Whole exome sequencing of independent lung adenocarcinoma, lung squamous cell carcinoma, and malignant peritoneal mesothelioma

A case report

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Abstract

The presence of multiple primary tumors (MPT) in a single patient has been identified with an increasing frequency. A critical issue is to establish if the second tumor represents an independent primary cancer or a metastasis. Therefore, the assessment of MPT clonal origin might help understand the disease behavior and improve the management/prognosis of the patient.

Herein, we report a 73-year-old male smoker who developed 2 primary lung cancers (adenocarcinoma and squamous cell carcinoma) and a malignant peritoneal mesothelioma (PM).

Whole exome sequencing (WES) of the 3 tumors and of germline DNA was performed to determine the clonal origin and identify genetic cancer susceptibility.

Both lung cancers were characterized by a high mutational rate with distinct mutational profiles and activation of tumor-specific pathways. Conversely, the PM harbored a relative low number of genetic variants and a novel mutation in the WT1 gene that might be involved in the carcinogenesis of non-asbestos-related mesothelioma. Finally, WES of the germinal DNA displayed several single nucleotide polymorphisms in DNA repair genes likely conferring higher cancer susceptibility.

Overall, WES did not disclose any somatic genetic variant shared across the 3 tumors, suggesting their clonal independency; however, the carcinogenic effect of smoke combined with a deficiency in DNA repair genes and the patient advanced age might have been responsible for the MPT development. This case highlights the WES importance to define the clonal origin of MPT and susceptibility to cancer.

Abbreviations: ADC = adenocarcinoma, COPD = chronic obstructive pulmonary disease, CT = computed tomography, Genomic DNA = gDNA, IHC = immunohistochemistry, MNV = multiple nucleotide variant, MPLC = multiple primary lung cancers, MPT = multiple primary tumors, NGS = next generation sequencing, PET = positron-emission tomography, PM = peritoneal mesothelioma, SCC = squamous cell carcinoma, SNP = single nucleotide polymorphism, SNV = single nucleotide variant, WES = whole exome sequencing.

Keywords: clonal origin, mesothelioma, multiple lung cancers, tumor susceptibility, whole exome sequencing.
1. Introduction

The incidence of multiple primary tumors (MPT) during an individual’s lifetime is increasing, mainly due to the advent of accurate cancer secondary prevention programs and the increase of life expectancy for cancer patients. The development of multiple primary lung cancers (MPLC) is an uncommon event, although the improvement in the diagnostic tests and novel therapies able to influence survival after the first diagnosis of cancer have led to an incidence peak that has grown up to 20% over the past 10 years. A correct understanding whether the second tumor is an independent primary lesion or a metastasis is fundamental for an adequate therapeutic management of these patients. Currently, the main criteria for defining the lineage of multiple unrelated intrapulmonary tumors compared with metastatic lesions are based on pathological and clinical assessments. To date, several studies have described MPLC cases, but most of them have analyzed a limited number of genetic markers, resulting in a low accuracy and limited ability to establish cancers clonality. Next generation sequencing (NGS) is a recent technology that can contribute to understanding the molecular mechanisms underlying tumor development by screening the whole DNA mutational profile. Recently, Murphy et al. applied the NGS approach to define the lineage of MPLC, demonstrating how genomic rearrangements were able to distinguish MPLC from metastatic lesions; however, the authors did not evaluate somatic and germline mutational profiles. Once established that MPLC are primary and independent tumors, understanding the intrinsic genetic susceptibility to develop multiple cancers during the lifetime is crucial; indeed, those subjects with high predisposition might be enrolled in prevention programs and benefit from personalized follow-ups. Herein, we report an interesting case of a patient that developed 2 primary histologically distinct lung tumors and a malignant PM after 6 years. WES allowed us to deeply screen the 3 tumors, in order to identify a mutational signature specific for each malignancy and to establish the clonal origin of cancers. Concomitantly, the sequencing of normal genomic DNA (gDNA) allowed the identification of germline genetic variants potentially correlated with an individual risk of developing multiple cancers.

2. Case report

We describe the case of a Caucasian male patient with a medical history of heavy smoking habit (100 pack-years), chronic obstructive pulmonary disease (COPD), and no exposure to asbestos. Before being referred to our unit, the patient was initially followed and treated in a different institution; hence, part of the patient’s oncologic history was retrospectively retraced when he came to our attention (Fig. 1). In January 2009, the patient, aged 73 years, was subjected to a chest X-ray as the only clearly detectable active site of disease (SUV max: 12.6, increased from the previous examination), while no distant metastases were identified; therefore, surgery with potential curative intent for oligo-metastatic disease was proposed. Hence, in January 2012, the patient underwent right upper lobectomy and radical lymphadenectomy with postoperative diagnosis of keratinizing and moderately differentiated squamous cell carcinoma (SCC) of the lung with positivity for p63 at IHC (pT2a G2 pN0 Mx, stage IB) (Fig. 2B). Although the clinical presentation could initially suggest a possible correlation between the 2 lung lesions, the IHC led to define 2 histologically distinct primary lung tumors. After surgery, the patient did not receive further treatments. In February 2014, metabolically active gastric lymphadenopathies and ascites were detected during follow-up, although no suspicious lesions were identified with esophagogastroduodenoscopy. Between October 2014 and January 2015, diffuse nodulations within the abdomen, morphologically compatible with peritoneal carcinomatosis, and a new lesion in the middle lobe of the right lung were identified. In February 2015, the patient was referred to our institution (Lung Cancer Unit; IRCCS AOU San Martino - IST, Genova, Italy), wherein he underwent biopsy of an easily accessible abdominal lesion located at the level of the right iliac fossa. At microscopic examination, the specimen was consistent with several small fibrous fragments diffusely infiltrated by an epitheliomimetic neoplasm composed of atypical cells, ranging from middle to large dimension, with well-represented eosinophilic cytoplasm, sometimes microvacuolated, and large nuclei, with prominent eosinophilic nucleoli; rare “hobnail cells” were identified and the neoplastic elements were arranged in solid nests, ribbons, and papillary structures. At IHC, expression of CK7, CK5&6, calretinin, and WT-1 was detected in neoplastic cells, whereas no expression of CK20, p63, MOC-31, TTF-1, and napsin-A was reported (Fig. 2C-H). On the basis of the morphology and the IHC pattern, the diagnosis of epithelioid PM was posed and, subsequently, the patient received chemotherapy with pemetrexed (500 mg/m2), which was discontinued after 2 cycles due to poor tolerance. Then, the patient experienced progressive worsening of clinical conditions and died in March 2015. Relevant images from CT-scans collected throughout the clinical history of the patients have been reported in Fig. 3.

In order to understand whether ADC, SCC, and PM were unrelated cancers or shared a common clonal evolution, WES analysis was performed on the 3 tumors by HiSeq 2500 sequencer (Illumina Inc, San Diego, CA, USA) as already described.
Simultaneously, the WES of germinal gDNA obtained from peripheral blood was performed to subtract the germline background for the identification of somatic variants (see text, Supplemental Content 1, http://links.lww.com/MD/B418, which illustrates samples processing and WES analysis). For this analysis, the ADC and the SCC samples were collected from stored surgical specimens (acquired during potentially curative surgery), while the PM sample derived from the tissue collected during the abdominal biopsy.

We firstly extracted the somatic mutational signature from all the tumors according to base substitutions, as already described by Alexandrov et al. This analysis displayed a predominance of C>A transversions in both lung cancers (ADC and SCC) (Fig. 4A), corresponding to a specific cancer signature related to tobacco consumption. In contrast, the PM did not exhibit any specific mutational signature, probably as a consequence of the few observed somatic variants (Fig. 4A). Then, we found that each tumor reported a specific set of somatic variants (358, 405, 28 in ADC, SCC, and PM, respectively; Fig. 4B; See Table, Supplemental Content 2A, http://links.lww.com/MD/B419, Supplemental Content 2B, http://links.lww.com/MD/B420, and Supplemental Content 2C, http://links.lww.com/MD/B421, which list all somatic mutations found in ADC, SCC, and PM, respectively), which were not shared across the 3 tumors. Both ADC and SCC showed lung tumor hotspot mutations reported in the Catalogue of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cosmic) database and described in lung cancers: EHHADH (COSM5247826), KRAS (COSM512), OR4K2 (COSM1515038), and TP53 (COSM6549) in ADC; KIAA1324L (COSM396629), NFE2L2 (COSM396629), PEG3 (COSM5284477), POM121L12 (COSM393793), and WAC (COSM5311283) in SCC. Moreover, both histotypes carried mutations associated with potential therapeutic targets (FLT3 and HGF in ADC; MTOR in SCC), or in a predictor of resistance to EGFR tyrosine kinase inhibitors (KRAS in ADC).

The enrichment analysis using Reactome 2015 (http://amp.pharm.mssm.edu/Enrichr) also showed that different pathways were deregulated in ADC and SCC. Specifically, ADC was enriched with altered genes belonging to the MAPK pathway (p.Gly12Phe KRAS; c.*76delC MAP2K; c.*30C>T MAP3K4),
whereas the mutations observed in SCC mostly affected genes involved in collagen modification, in extracellular matrix organization (p.His1331Gln ADAMTS3; p.Phe486Ser COL19A1; p.Ala75fs LOX; c.93+567C>A SPP1; p.Pro947Ser LAMB1; p.Met688Ile A2M), and in the meiotic synapsis pathway (p.Ser1801Gly ATR; p.Gln1747Glu DIDO1; c.1961+53A>T SUN1; c.17542-41A>C SYNE1). Conversely, the PM did not display COSMIC mutations or pathways associated with the carcinogenesis, probably due to the low number of somatic mutations (28); however, among these mutations, we identified 3 novel variants including 2 frameshift variants (p.Glu673fs BAP1; p.Glu1595fs SETD2) and a missense variant (p.Ser71Phe WT1).

Germline analysis was also performed in order to discover genetic variants potentially linked to cancer predisposition. Germinal gDNA sequencing identified a total of 31,608 genetic variants of which 15,790 and 15,818 occurred in exons and nonexons regions, respectively (Fig. 4B). In particular, 49% (7784/15,790) of the exon variants showed a high/moderate effect on the protein, whereas the 66% (10,397/15,818) of nonexon variants potentially modified the protein regulation based on effect prediction of SnpEff tool (http://snpeff.sourceforge.net).

As pathway analysis did not disclose enrichment pathways linked to tumor susceptibility, we focused on genes related to DNA repair or associated with cancer predisposition. The analysis identified 74 genetic variants in 59 genes related to DNA repair/cancer predisposition. Specifically, 21 out of 74 genetic variants have already been described to confer a high risk of cancer development and 7 of them were homozygous (rs3760413, EME1; rs26279, MSH3; rs8305, POLI; rs373572, RAD18; rs462779, REV3L; rs25487, XRCC1; rs1143634, IL1B) (Table 1). Finally, we found 5 single nucleotide polymorphisms (SNPs) (rs1948, CHRNBB4; rs1051730, CHRNA3; rs16969968, CHRNA5; rs4950, CHRNA3;
and ADC, we found 6 (tumors occurred in our case. Across 358 altered genes in the smoking and the development of the 2 clonally unrelated lung patients. These data support the association between extensive ADC and SCC tumors were mainly identified in primary lung tumors demonstrating that clonally independent lung ADC.

Of note, mutation in among 18 genes found significantly mutated in 484 lung SCC tumors. Recently, Warth et al. analyzed a set of synchronous mutations. Interestingly, the signature characterized by Cancer Genome Atlas Research Network has been also observed in a comprehensive genome-wide characterization by DNA leading to the accumulation of somatic mutations. Recently, Warth et al. analyzed a set of synchronous primary lung tumors demonstrating that clonally independent ADC and SCC tumors were mainly identified in heavy smoker patients. These data support the association between extensive smoking and the development of the 2 clonally unrelated lung tumors occurred in our case. Across 358 altered genes in the ADC, we found 6 (KRAS, MAP2K1, MGAM, NFI, PPP3CA, and TP53) of 38 genes significantly mutated in a cohort of 660 lung ADC. Of note, mutation in PPP3CA co-occurred with an activating KRAS mutation (COSM512) as already described by Campbell et al. In addition, the mutation in the MGAM gene has been also observed in a comprehensive genome-wide characterization by Cancer Genome Atlas Research Network among 18 genes found significantly mutated in 230 lung ADC tumors. Across the 405 SCC-mutated genes, we found only 1 gene (NFE2L2) of 20 genes recurrent mutated in 484 lung SCC tumors, moreover, mutations in NFE2L2 gene have also been identified in 34% of 178 lung SCC tumors profiled by Cancer Genome Atlas Research Network.

Furthermore, both lung tumors showed a specific mutational signature linked to distinct pathways of activation. Specifically, the ADC harbored mutations in genes involved in EGFR signaling pathway, such as 2 novel genetic variants in the 3’UTR regions of MAP3K4 and MAP2K1 genes, and a hotspot mutation in the KRAS codon 12; as it is known, the EGFR signaling pathway is one of the most frequently altered pathways in this histology. On the contrary, the SCC carried several mutations in genes involved in the extracellular matrix organization, a pathway often deregulated in cancer. In particular, we found a novel frameshift deletion (c.221delC; p. Ala75fs) leading to a potential LOX protein destruction. LOX downmodulation has been found in SCC and its lack has been shown to induce the extracellular matrix disorganization leading to tumor development. Furthermore, in addition to being potentially involved in tumor development, some of the affected genes that were observed in this case might also play a relevant role in a targeted therapy approach in patients affected by lung cancer, possibly reducing sensitivity to currently registered agents or eventually representing potential targets for drugs that might become available for lung cancer in future. Although it is still unclear whether KRAS mutations are actually associated with resistance to EGFR inhibitors in lung cancer, aberrations of HGF signal are apparently involved in resistance to anti-EGFR and anti-VEGF targeted therapies. Contrarily, FLT-3 and mTOR might represent potentially actionable targets, as the former is sensitive to drugs such as dovitinib, while the latter is sensitive to everolimus.

Conversely, in PM, the distribution of base substitutions did not match any specific mutational signature, probably as a consequence of a relatively limited number of observed mutations (28 variants in PM vs > 350 in the lung cancer lesions). Peritoneal mesothelioma is an extremely rare tumor and our sequencing data were in accordance with a previous study in which the authors performed WES on 7 PM finding a low mutational rate...
| dbSNP | Gene ID | Locus | Reference sequence | Coding DNA sequence | Protein sequence | MAF | Genotype | Effect | PMID/DOI |
|-------|---------|-------|-------------------|---------------------|------------------|-----|----------|--------|----------|
| rs8305 | POLI    | chr18:51820805 | NM_007195.2 | c.2191G>A | p.Ala731Thr | 0.77 | HOM | Lung cancer susceptibility | 15603917 |
| rs25487 | XRCC1   | chr19:44055726 | NM_002987.2 | c.1196A>G | p.Gln399Arg | 0.74 | HOM | Lung cancer susceptibility | 25663194; 26634519; 26767006 26199902 |
| rs3760413 | EME1 | chr17:48452776 | NM_01166131.1 | c.207A>C | p.Glu69Asp | 0.74 | HOM | Lung cancer susceptibility | http://dx.doi.org/10.6000/1929-2279.2014.03.04.1 |
| rs1143634 | IL1B | chr2:1:13580390 | NM_000576.2 | c.315C>T | p.Phe105Thr | 0.13 | HET | Lung cancer susceptibility | 16193237 |
| rs3087386 | REV1 | chr2:100055506 | NM_001166131.1 | c.770T>C | p.Phe257Ser | 0.57 | HET | Lung cancer susceptibility | 24012694; 16774934; 15609317 |
| rs175080 | MLH3 | chr14:7551382 | NM_014381.2 | c.2531C>T | p.Pro844Leu | 0.36 | HET | Lung cancer susceptibility | 17494092 |
| rs2395655 | CDKN1A | chr19:44055726 | NM_002987.2 | c.83A>G | p.Asp28Glu | 0.51 | HET | Lung cancer susceptibility | 21140615 |
| rs1047840 | EXO1 | chr1:242042301 | NM_001184.3 | c.632T>C | p.Met211Thr | 0.62 | HET | Lung cancer susceptibility | 16193237 |
| rs3760413 | EME1 | chr17:48452776 | NM_01166131.1 | c.315C>T | p.Glu69Asp | 0.74 | HOM | Lung cancer susceptibility | http://dx.doi.org/10.6000/1929-2279.2014.03.04.1 |
| rs1143634 | IL1B | chr2:1:13580390 | NM_000576.2 | c.315C>T | p.Phe105Thr | 0.13 | HET | Lung cancer susceptibility | 16193237 |
| rs3087386 | REV1 | chr2:100055506 | NM_001166131.1 | c.770T>C | p.Phe257Ser | 0.57 | HET | Lung cancer susceptibility | 24012694; 16774934; 15609317 |
| rs175080 | MLH3 | chr14:7551382 | NM_014381.2 | c.2531C>T | p.Pro844Leu | 0.36 | HET | Lung cancer susceptibility | 17494092 |
| rs2395655 | CDKN1A | chr19:44055726 | NM_002987.2 | c.83A>G | p.Asp28Glu | 0.51 | HET | Lung cancer susceptibility | 21140615 |
| rs1047840 | EXO1 | chr1:242042301 | NM_001184.3 | c.632T>C | p.Met211Thr | 0.62 | HET | Lung cancer susceptibility | 16193237 |

**dbSNP** = SNP database (http://www.ncbi.nlm.nih.gov/SNP), **DOI** = Digital Object Identifier (article published and electronically available), **HET** = heterozygote, **HOM** = homozygote, **Locus** = base position relative to GRCh37/hg19, **MAF** = minor allele frequency (http://www.1000genomes.org/), **NA** = not available, **PMID** = PubMed Identifier (article published and available in PubMed database).
and BAP1 as the most altered gene.[24] We also found an insertion in BAPI, potentially associated with a loss-of-function, and a deletion changing the reading frame in SETD2, a gene found altered in malignant pleural mesothelioma.[25] In addition, we detected a novel mutation in the WT1 transactivation domain (NM_000378.4; c.212C>T; p.Ser71Phe). Mutated WT1 has been already described in mesothelioma; interestingly, Park et al.[26] reported a patient with PM that harbored a point mutation within the transactivation domain of WT1 gene, demonstrating that this variant conferred an activation of its transcriptional role. However, the authors did not find any WT1 mutations in a further set of 32 asbestos-related mesothelioma patients, thus concluding that the WT1 pathway could be involved in the malignant transformation of non-asbestos-related mesothelioma. These data suggest that the p.Ser71Phe WT1 mutation might be implicated in the PM carcinogenesis process through the WT1 downstream pathway activation. Indeed, the mutation serine-71-phenylalanine (p.Ser71Phe) in WT1 gene is a nonconservative mutation that alters the properties of the protein by replacing the small and polar serine with the large and bulky side chain of a phenylalanine.

According to the previous data and excluding a common lineage across the 3 tumors, we hypothesized that this patient could have an intrinsic predisposition to develop MPT. Indeed, the germinal gDNA sequencing showed that more than half of the variants were potentially associated with protein alterations. Notably, the analysis identified 21 genetic variants that were already described; of these, 62% were related to increased lung cancer risk. Among such variants, the association of the p. Glu589Lys in EXO1 gene (rs1047840) with cigarette smoking has been described as conferring a significantly increased lung cancer risk, with a reported odds ratio equal to 1.72.[28]

To the best of our knowledge, this is the first study that investigates the whole exome mutational profile of 3 MPT aimed at defining the clonal origin of the tumor lesions and also the germline assets in order to discover an individual genetic susceptibility to cancer predisposition. Our data support the hypothesis that the development of the 3 tumors was clonally independent, as they do not share a common mutational profile; however, we could not exclude the presence of mutations in regulatory regions, omitted by WES. The patient also carried several SNPs involved in nicotine dependence and DNA repair. The carcinogenic effects of tobacco smoke together with both a DNA repair deficiency and the advanced age of the patient may have led to a high mutation rate in the lung cancer lesions. It is also known that chemotherapy might affect the mutational status of eukaryote cells.[29] Despite the only 2 cycles of carboplatin, considering the interval between treatment and SCC tumor collection (about 14 months), we cannot exclude the mutagenic effect induced by carboplatin.

On the contrary, the low number of somatic mutations in PM suggests that its development is mainly caused by onset of mutations in driver genes (BAP1 and SETD2) and that other mechanisms, such as microRNA deregulation, might be involved.[30] In addition, the novel missense mutation in WT1 gene may also explain the PM development regardless of asbestos exposure.

In conclusion, this study underlines how the germline assets could influence the cancer predisposition and how future WES studies on patients with MPT should be directed toward the genetic variants identification leading to cancer susceptibility. Our findings highlight the power of WES analysis in screening the mutational landscapes of patient with MPT in order to define the clonal feature and identify novel potential molecular targets for treatment.

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