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Case Report

Comparative Genomic Analysis to Distinguish Triple Metachronous Lung Carcinoma from Metastatic Recurrence: Brief Report on First Case Treated with Three Consecutive VATS Lobectomies

Kersti Oselin[1], Lauris Kaplinski[2], Andri Jääger[3], Ann Valter[1], Tarmo Puurand[2], Ingemar Almre[3], Maido Remm[2], Tõnu Vanakesa[3*]

[1]Department of Chemotherapy, Clinic of Oncology and Haematology, North Estonia Medical Centre, Tallinn, Estonia

[2]Institute of Cell and Molecular Biology, Tartu University, Tartu, Estonia

[3]Department of Thoracic Surgery, North Estonia Medical Centre, Tallinn, Estonia

[*]**Corresponding author**: Tõnu Vanakesa, Department of Thoracic Surgery, North Estonia Medical Centre, Tallinn, Estonia

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Abstract

Introduction: Multiple metachronous lung cancer has a very low incidence. Discrimination of metachronous tumors from metastatic spread is important to optimal patient management. **Methods:** We present here a case of three consecutive lobectomies performed on a 72-year-old male patient. To characterize intertumoral heterogeneity, we applied whole exome sequencing and phylogenetic analysis. **Results:** This is the first reported case of triple metachronous primary lung cancers treated using video-assisted thoracoscopic surgery (VATS) anatomical resection. The surgeries resulted in microscopically margin-negative (R0) resection with no postoperative complications. According to Martini and Melamed criteria, the tumors can be considered as triple metachronous primary lung cancers. After the third lobectomy in 2019 and adjuvant chemotherapy, the patient has no signs of metastases or tumor recurrence. Our results suggest that the use of genomic profiling can identify key somatic alterations in lung cancer evolution and distinguish metachronous cancers from intrapulmonary recurrences. All three tumors had very distinct non-overlapping mutation profiles. The small cell carcinoma in 2019 showed hallmark RB1 and KEAP1 mutations, the squamous cell carcinoma in 2018 had PIK3CA, NOTCH1, and LRP1B mutations, while the large cell carcinoma in 2015 had SMAD4 and CBL mutations. All cases had independent mutations in TP53. **Conclusions:** This is an example where cancer genome sequencing can resolve diagnostic difficulties and to inform treatment decisions.

Keywords: Triple metachronous lung cancer; Video-assisted thoracoscopic surgery (VATS); Comparative genomic analysis

Introduction

Lung cancer is the leading course of cancer death worldwide. Approximately 25% of lung cancer cases are amenable to curative resection. Risk of developing second lung cancer is at least 2-fold higher in these individuals than in the general population [1]. The frequency of diagnosed and treated multiple metachronous lung cancers has increased as a result of advances in radical cancer therapy, understanding the importance of regular follow-up and developing novel detection techniques.

Discrimination of metachronous tumors from metastatic spread, which remains a major challenge of treatment failure, is important to optimal patient management. Genomic studies have revealed that different mutational processes act over time to generate the specific mutational landscape of a tumor [2,3]. Cancer evolution with spread from primary tumor to form metastases or development of a new primary tumor exhibit divergent evolutionary trajectories. Applying sequencing analyses a specific genomic pattern generated by different mutational processes can be identified.

We report a 72-year-old male who was diagnosed with three metachronous lung carcinomas, that resulted in three consecutive lobectomies and systematic lymph node dissection (SLND) using video-assisted thoracoscopic surgery (VATS). Whole exome sequencing (WES) was carried out on formalin-fixed paraffin-embedded (FFPE) resection specimens. In addition, phylogenetic analysis between these tumors was carried out to differentiate whether these are metastatic lesions or primary tumors.

Methods

DNA extraction, tumor whole exome sequencing and germline whole genome sequencing

Archived primary tumors from resection specimens were used for tumor DNA extraction. Tumor DNA extraction and WES was carried out by the Intermountain Genomics Precision Medicines, Utah, US. Germline whole genome sequencing was carried out from the commercial saliva kit provided by the DanteLabs S.r.l., L'Aquila (AQ), Italy. For full protocol of DNA extraction and sequencing, see the Supplementary Materials.

Bioinformatic analysis

The mutations were called from raw FASTQ files. Two independently developed software packages that use completely different algorithms for variant calling were used. One software is commonly used GATK, which uses mapped sequencing reads to call variants [4]. Another software was recently developed KATK, which uses an alignment-free approach [5]. GATK and KATK have approximately the same accuracy, but due to the differences in their algorithms they report slightly different sets of variants. For full description of bioinformatics analysis, see the Supplementary Materials.

Results

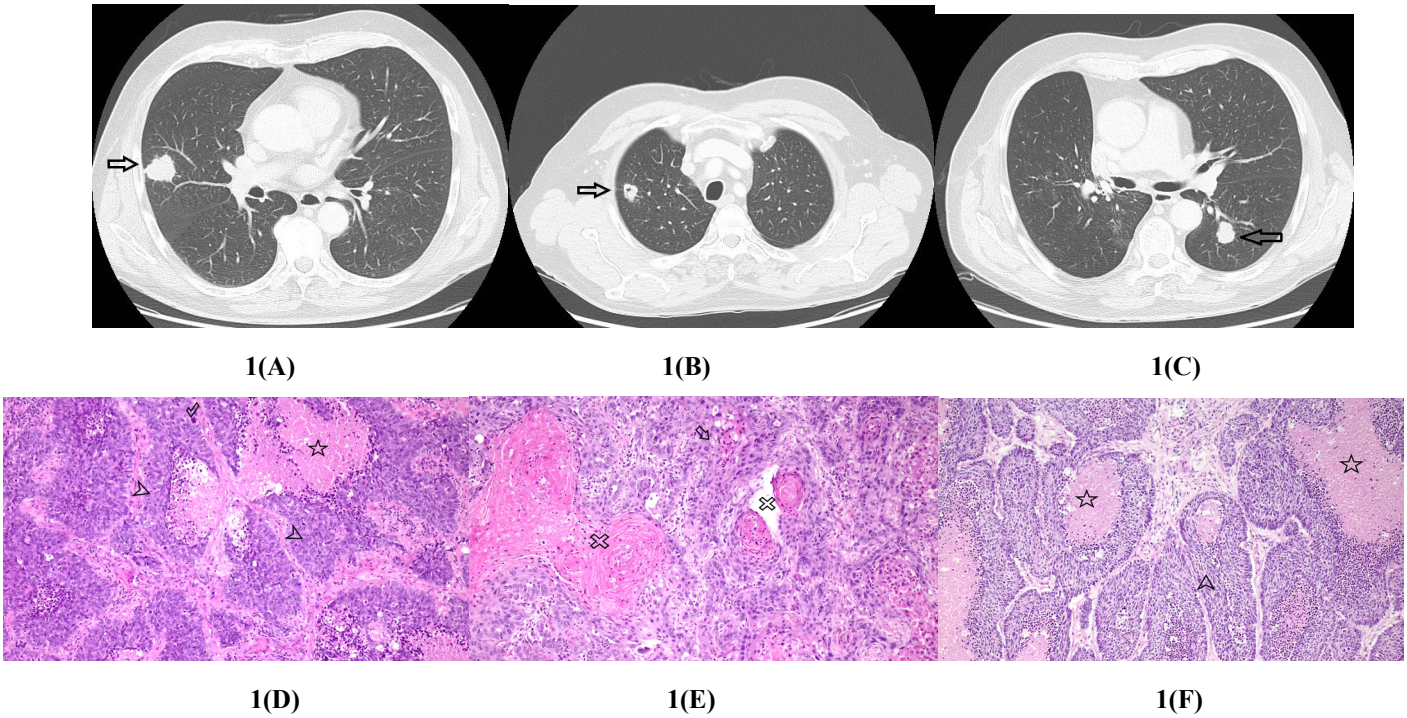
Clinical case presentation

A 72-year-old ex-smoker male with 50 pack-year smoking history was diagnosed with third primary lung cancer during regular surveillance after two VATS lobectomies. The patient quit smoking before the first surgery.

He was first referred for surgical treatment in 2015 after diagnosis of right middle lobe solitary pulmonary nodule on routine chest x-ray. Diagnostic PET/CT showed a 2.5 cm lesion (SUVmax 11.9) (Figure 1A). VATS right middle lobectomy with SLND was performed and the patient was discharged on the 3rd postoperative day. Histological study showed a pT1c pN0 M0 G3 IA3 (TNM 8th edition) large cell carcinoma (Figure 1D).

In 2018 during regular follow-up, 32 months after the first surgery, a second metachronous tumor with a diameter of 2 cm was found on chest CT (Figure 1B). VATS right upper lobectomy with SLND was performed and the patient was discharged on the 3rd postoperative day. Histological findings showed a pT1b pN0 M0 G2 IA3 squamous cell carcinoma (Figure 1E).

In 2019, a chest CT was performed 52 months after the first surgery, a third solid tumour with a diameter of 2.1 cm was found in left lower lobe. PET/CT showed a cT1c N0 M0 peripheral tumor (SUVmax 20.9) (Figure 1C). Uniportal VATS left lower lobectomy was performed using a single 3 cm incision in the 5th intercostal space. The patient was discharged on the 4th postoperative day. Histologically the tumor was pT1c pN0 M0 G3 IA3 small cell carcinoma (SCLC) (Figure 1F). The patient received 3 cycles of adjuvant systemic therapy, fourth cycle was omitted due to atypical lung infection. 42 months after third VATS lobectomy the patient is physically active without recurrence and remains under active surveillance.



1(A) **1(B)** **1(C)**

1(D) **1(E)** **1(F)**

Figure 1: Radiological and histological findings of three primary carcinomas of the lungs; Chest computed tomography scans of three tumors; (A) solid lesion (arrow) 2.5 cm in the right middle lobe in 2015; (B) peripheral nodule (arrow) 2.0 cm in the right upper lobe in 2018, and (C) soft tissue mass (arrow) 2.1 cm in the left upper lobe in 2019. No regional metastases were detected in any of the scans; H&E (x20) stains of three resection specimens, (D) and (F) indicate morphologically large and small cell carcinoma in 2015 and 2019, respectively, with necrosis (star), cancer cells with neuroendocrine morphology (arrow), and atypical mitoses (check mark), (E) indicate squamous cell carcinoma in 2018 with keratinization (cross), and cancer cells with squamous cell morphology (arrow).

Genetic relationship of cases

To investigate the genetic relationship between three cancer cases WES was performed. For comparison, the normal genomic DNA of the same patient was sequenced from a saliva sample. Pairwise comparison of tumors' exome sequences with each other and with the normal tissue sample can reveal whether the cancer cases are independent or if some of them have a common origin. The number of common mutations between any pair of samples is shown in Table 1. The lack of common mutations from different cases strongly suggests the independent origin of all three cases. As expected, a large number of *de novo* mutations were detected in the cancer samples.

	NormalTissue	**2015**	**2018**	**2019**
Normal Tissue	2			
2015	0	152		
2018	0	2	115	
2019	3	0	0	115

Table 1: Mutation matrix of samples. Each cell indicates the number of common mutations (differences from reference genome) detected in exomic regions present in all sequenced samples. Only mutations that were called by both GATK and KATK are reported.

Germline mutations in cancer predisposition genes

We investigated which potential cancer disposition genes (driver genes) are affected by *de novo* mutations. First, the potential effect of all mutations detected by either GATK or KATK was predicted using Variant Effect Predictor software [6]. We selected the list of driver genes from a previously published study [7]. and investigated which of them have mutations whose effect on phenotype is classified as MODERATE or HIGH by Variant Effect Predictor.

The results are shown in Figure 2. Each cancer case had a different mutation in TP53: 157V->F, 258E->G and a change in the splice donor region. Two samples had mutations in RB1 (779L->F and frameshift mutation at codon 299). Interestingly, all tumor samples had common amino acid changes in FAT3 3686R->H (present also in saliva sample) and in KMT2C 339K->N (not present in the saliva sample). It remains unclear whether these mutations had any predisposing effect on lung cancer development in this patient or not.

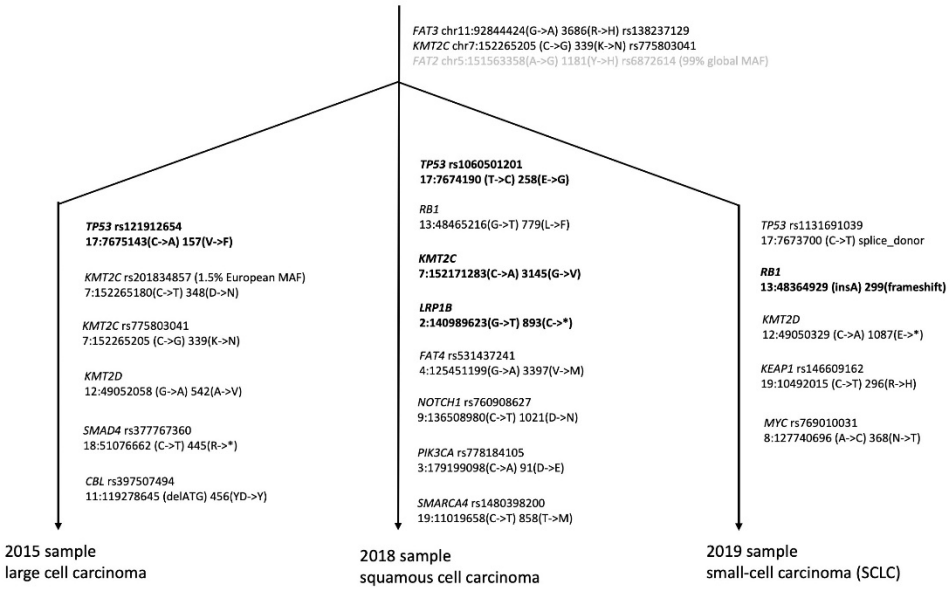


Figure 2: Driver gene mutations with moderate or high effects. Mutations that were present in all three samples or in the normal saliva sample are shown above, before the branching of cancer cases. FAT2 mutation is greyed out because it is a common variant that is very frequent (>99% MAF) in all populations. Mutations shown in bold were identified by both GATK and KATK software, the rest were identified by one of them.

Discussion

According to Martini and Melamed clinical-pathological criteria first introduced in 1975 [8], the tumors in our patient can be considered as metachronous primary tumors. The risk of developing a second primary lung cancer after radical surgery is up to 27% [9]. Younger patients with early stage cancers, who are expected to survive the longest, are at highest risk of developing a new primary metachronous lung cancer. The incidence of triple primary lung cancers is very low. Several cases of triple metachronous lung carcinomas have been reported, but the authors did not find any cases that were treated using VATS anatomical resection with curative intent.

Lobectomies using VATS have a significantly lower risk of pulmonary morbidity, operative mortality and mortality compared to open surgery [10,11] .The uniportal technique used in the 3rd surgery has been proven to give relatively similar or improved perioperative outcomes compared to multiportal VATS without compromising safety or oncologic principles [12]. In our case, all three surgeries resulted in R0 resection postoperative complications. Based on the randomised IFCT-0302 trial, surveillance with CT imaging versus chest x-ray could potentially improve overall survival due to the detection of other cancers, including second primary lung cancer [13]. Our case confirms the importance of active surveillance in patients who can benefit from further curative treatment of a metachronous cancer.

Several studies published previously have examined only few genes for molecular profiling to address multiple pulmonary lesions. A comprehensive genomic analysis of the three resected tumors was conducted in our study to provide insights into the evolutionary processes of cancer development. We observed persistent FAT2/FAT3 mutations in all three cancers. FAT2 and FAT3 are members of the cadherin superfamily, a group of integral membrane tumor suppressor proteins controlling cell proliferation and migration. FAT2 missense variant 1181(Y->H) has unknown clinical significance, moreover the chr5:151563358 A->G allele alteration reported here represents the most common allele (99%) in European populations, including Estonians. FAT3 3686(R->H) (chr11:92844424 G->A alteration) has very low frequency in Europeans, but has been commonly reported in lung cancer and rises susceptibility to tobacco induced mutations in our patient.

Genomic alteration in TP53 was present in all three cases, although three different missense alterations occurred in different positions over years. Previous studies have shown TP53 mutations are highly consistent between matched primary tumor and metastatic lesion, supporting TP53 significance as an early truncal and driver event [2]. Divergent mutations detected in the three tumors also support metachronous cancers in our patient. Tumor suppressor gene TP53 is the most frequently mutated gene in lung cancer. In 1518 surgically treated patients with localized-stage lung cancer, TP53 mutations were negative prognostic markers for disease free and overall survival [14]. TP53 mutations may also confer higher resistance to chemotherapy compared to TP53 wild type tumors. Furthermore, TP53 mutated tumors have increased shedding of cfDNA and numerically higher VAF for approximated tumor volume, both associated with worse outcome [15]. Although, TP53 seems crucial oncogene in our patient, the patient remains recurrence free after 42 months of last surgery.

Based purely on morphology and histological diagnoses the aggressiveness of a cancer in our patient increased from a large cell carcinoma in 2015, to squamous cell carcinoma in 2018, and finally SCLC in 2019. All three tumors had very distinct non-overlapping mutation profiles. The first tumor in 2015 harboured TP53, KMT2C, KMTSD, SMAD4 mutations, and CBL deletion. The classification of large cell carcinoma, large cell neuroendocrine carcinoma and small cell carcinoma has been controversial, as they exhibit similar morphological and immunohistochemical features. Distinct genomic profiles between 2015 large cell carcinoma and 2018 squamous cell carcinoma do not support gain of squamous cell lineage alterations in our patient, rather development of a new primary lung cancer. Furthermore, the absence of RB1 mutations in 2015 large cell carcinoma likely indicates correctness of the pathological diagnoses. The squamous cell carcinoma in 2018 had LRP1B mutation, PIK3CA, and NOTCH1, highly established squamous cell cancer susceptibility genes. Finally, the SCLC in 2019 harbored characteristic small cell alterations, including small insertion in RB1, KEAP1, and MYC mutations in addition to splice donor mutation in TP53.

Conclusion

In conclusion, our results suggest the use of genomic profiling can identify key somatic alterations in lung cancer evolution and distinguish metachronous cancers from intrapulmonary recurrences. Genomic profiling may solve diagnostic obstacles of cases with mixed morphology. The study also supports Martini and Melamed clinical criteria for a diagnoses of new primary lung cancer. Minimally invasive surgical approach provides optimal care in patients who are candidates for repeated surgery for lung cancer.

Disclosure

The Authors have no conflict of interests to declare in relation to this manuscript.

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Informed Consent

Written informed consent was obtained from the patient for publication of this manuscript and any accompanying images.

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Supplementary Materials

Tumor DNA extraction

Six 15 µm scrolls were cut from FFPE tissue sample blocks for DNA extraction. DNA was extracted from FFPE sample scrolls using the Promega Maxwell FFPE Plus DNA Kit on the Promega Maxwell RSC instrument. DNA was quantified via the Quant-iT PicoGreen dsDNA Assay Kit (Thermo). 2 µg of DNA for each sample was fragmented using the Covaris LE220-plus instrument. The fragmented DNA underwent a 0.7X bead cleanup using AMPure XP size-selection beads (Beckman Coulter) to remove smaller fragments. Subsequent quantitation of the fragmented DNA allowed for an input of 500 ng of fragmented DNA for library prep using the KAPA HyperPrep Library Prep kit (Roche). Library quality was determined using the Fragment Analyzer DNA/NGS Kit (Agilent). Libraries were quantified by qPCR using the KAPA Library Quantification Kit (Roche). Libraries were then pooled according to concentrations obtained from qPCR.

Tumor whole exome sequencing and variant calling

The library pools underwent exome capture using the IDT Exome Research Panel. After exome capture, captured pools were then quantified by qPCR as described above. Capture pools were then balanced and pooled via qPCR concentration and loaded for sequencing at 1.5 nM. 150 bp paired-end sequencing was performed. After ligation, ligated DNA products underwent 0.7X bead cleanup with AMPure XP beads. The cleaned up ligation products were amplified with 8 cycles of PCR followed by another 0.7X bead cleanup. Following exome capture the captured DNA was amplified with 12 cycles of PCR followed by a 0.8X bead cleanup.

Tumor DNA extraction and WES was carried out by the Intermountain Genomics Precision Medicines, Utah, US.

Germline whole genome sequencing

Commercial saliva swabs were used for germline DNA collection provided by DanteLabs S.r.l., L'Aquila (AQ), Italy. Entire DNA was sequenced with average 30X coverage using next generation sequencing by Illumina technology. FASTQ files were downloaded from DanteLabs for inhouse bioinformatics analysis.

Bioinformatics analysis

The mutations were called from raw FASTQ files with two different methods. First, with KATK all parameters were left at default values, except that for exome sequences (2015, 2018 and 2019 cancer samples) the –exome argument was used.[1] The per-nucleotide calls of KATK were converted to Variant Call Format (VCF) file using katk2vcf.pl script included in package. Secondly, with GATK all FASTQ files were mapped to human reference genome GRCh38 and then called jointly with default parameters.[2] From joint VCF file, individual per-sample calls were extracted. The calling thus generated 8 VCF files (2 per sample). As exome sequencing is known to have many errors due to uneven coverage, these were further analyzed to find high-quality consensus calls. For phylogeny analysis only single-nucleotide variations (SNPs) were used. All SNVs previously recorded in dbSNP build 151 were removed.[3] Then only positions where both calling methods (KATK and GATK) gave the same genotype were kept (total 520 positions). For effect prediction all mutation types (substitutions, insertions and deletions) were used and only the dbSNP variants with MAF >= 0.05 were discarded. Then VCF files were analyzed with Variant Effect Predictor (VEP).[4] From VEP prediction output the sites with moderate and high impact were chosen. For each such mutation, the confidence was assigned based on whether both calling methods gave the same call (high confidence), different calls (low confidence) or one of the calls was missing due to low coverage (medium confidence).

To investigate the genetic relationship between three cancer cases we performed exome DNA sequencing of all three samples. For comparison, the normal genomic DNA of the same patient was sequenced from a saliva sample. Pairwise comparison of those exome sequences with each other and with the normal tissue sample can reveal whether the cancer cases are independent or if some of them have a common origin. Two independently developed software packages, as described above, that use completely different algorithms for variant calling were used. To avoid biases potentially caused by sample processing during the exome library preparations we used a very conservative approach to variant calling. Only mutations that were reliably detected by both algorithms were used for inferring the genetic relationship of

cases. Also, we made efforts to ensure that exactly the same subset of genomic regions were compared between all pairs of samples. The mutations that were not callable in at least one sample were removed from all four samples.

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