transcription factors. ARID1A is involved in SWItch/Sucrose Non-Fermentable (SWI/SNF) chromosome remodeling complex [26]. *TP53*, *KMT2D* (coding for *MLL2*) and *CREBBP* code proteins that are involved in histone acetylation, Rb1 protein represses transcription through interaction with histone deacetylase [27-29]. In addition, there are structural homology between the SWI/SNF complex B/MDM2 domain and MDM2 protein [30], possibly interacting with p53 protein. Inactivation of Rb1 leads to activation of transcription factor E2F [31].

MYC and MYCL are other transcription factors frequently altered in this study. NOTCH protein alterations lead to changes in chromosome remodeling [32]. In addition, altered receptor tyrosine kinases (FGFR and EGFR) and *PIK3CA*, *PTEN*, *AKT*, *RICTOR* and *KRAS* gene show altered *PIK3CA*/*PTEN*/*AKT* and *RAS*/*MEK* signaling pathways in these tumors (Supplemental figure).

Multiple potentially targetable genetic alterations were identified. It should be noted that an alteration may not be readily targetable or applicable in the clinic. Potential as therapeutic alteration also depends on what domain of the protein is altered, whether there is enough gene expression, role of the altered gene as a driver mutation, availability of effective medication for specific mutations, accessibility of the altered protein domains, among many important factors. Genetic alterations with available targeted therapies were frequently found. The most common alteration was PIK3CA/PTEN/AKT pathway, confirming prior reports [33,34]. NOTCH alterations were frequently seen in both SCLC and SCUP, suggesting their potential role in the future targeted therapies. NOTCH family alterations *CREBBP*, *EP300*, *TP73*, *RBL1*, and *RBL2* have been reported to be mutually exclusive in SCLC [12].

Despite prior reports of high *hTERT* expression in small cell neuroendocrine cancers [35-37], current study did not find a high rate of alteration in *hTERT* promoter. Prior studies have also reported high rate of *hTERT* promoter mutation in Merkel cell tumors, which is an aggressive small cell neuroendocrine cancer of skin [37]. Similarly, another study reported low rate of *hTERT* promoter mutation in small cell neuroendocrine cancer, except for those in the bladder [15]. Cell culture studies have shown that *hTERT* expression is needed for continued cell growth and overcoming crisis in cells with disrupted *p53* and *Rb1* pathways [38]. Additionally, SWI/SNF complex has been shown to be involved in expression of *hTERT* [39]. These findings suggest that different mechanisms can be involved in *hTERT* expression in tumors of different origins.

Current study also reported frequent alteration of *KMT2D* (12.4%). The prior studies reported that inactivation of this gene is associated with decreased proliferation of malignant cells and improved patient survival [27,40-43]. If similar correlation exists in SCLC, then there is a potential to target this gene as a therapeutic target.

High tumor mutation burden (TMB-high) was not a common finding and there was a low incidence of the genetic alteration in the genes associated with microsatellite instability (*MSH2, MSH6, MLH1, PMS2*), resembling other solid tumors [25]. Most of the patients with significant microsatellite instability (MSI-high) have high tumor mutation burden (TMB-high) and is associated with response to immunotherapy [25,44,45]. Low incidence of high TMB has been reported in previous SCLC studies [46]. Low incidence of high TMB in these cancers is happening despite high association with smoking and *TP53*, which is known as guardian of DNA. Interestingly, it seems that survival (progression free survival and overall survival) and not response rate, correlate with TMB in SCLC patients treated with nivolumab alone or combined with ipilimumab, does not correlate with TMB [47].

Notably, there was significantly enriched co-occurrence of *TP53* and *RB1* alterations, suggesting that their concurrent alteration is crucial in this type of cancer. Despite frequent alteration of the genes involved in membrane signaling and transcription machinery, these alterations did not show enriched co-occurrence. The independent alterations of the genes involved in parallel pathways and common mechanisms provide variety of mechanisms for aggressive, resistant and relapsing nature of SCLC and SCUP. It also identifies multiple new potential therapeutic targets and pathways, which necessitate further evaluation and clinical trials in these cancers. In addition, similar gene alterations and tumor mutation burden were found in the SCLC lung lesions and distant metastatic foci [48-50]. It seems that the presence of *TP53* and *RB1* alteration is preserved in combined small cell lung cancer and non-small cell lung cancer [51].

Conclusion

This study showed similar genetic alteration and tumor mutation burden in the lung lesions and in distant metastatic foci. *TP53* and *RB1* were the frequently altered concurrently.

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