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ORIGINAL ARTICLE

Mutational Diversity of Lung Cancer and Associated Lymph Nodes. An Exploratory Prospective Study of 4 Resected cIIIA-N2

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Abstract Mutational heterogeneity could explain different metastatic patterns among IIIA-N2 lung cancer and influence prognosis. The identification of subclonal mutations using deep sequencing to evaluate the degree of molecular heterogeneity may improve IIIA-N2 classification. The aim of this prospective study was to assess mutational and immunohistochemical characteristics in primary tumours and involved lymph nodes (LN) in operated patients. Four patients operated for primary lung carcinoma and unisite N2 mediastinal involvement were consecutively selected. Samples (tumour and paired LN) were analysed for PD1, PD-L1 and CD8 immunostaining. Somatic mutation testing was performed by deep targeted next generation sequencing (NGS), with the AmpliSeq™ Colon and Lung Cancer Panel (LifeTechnology). A total of 9 primary lung cancer samples and 10 LN stations were analysed. For each cancer, we found 2 mutations, with allelic ratios from 3% to 72%. Mutational patterns were heterogeneous for 2 primary tumours. In 3 cases, mutations observed in the primary tumour were not found in LN metastases (*ALK*, *FGFR3*, *MET*). Inversely, in 1 case, a *KRAS* mutation was found in LN but not in the primary tumour. All primary tumours were found PD-L1 positive while CD8+ T cells infiltrate varied. In the different examined LN samples, PD-L1 expression, CD8+ and PD1+ T cells infiltrate were not similar to the primary tumour. This preliminary prospective study shows the diversity of intra-tumour and LN mutations using routinely-used targeted NGS, concerning both mutated gene and allelic ratio. Further studies are needed to evaluate its prognostic impact.

Keywords Lung neoplasms · Lymph nodes · Genetics · Biomarkers, tumour · High-throughput nucleotide sequencing

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Introduction

Mediastinal lymph node involvement (N2) in non-small cell lung cancer (NSCLC) concerns 15% of patients and is associated with a poor prognosis and an overall 5-year survival ranging from 9 to 34% [1, 2]. Literature fails to provide any definitive consensus regarding the management of these patients, except for the platinum-based doublet chemotherapy. Options for locoregional treatment are surgery and radiation therapy. Adjuvant checkpoint inhibitors are also currently evaluated. Optimal management of cIIIA-N2 NSCLC remains controversial but complete surgical resection is related to long-term survival, 10 years or more, in some patients [3–5]. Thus, we need to enhance our ability to select cIIIA-N2 patients who will benefit from surgery. Multisite molecular

testing has been investigated in a few studies and showed diverse patterns of evolution [6–8]. Most of these studies investigated exome sequencing to characterize molecular heterogeneity, which is not compatible with large-scale routine care screenings. Moreover, in most cases a small set of genes was retained to classify tumours. It is clear that tumour heterogeneity influences outcome and treatment responses therefore addressing this question using deep, targeted NGS sequencing seems important as it could easily be implemented to the clinics. We performed this exploratory study on 4 patients with surgically resected cN2-NSCLC to assess differences between the primary site and the metastatic LN, from an anatomical, molecular and immunophenotypic point of view.

Patients and Methods

Patients

In July 2015, we included 4 consecutive patients after surgery for NSCLC with uni-site cN2 disease. Mediastinal staging included positron emission tomography and trans-bronchial needle aspiration. This study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee (CPP Ile De France 2 n°2012–08-09 A1). Informed consent was obtained from all individual participants included in the study. Exclusion criteria were consent withdrawal, induction chemotherapy and incomplete resection. Lobectomy or pneumonectomy was performed, with systematic radical hilar and mediastinal lymphadenectomy, including en-bloc resection of the stations 9, 7, 8, 4R and 2R in case of right resection or 9, 7, 8, 5 and 6 in case of left resection. The stations 10 (hilar) and 11 (interlobar) were also systematically removed. Patients were pathologically classified according to the latest 2015 IASLC and 2017 UICC TNM classification, after full systematic slicing.

Tumour Samples

For each patient, 2–3 samples of the primary tumour and 2–4 samples of metastatic lymph nodes were addressed, after formalin fixation and paraffin embedding, for sequencing and immunohistochemistry (IHC). All analysed samples contained at least 50% of tumour cells. All samples were surgically resected, except for patient #D who had had a preoperative biopsy, included in the analysis (Ta).

Sequencing

DNA was extracted using the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega Corporation, USA). Paraffin embedded samples were characterized using Next-Generation Sequencing (NGS) (AmpliSeq™ Ion Torrent, Life Technologies) designed to amplify small DNA fragments, after multiplex PCR library preparation of the regions of interest (AmpliSeq™ Colon Lung Cancer Panel, Life Technologies) covering more than 500 COSMIC hotspot mutations within the following 22 genes (*KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBXW7, NOTCH1, ERBB4, FGFR1, FGFR2, FGFR3*) by AmpliSeq™ technology (Ion AmpliSeq™ library kit V2, Ion library equalizer kit, Life Technologies) [9] . Librairies were equalized using the Ion Library Equalizer™ Kit. Clonal amplification was done using Ion Chef™ System (Ion PI™ Hi-Q™ Chef, Ion PI™ Chip Kit v3, Life Technologies). Sequencing was performed on Ion Proton™ system, data analysis by Torrent Suite 5.0.2 and variants annotation with SAFIR 2 report tool on a galaxy plateform. Variant detection threshold was 2%, minimal deepness for negative results was 300X. Called variants underwent subsequent quality control taking into account the coverage of each amplicon (≥300X) and the allele frequency (≥2%). The amplicon list is available on www.thermofisher.com. The identification of gene amplification was performed using coverage depth data and a locally developed algorithm using R. For MET the algorithm was validated by FISH testing, for ERBB2, EGFR, KRAS and MET it was validated by qPCR. Suspected mutations were controlled on Alamut® Visual (Interactive Biosoftware, Rouen, France).

Immunohistochemistry

Four-micron sections of paraffin-embedded samples of the primary lung tumour were used for IHC. Immunostainings were performed on the Benchmark ULTRA™ Ventana autostainer with the primary rabbit monoclonal PD-L1 antibody (CD274, clone 28.8, ab205921 Abcam, Cambridge, UK) using OptiView| Universal DAB detection kit (Ventana Medical Systems, Roche), the mouse monoclonal PD-1 antibody (clone NAT105, ab59587 Abcam, Cambridge, UK) and the mouse monoclonal CD8 antibody (clone C8/144B, M7103, Dako Cytomation; Dako Carpinteria, CA, USA) using UltraView Universal DAB detection kit (Ventana Medical Systems, Roche). Human tonsil was used as an external positive control. Isotype-matched antibodies were used as negative controls. Two well-experienced pathologists (HR, LG) examined the immunohistochemical slides. Percentage of PD-L1 positive tumour cells was evaluated for each sample. Only membrane staining was considered. In the absence of any standardized system, we evaluated the positivity according to Garon et al.'s system [10]: membrane staining in more than 1% of tumours cells were considered as weak positive staining, and membrane staining in more than 50% of tumours cells were considered as strongly positive. The

intra-tumour density of CD8 + T cells and PD-1+ cells was quantified with a qualitative approach (+ = minor infiltrate, ++ = moderate infiltrate, +++ = significant infiltrate).

Results

Patients

Clinical and preoperative characteristics are presented in Table 1. Mean age was 69. All patients were PS 0–1. Pathological characteristics are presented in Table 2. Cases #A,B were adenocarcinomas, case #C a squamous cell carcinoma and case #D a sarcomatoid carcinoma. Surgical resections were complete in all cases (R0). Mean follow-up was 11 months [3–18]; 2 patients relapsed and died of their tumour, both had a rather clonal tumour evolution with strong oncogenic drivers present in all samples at relapse, *MET* p.D1028H that is associated with a *MET* exon14 skipping for patient #D and *KRAS* p.G13C for patient #B.

Sequencing

Nine samples of primary tumour and 10 metastatic LN stations were assessed (Table 3). Mutations observed were *KRAS* p.G12A and p.G13C, *ALK* p.C1182*, *STK11* p.G196 V, *TP53* (a splice of exon 6 and a deletion: p.D281-E285del), *FGFR3* p.S249C, *MET* p.D128H. For each patient, we found 2 mutations, with allelic ratios from 7% to 60%. Mutational patterns were heterogeneous (considering gene and allelic ratios) within primary tumour for patients #A,D. Heterogeneity was also observed comparing each pair "tumour-LN" for a single patient (Table 3). Uninvolved LN did not harbor any mutation (minimal coverage 2378 reads). Furthermore, a *MET* amplification was found in the primary tumour of case #D but it was lost in LN.

Immune Cells Infiltration and PD-L1 Expression

One sample of each primary tumour and 2–3 metastatic LN were assessed by IHC. All primary tumours were found PD-L1 positive, 3 patients (#A,C,D) with a strong expression of PD-L1 (>50%),and one (#B) with a weak and heterogeneous expression of PD-L1 (20%) (Fig. 1). In primary tumours the CD8+ T cells infiltrate varied from high (#A,B), moderate (#C) and weak (#D). In the 4 patients, the PD1 staining was equivalent to CD8 staining (data not shown).

In the different examined LN, for patients #A,B, expression of PD-L1 was weak in intrapulmonary LN (11 and 11i areas) and absent in mediastinal LN (4R and 5 areas). For patients #C,D, the expression of PD-L1 was strong in LN, as in primary tumour (Table 4).

In the same way, if CD8+ and PD1+ T cells infiltrate was conserved low in LN metastasis as in primary tumour for the patient #D, a discordance was observed between LN metastasis and primary tumour for the patient #A. However, patients #B and #C presented a high or moderate CD8+ and PD1 T cells infiltrate in both LN metastasis and primary tumour (Table 4).

Discussion

Main Results

At least 2 mutations were found in all tumours, with allelic ratios from 3% to 72%. Mutational pattern was heterogeneous for 1 primary tumour (#A), and for patient #D the amplification of MET was also restricted to a specific tumour area. Comparing each pair "tumour-LN" for a single patient, in 3 cases, alterations observed in the primary tumour were not found in LN (*ALK*, *FGFR3*, *METamp*). Inversely, in 1 case, a *KRAS* mutation was found in LN but absent in primary tumour. All primary tumours were found PD-L1 positive, and associated LN in only 2 cases (#C,D).

Table 1 Clinical, radiological and surgical characteristics

Case	Age	Tobacco (pack-year)	T	T (PET, SuVmax)	N	N (PET, SuVmax)	Surgery
#A	71	25	3.5 cm RIL	7.9	< 1 cm	4R area: 6 7 area: 3	Lobectomy
#B	61	15	4 cm LIL + culmen	4.1	< 1 cm	10 L area: 6 4 L area: 4.7 5 area: 4.5	Pneumonectomy
#C	58	75	2.5 cm hilar mass	13.6	within the mass		Pneumonectomy
#D	87	15	3.7 cm RUL	15.3	< 1 cm	7 area: 3.6 10R area: 5.3	Lobectomy + Chest wall resection

LIL, left inferior lobe; *RUL*, right upper lobe; *PET*, positron emission tomography

Table 2 Pathological characteristics

Case	Pathology	pTNM	Invloved lymph nodes
#A	Adenocarcinoma	pT2aN2b	11i area: 2+/4
	acinous well-differentiated predominant minor less-differentiated solid component		4R area: 3+/7
#B	Adenocarcinoma	pT3N2c	12 area: 1+/1
	acinous-predominant		11 area: 5+/9
	solid and micropapillary components		4 L area: 3+/3
	intra-vascular tumour emboli		5 area: 9+/9
			pre-carinal area: 2 N+/2
#C	squamous cell carcinoma	pT2aN1a	10 L area
#D	sarcomatoid carcinoma	pT3N2b	4R area: 3+/5
			pre-carinal area: 2+/2
			10R area: 1+/1

cIIIA-N2

The N2 involvement remains a matter of debate because of its not yet well-classified heterogeneity. Regarding anatomy, the Mountain and Dresler's regional LN classification for lung cancer staging was widely the reference [11] . Different studies classified IIIA-N2 disease into 4 groups, in addition to the skip-N2 phenomenon: minimal-N2, N2 single station, N2 multiple stations, and bulky-N2 [2, 5, 12] . Other subgroups were proposed for the 8th edition of the TNM: N2a1 – single station skip, N2a2 – single station non skip, N2b – multiple stations [1].

Table 3 Molecular characteristics

Case	Tumour site	Histological subtype	% of tumour cells	Mutation 1		Mutation 2	
				Allelic ratio	Coverage (reads)	Allelic ratio	Coverage (reads)
				ALK p.Cys1182*		*KRAS* p.G12A	
#A	Ta	acinous and solid	>50	–	3595	–	2808
	Tb	acinous	>50	13%	6050	–	9644
	Tc	solid	>50	–	6427	–	4285
	11i area	solid	>50	–	16,285	7%	9644
	4R area	solid	>50	–	16,806	–	7250
				STK11 p.G196 V		*KRAS* p.G13C	
#B	Ta	micropapillary	>50	11%	10,216	11%	18,382
	Tb	solid	>50	28%	8637	18%	12,894
	11 area	solid	>50	35%	1753	21%	3105
	11 area	solid	>50	21%	2731	14%	5734
	pre-carinal area	solid	50	13%	4636	9%	6892
	5 area	solid	>50	57%	7926	31%	16,332
				TP53 splice ex6		*FGFR3* p.S249C	
#C	Ta	squamous	>50	59%	3637	26%	3489
	Tb	squamous	>50	60%	3565	26%	3307
	10 L area (contiguous LN)	squamous	>50	28%	4876	13%	4528
	10 L area	squamous	50	6%	7164	–	6571
				MET p.D1028H		*TP53* p.D281-E285del	
#D	Ta (preoperative biopsy)	sarcomatoid	>50	68%	95743*	34%	3914
	Tb	sarcomatoid	80	72%	9668	46%	4006
	4R area	sarcomatoid	>50	7%	7946	3%	7788
	pre-carinal area	sarcomatoid	>50	29%	9163	15%	3950

Ta, Tb and Tc are samples of the primary tumour. *: *MET* amplification

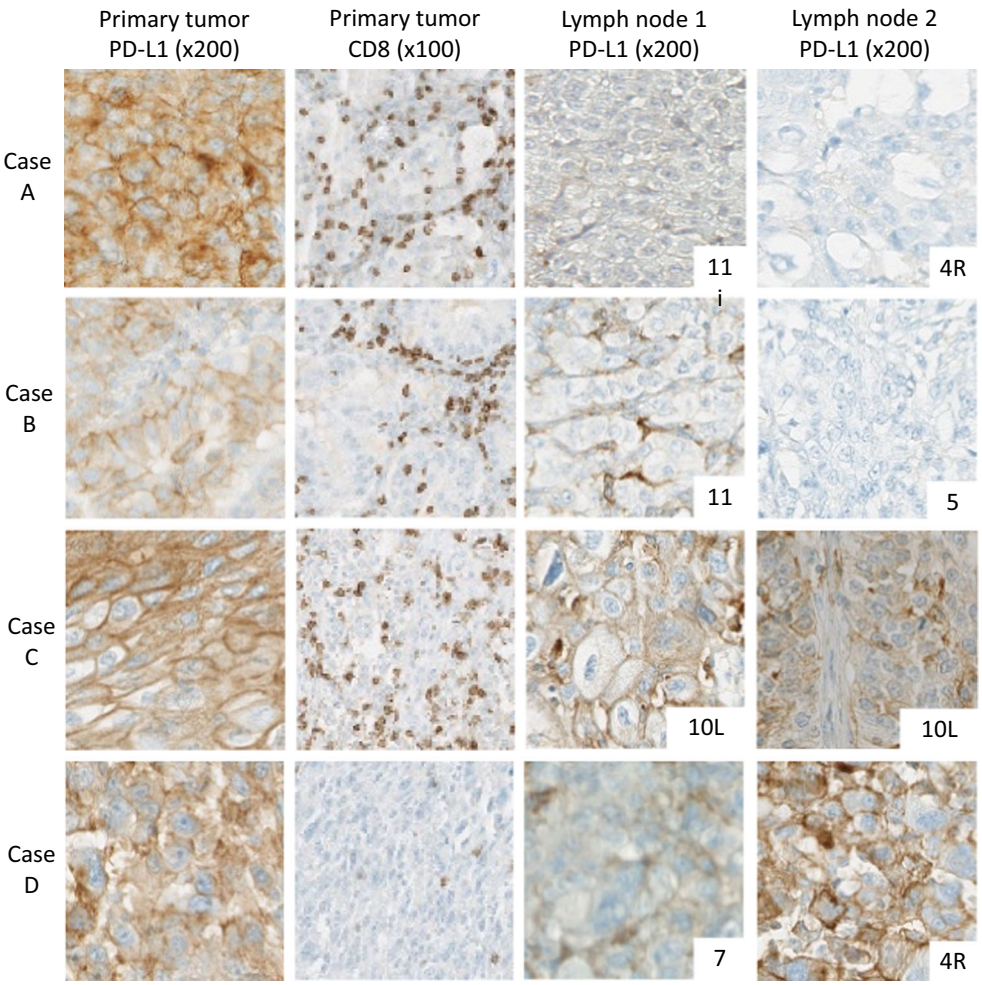

Fig. 1 Immunostaining for PD-L1 and CD8+ tumour infiltrating T cells in tumours and lymph nodes

The French National Cancer Institute proposed guidelines based on the American College of Chest Physicians expertise [13, 14], but in case of cN2 preoperatively diagnosed but not infiltrating, guidelines remained imprecise ("resectability should be discussed for each case") and suggested surgery first, or induction chemotherapy, or concomitant chemoradiation [15] .

Thus, optimal management of cIIIA-N2 remains controversial but complete tumour resection can be related to long-term

Table 4 Immunostaining of PDL1 and CD8 in tumours and lymph nodes

Case	Tumour site	PD-L1 (% of tumour positive cells)	CD8+ infiltrate	PD-1 + infiltrate
#A	T	100%	+++	++
	11i area	10	+	+
	4R area	<1%	+	+
#B	T	20%	+++	++
	11 area	20%	++	+
	5 area	<1%	+	+
#C	T	60%	++	+
	10 L area (contiguous LN)	>50%	+++	++
	10 L area	>50%	+++	++
#D	T	>80%	+	+
	7 area	>50%	+	+
	4R area	20%	+	+

survival in some patients, including 10 years after surgery [4, 5]. This leads to the need to improve our knowledge to select IIIA-N2 patients who will benefit from surgical resection. Furthermore, the management strategy of IIIA-N2 patients does not consider various histological types, justifying that we included 4 consecutive cIIIA-N2 patients, regardless of histology.

Molecular Approach

Advances in whole-genome or exome sequencing have led to consider the complexity and the heterogeneity of the cancer genome and its evolution over time [16]. High throughput sequencing studies have shown that mutations and copy number alterations can occur early during tumour evolution, such somatic events being quasi-ubiquitous in tumour cells, or later, leading to different tumour sub-clones [17]. During metastatic spreading to the LN, tumour cells might gradually diverge from the primary tumour but if specific molecular alterations or changes in tumour microenvironment might be related to the pattern of LN metastasis network remains unknown. Moreover, although physical distance might be related to molecular and/or immunophenotypic differences, no study has shown that genetic or microenvironment heterogeneity drives prognostic differences between patients with IIIA-N2 NSCLC. Furthermore, molecular heterogeneity has been proved in localized NSCLC but only 3 metastatic LN were assessed in these small series [6, 7].

Observed Mutations

Tumour #A was heterogeneous, from a pathological and a molecular point of view. The impact of the *ALK* mutation (stop located in the protein kinase domain) is unclear. The *KRAS* mutation (p.G12A) found in LN may be a de novo mutation in the LN, or part of tumour contingent not found in primary tumour. Its absence in mediastinal LN might be explained by a skip phenomenon. Furthermore, the mutation heterogeneity should arise from the different pathological subtype components located in detected specimens ([*ALK* in Tb, acinar] and [*KRAS* in 11i area, solid]). Tumour #B was characterized by the homogeneity of the mutational pattern, but the highest allelic ratios in the subaortic LN suggests some selective advantages in cells harbouring these mutations for lymphatic spread. The *FGFR3* p.S249C mutation (case #C) is pathogenic and targetable [18]. However, considering low allelic ratios in LN, inhibitor of FGFR3 might be inefficient on metastatic sites, such information being relevant for adjuvant drug choice. Furthermore, *TP53* exon-6 truncating mutations promote cancer proliferation [19], this ubiquitous mutation might lead to specific adjuvant strategies. Tumour #D was homogeneous between the biopsy and surgical sample. The *MET* mutation is a receptor tyrosine kinase activating mutation described in sarcomatoid carcinoma [20, 21]. The *TP53* in-phase deletion is disruptive. The *MET* amplification was lost in metastatic 4R LN, associated with a low allelic ratio of the D1028H variant, suggesting a lower sensitivity to anti-MET therapy on distant metastases.

PD1 – PD-L1 Immunostaining

In NSCLC, PD-L1 expression is correlated with different pathological subtypes (solid, poor differentiated, sarcomatoid subtype), *KRAS* and *MET* mutations and tobacco consumption [22–24]. Furthermore, PD-1/PD-L1 inhibitors demonstrated durable clinical responses in patients with metastatic NSCLC [24]. Trials are on-going to explore the role of these treatments in an adjuvant setting. PD-L1 expression by tumour cells, as well as CD8+, PD1+ tumour-infiltrating T cells and a high mutational load seems to be associated with response to treatment [10]. Here, the correlation between PD-L1 expression and the tumour infiltration by CD8+ lymphocytes suggests a possible role of adaptative immunity in the regulation of PD-L1 on tumour cells and the predictive role of PD-L1 expression in anti-PD-1/PD-L1 therapies. Furthermore, PD-L1 expression was heterogeneous between primary tumour and LN, consistent with literature data [25, 26]. This inter-tumour heterogeneity of PD-L1 expression raises a concern about selecting the most appropriate sample for PD-L1 IHC. Impact of microenvironment on lymphatic spread and prognosis is far from being understood [27].

Strengths and Weaknesses

Interpretation of such data should consider that we performed a molecular and phenotypical snap-shot of the tumour, without consideration of the evolution over time of tumours [17]. Prognostic impact was not assessed due to the small number of patients and heterogeneity of histological types, but we showed that a validated, available and ready-to-use tool allowed evaluation of tumour heterogeneity. This evaluation should lead to a better classification of the subgroup of surgically resectable cIIIA-N2 NSCLC, with specific treatment. A French national prospective study will start soon (MET-N2 study, grants from French National Cancer Institute and French Health Ministry).

Conclusions

This preliminary prospective study demonstrated the diversity of intra-tumour and LN mutations using routinely-used targeted NGS, concerning both mutated gene and allelic ratio. Prognostic impact will be assessed in a French national prospective study, leading potentially to new management strategies for the heterogeneous subgroup of cIIIA-N2 NSCLC patients.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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