Convergent loss of PTEN leads to clinical resistance to a PI(3)Kα inhibitor

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Broad and deep tumour genome sequencing has shed new light on tumour heterogeneity and provided important insights into the evolution of metastases arising from different clones1,2. There is an additional layer of complexity, in that tumour evolution may be influenced by selective pressure provided by therapy, in a similar fashion to that occurring in infectious diseases. Here we studied tumour genomic evolution in a patient (index patient) with metastatic breast cancer bearing an activating PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha, PI(3)Kα) mutation. The patient was treated with the PI(3)Kα inhibitor BYL719, which achieved a lasting clinical response, but the patient eventually became resistant to this drug (emergence of lung metastases) and died shortly thereafter. A rapid autopsy was performed and material from a total of 14 metastatic sites was collected and sequenced. All metastatic lesions, when compared to the pre-treatment tumour, had a copy loss of PTEN (phosphatase and tensin homolog) and those lesions that became refractory to BYL719 had additional and different PTEN genetic alterations, resulting in the loss of PTEN expression. To put these results in context, we examined six other patients also treated with BYL719. Acquired bi-allelic loss of PTEN was found in one of these patients, whereas in two others PIK3CA mutations present in the primary tumour were no longer detected at the time of progression. To characterize our findings functionally, we examined the effects of PTEN knockdown in several preclinical models (both in cell lines intrinsically sensitive to BYL719 and in PTEN-null xenografts derived from our index patient), which we found resulted in resistance to BYL719, whereas simultaneous PI(3)Kα p110β blockade reverted this resistance phenotype. We conclude that parallel genetic evolution of separate metastatic sites with different genomic alterations leads to a convergent PTEN-null phenotype resistant to PI(3)Kα inhibition.

We are currently engaged in testing the antitumour activity of a novel PI(3)Kα inhibitor, BYL719, in patients with tumours harbouring activating PI(3)Kα mutations. The PI(3)K pathway is essential for cell growth, proliferation, survival, and metabolism4,5. The PI(3)K family of enzymes is divided into three main classes (I to III), with class I being the most often implicated in human cancer6. Class IA PI(3)K is a heterodimer composed of a catalytic subunit (p110α, β or δ) and a regulatory subunit7,8. PIK3CA, the gene encoding p110α, is mutated in up to 40% of oestrogen receptor (ER) and/or HER2 positive breast tumours9,10. In our ongoing phase I clinical study of BYL719, we have observed clinical responses in breast, head and neck and other tumours1, providing proof of principle that PI(3)Kα targeting is active against tumours harbouring PIK3CA mutation.

We present the case of a 60-year-old breast cancer patient (index patient) diagnosed with invasive ductal carcinoma who underwent surgery followed by adjuvant treatment with chemotherapy and the aromatase inhibitor exemestane. Four years later, the patient developed bone metastases and started therapy with the ER antagonist fulvestrant, achieving stable disease. After 18 months on therapy, her disease progressed in the liver, bone and lymph nodes. The archival tissue of the primary tumour was subjected to PCR-based genetic analysis11 and a hot spot mutation in PIK3CA (ES42K) was detected. This finding led to the patient’s enrolment in a phase I clinical trial testing the tolerability and antitumour activity of BYL719 (NCT01219699). The patient rapidly achieved a confirmed partial response according to the RECIST 1.0 criteria12 that lasted 9.5 months (Table 1 and Extended Data Fig. 1). At that point, while the tumour remained stable in multiple sites including a peri-aortic lymph node location, progression occurred in the lungs (Fig. 1) and consequently the patient discontinued BYL719 therapy.

Figure 1 | Clinical response of index patient treated with BYL719. CT scans showing stable (responding) peri-aortic lymph node metastasis (yellow circles, left column) and the appearance of new lung metastatic lesions (yellow circles, right column) after the completion of the tenth cycle of BYL719 therapy. Arrow, pleural effusion.

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Figure 2 | Loss of PTEN upon BYL719 resistance. a, Circos plots from WGS analysis of primary tumour (before BYL719 treatment) and a lung metastasis appearing after the tenth cycle of BYL719 therapy. b, Copy number variation of chromosome 10. c, WES of the peri-aortic lymph node showing durable stable disease during BYL719 therapy compared to both primary tumour and the progressing lung lesion. The diagram shows the variant allele fractions (VAF) of the listed gene mutations in the three lesions. The estimated tumour purities are 44% for the breast primary tumour, 50% for the lung metastasis, and 59% for the lymph node metastasis. d, PTEN IHC of primary tumour, peri-aortic lymph node, and lung metastasis. Images were taken from Servier Medical Art (licensed under a Creative Commons Attribution 3.0 Unported License).
therapy with BYL719 was discontinued. The clinical status of the patient deteriorated rapidly and she died two months after termination of the BYL719 treatment. A rapid autopsy was performed three hours after death and a total of 14 metastases with tumour cells present were identified and collected for sequencing (Extended Data Table 1).

In order to proceed systematically to identify possible genetic determinants of acquired resistance to PI(3)K inhibition, we took a three-step approach. First, we examined both the primary tumour (before BYL719 treatment) and the new lung metastasis by whole genome sequencing (WGS). Although both samples shared many somatic genetic aberrations (Fig. 2a and Extended Data Fig. 2), PTEN copy number loss was detected only in the lung metastasis (Fig. 2b). Second, we analysed by whole exome sequencing (WES) the primary tumour, lung metastasis, and peri-aortic lesion that remained stable (responding) at the time of progression to BYL719 therapy (Fig. 2c). This analysis revealed that both peri-aortic and lung lesions harboured mutations in PIK3CA, ESR1 and BRCA2, and single copy loss of PTEN. Importantly, in addition to the PTEN copy number loss, we identified a PTEN del339FS (frameshift) mutation only in the lung metastasis (Fig. 2c). By immunohistochemistry (IHC), we observed that PTEN protein expression was lost in the lung metastasis but was present in both the primary tumour and peri-aortic lesion (Fig. 2d).

Third, to confirm and expand our findings, we sequenced the primary tumour and all the metastatic lesions to >500-fold coverage using a custom targeted deep-sequencing assay, IMPACT13,14 (Methods). A number of mutations were shared by the primary tumour and the metastatic sites, whereas others were observed only in all or in selected metastatic lesions (Fig. 3a and Supplementary Table). We confirmed that the PIK3CA E542K mutation in the primary tumour was conserved in the metastatic samples and detected the presence of another PIK3CA mutation (D725G). Moreover, we found increased copy number of FGFR1 and E1A4BP1 in all tumour samples, consistent with the relatively frequent 8p11-12 amplification described in breast cancer15,16. ESR1 Y537N and BRCA2 L971S alterations were present in all the metastatic lesions but not in the primary tumour. We speculate that the ESR1 Y537N mutation, reported to promote ligand-independent ER activation1, was selected upon anti-oestrogen therapy received by the patient before BYL719 treatment.

Central to our work, all metastatic lesions appeared to harbour a single copy loss of PTEN (Extended Data Fig. 3). Furthermore, we found that 10 of the 14 metastatic lesions harboured additional genomic alterations within PTEN. The spectrum of PTEN alterations was heterogeneous across the 10 samples and included a splice site mutation at K342, a frameshift indel at P339 (confirming the WES result), and 4 different exon-level deletions (Fig. 3a and Extended Data Fig. 4). All 10 specimens with either secondary PTEN mutations or copy number loss were confirmed negative for PTEN staining by IHC, whereas the four specimens that retained a PTEN wild-type allele were positive for PTEN protein expression (Extended Data Fig. 5). In addition, for those lesions

**Figure 3 | Loss of PTEN by different genetic alterations.** a, Heatmap of the non-silent genetic alterations across the primary tumour and the 14 metastases (M) collected during the autopsy of the index patient. Gene mutations are depicted in green, gene amplifications in red, and gene copy number loss in blue. b, Dendrogram showing the proposed phylogenetic evolution of the metastases in the index patient. Shaded circles represent metastatic lesions with bi-allelic loss of PTEN and lack of PTEN expression by IHC.

| Table 1 | Patient information |
|----------------|------------------|
| Patient Site  | PIK3CA baseline | Dose (mg) | Response | DOT (days) | PTEN | PIK3CA Post-T |
| Index         | breast          |          |          |            |      |              |
| 1             | Breast          | E542K    | 400      | PR (-52.4%) | 285  | Loss | E542        |
| 2             | Breast          | H1047R   | 400      | SD*         | 181  | Unch. | H1047R      |
| 3             | Breast          | H1047R   | 400      | SD (-26.3%) | 424  | Loss | H1047R      |
| 4             | Breast          | H1047R   | 400      | SD (-28.5%) | 179  | Unch. | WT          |
| 5             | Breast          | H1047L   | 400      | SD (-11.3%) | 504  | Unch. | H1047L      |
| 6             | Breast          | H1047R   | 400      | SD (-24.9%) | 110  | Unch. | H1047R      |
| 7             | Salivary        | E545K    | 400      | SD (-17%)   | 112  | Unch. | WT          |

* With internal decrease in left breast and left chest wall skin thickening.

**RECIST, Response Evaluation Criteria in Solid Tumors; DOT, duration of treatment; PR, partial response; SD, stable disease; Unch., unchanged; WT, wild-type; Amp., amplified; Post-T, post-treatment.**
that were visualized by CT (computerized tomography) scan, there was a tight correlation between progression of disease and loss of PTEN expression. The peri-aortic lesion (M02) that was responding at the time of disease progression still contained one PTEN wild-type allele and protein expression. Conversely, the lung lesions (M04, M06, M09 and M11) and liver lesion (M12) with documented progression to therapy had bi-allelic PTEN alteration and lack of expression.

In an effort to integrate the genomic data from our patient, we constructed a dendrogram mapping the phylogenetic evolution of the disease. Our findings suggest that all the lesions were derived from the PTEN wild-type primary tumour, and that there was a progressive and parallel loss of PTEN under BYL719 selective pressure (Fig. 3b). Of note, the two-month duration between progression to BYL719 and autopsy needs to be considered as well.

In order to expand our observations, we analysed paired samples (pretreatment and at progression) from six additional patients enrolled in the same study at our institution (Table 1). Targeted sequencing identified homozygous loss of PTEN in a post-treatment sample of a breast cancer patient who developed resistance to BYL719 after initially experiencing a durable response to therapy (Table 1). We also confirmed lack of PTEN expression by IHC in the post-treatment sample (Extended Data Fig. 6). We found no detectable PIK3CA mutations in the post-treatment samples of two patients (Table 1). Given that the presence of PIK3CA mutations drove sensitivity to BYL719 in our cell line screens17, positive selection of clones bearing wild-type alleles of PIK3CA may explain the emergence of resistance to BYL719 in these two additional cases. These results may be an indication that in some cases PIK3CA mutations are not early founder/truncal events but branched subclonal drivers that are cleared from the tumours under the selective pressure of PI(3)K inhibition. In any case, the fact that loss of PTEN expression and emergence of PIK3CA wild-type clones are mutually exclusive in our patient samples indicates that both events may be important in opposing the therapeutic efficacy of BYL719.

No other alterations with an obvious connection to BYL719 resistance were found in the responding cases, with the exception of a mutation (E1490*) and an in-frame deletion in MAP3K1 in one of the three patients for whom neither PTEN nor PIK3CA status changed during BYL719 treatment. Further characterization is needed to determine whether these mutations lead to increased MEK and ERK signalling and limit the effects of PI(3)K inhibition.

PTEN encodes for a phosphatase that regulates the activity of PI(3)K by limiting the accumulation of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3 or PIP_3), a required mediator to initiate the PI(3)K/AKT/mTOR signalling cascade18. In the absence of PTEN, cancer cells become dependent mostly on the activity of the p110β isof orm of PI(3)K (PI(3)Kβ) to propagate signalling through downstream pathway

Figure 4 | Loss of PTEN expression and sensitivity to PI(3)Kα and PI(3)Kβ blockade. a, Western blot showing PTEN knockdown by two independent shRNAs and its effects on the PI(3)K/AKT/mTOR pathway. b, Cell viability assay in T47D cells with inducible PTEN knockdown (shPTEN no. 1 and no. 2) or PTEN expressing controls (shRenilla) treated with increasing concentrations of BYL719 or BKM120. Error bars, s.e.m. c, Antitumour activity of either BYL719 (25 mg kg\(^{-1}\) daily) or BKM120 (25 mg kg\(^{-1}\) daily) in PDXs subcutaneously grown in nude mice (n = 6 (vehicle) and n = 8 (treatments)). Error bars, s.e.m. d, Representative immunostaining for phosphorylated AKT (pAKT) and phosphorylated S6 (pS6) in PDXs treated as shown. Tumours were collected at the end of the experiment of c, 2 h after the last dosage. Scale bar, 100 μm.  

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effector(s). Therefore, we hypothesized that progressive decrease or loss of PTEN expression in the presence of PI(3)Kx inhibition might restore PI(3)K/akt signalling through PI(3)Kp activity. To test our hypothesis, we established cell lines expressing either doxycycline-inducible or constitutive short hairpin (sh)RNA against PTEN messenger RNA using T47D and MCF7 cells, known to be intrinsically sensitive to BYL719. As expected, induction of PTEN downregulation led to activation of AKT and the downstream effectors PRAS40, GSK3β, and S6 in both T47D (Fig. 4a) and MCF7 (data not shown) cells under basal conditions. PTEN downregulation markedly limited the effects of BYL719, both at the signalling and cell viability level. On the other hand, PTEN knockdown did not result in resistance to the pan-PI(3)K inhibitor BKM120, which blocks all the PI(3)K p110 isoforms (Fig. 4b and Extended Data Fig. 7a). Similar effects were observed in another BYL719-sensitive cell line (MDA-MB-453) with constitutive PTEN knockdown (Extended Data Fig. 7b).

From our patient's non-responding PTEN-null lung metastatic lesion, we were able to establish xenografts in nude mice. Consistent with the in vitro data, this patient-derived xenograft (PDX) was resistant to BYL719 treatment but sensitive to BKM120 (Fig. 4c). The degree of inhibition of phospho-AKT and phospho-S6 was also higher with BKM120 (Fig. 4d and Extended Data Fig. 7c and d). These results were complemented by the combination of BYL719 and the PI(3)K inhibitor AZD6482. Upon PTEN knockdown, only combined PI(3)Kz and β blockade was capable of reverting the resistant phenotype (Fig. 4e and Extended Data Fig. 8a). Similarly, the BYL719-resistant PDX was insensitive to AZD6482 alone but responded to the combination of both compounds (Fig. 4f). Profound inhibition of AKT and S6 phosphorylation was achieved only upon treatment with BYL719 in combination with AZD6482 (Fig. 4g and Extended Data Fig. 8b and c). Taken together, these data indicate that inhibition of the PI(3)Kβ isoform is required to achieve antitumour activity in cells/tumours that lost PTEN expression and become resistant to BYL719.

We have reported a case of parallel genetic evolution under selective therapeutic pressure leading to a progressive loss of PTEN expression and consequent gain of dependency on the PI(3)Kβ isoform. Parallel evolution under selective pressure has been described in conditions where treatments are highly efficacious, such as in HIV. Our case highlights that this tumour, despite its heterogeneity, was dependent on PI(3)K signalling, probably as a result of the presence of the same activating PIK3CA mutation in all the tumour sites. Upon continued suppression of PI(3)Kz, diverse genomic alterations emerged, leading to PTEN loss as an alternative mechanism of PI(3)K activation. Moreover, our study emphasizes the importance of tumour interrogation upon progression to therapy and the dynamic nature of tumour genomes under selective therapeutic pressure.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions D.J., P.C., M.F.B., J.B. and M.S. conceived the project, designed and analysed the experiments, and wrote the manuscript. M.G., O.L.G., B.J.A., A.R. and E.R.M. performed and analysed the WGS and WES data. T.H., M.M.-K., D.S., L.A.T., L.E., C.O., M.P., A.D., R.B. and A.H. collected and analysed patients’ samples. P.C., H.-E. S.H.E. and S.W.L. performed and supervised the laboratory experiments. H.H.W., G.I., R.H.S., D.B.S. and M.F.B. performed and supervised the IMPACT sequencing and analysis.

Author Information DNA sequences have been deposited in the European Genome-phenome Archive with accession number EGAS00001000991. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.S. (scatirim@mskcc.org), J.B. (basegieja@mskcc.org) or M.F.B. (bergerm1@mskcc.org).
METHODS

PIK3CA mutant cell lines MCF7 (E545K) and T47D (H1047R) (ATCC) were transduced with the retroviral TRMPV vector. Doxycycline (Sigma) was used to temporally activate the expression of a microRNA 30-embedded shRNA targeting Renilla luciferase (control) or PTEN mRNA. The hairpin sequences used were as follows.

**Renilla luciferase.** CTCGAGAAGGTATATTTGCTTTGACATGAGCCGAGAATTTAATGCTTATCTATATAGTGAAAGCGATGATAGTAGATCGAGTCCTGACAGTTGAGCTGCCTACTGCCTGCGAATTC

**PTEN no. 1.** CTCGAGAAGGTATATTTGCTTTGACATGAGCCGAGAATTTAATGCTTATCTATATAGTGAAAGCGATGATAGTAGATCGAGTCCTGACAGTTGAGCTGCCTACTGCCTGCGAATTC

**PTEN no. 2.** CTCGAGAAGGTATATTTGCTTTGACATGAGCCGAGAATTTAATGCTTATCTATATAGTGAAAGCGATGATAGTAGATCGAGTCCTGACAGTTGAGCTGCCTACTGCCTGCGAATTC

Cell viability was assessed using the tetrazolium-based MTT assay after 6 days of treatment. All cell lines resulted negative for mycoplasma contamination. Western blotting was carried out using previously described methods21. All the in vitro experiments were performed in triplicate.

**Patient-derived xenografts and IHC.** Animals were maintained and treated in accordance with Institutional Guidelines of Memorial Sloan Kettering Cancer Center (Protocol number 12-10-019). Tumours were implanted subcutaneously in accordance with Institutional Guidelines of Memorial Sloan Kettering Cancer Center Institutional Review Board. Autopsy in the index patient was performed within the first three hours post mortem.

**Targeted exome sequencing (IMPACT).** DNA derived from the primary tumour, 14 metastases, and matched normal spleen tissue was further subjected to deep-coverage targeted sequencing of key cancer-associated genes. Our assay, termed IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets), involves hybridization of barcoded libraries to custom oligonucleotides (Nimblegen SeqCap) designed to capture all protein-coding exons and select introns of 279 commonly implicated oncogenes, tumour suppressor genes, and members of pathways deemed actionable by targeted therapies14. The captured pool was subsequently sequenced on an Illumina HiSeq 2500 as paired-end 75-base pair reads, producing 513-fold coverage per tumour. Sequence data were analysed to identify three classes of somatic alterations: single-nucleotide variants, small insertions/deletions (indels), and copy number alterations.

Barcoded sequence libraries were prepared using 250 ng genomic DNA (Kapa Biosystems) and combined in a single equimolar pool. Sequence data were demultiplexed using CASAVA, and reads were aligned to the reference human genome (hg19) using BWA and postprocessed using the Genome Analysis Toolkit (GATK) according to GATK best practices25.

MuTect and GATK were used to call single-nucleotide variants and small indels, respectively26. Exon-level copy number gains and losses were inferred from the ratio in Tumour:Normal sequence coverage for each target region, following a loss-normalization to adjust for the dependency of coverage on GC content27.

**Statistical analysis.** Two-way t-tests were performed using GraphPad Prism (GraphPad Software). Error bars represent the s.e.m. *P* < 0.05. All cellular experiments were repeated at least three times. All the in vivo experiments were run with at least 6–8 tumours for each treatment arm. Sample size was chosen to detect a difference in means of 20% with a power of 90%. Animals were randomized in groups with similar average in tumour size. Investigators were blinded when assessing the outcome of the in vivo experiments.

For the cell viability graphs, nonlinear regression was applied to the experimental data sets. Curves were compared using the extra-sum-of-squares F test using *x* = 0.05. Hypothesis was rejected when nonlinear models were not nested within each other and was considered statistically significant.

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Extended Data Figure 1 | CT scan of index patient. CT scan showing a liver lesion (baseline) experiencing a partial response after 8 cycles (cycle 8) of BYL719.
Extended Data Figure 2 | Gene copy number variation in both primary tumour and lung metastasis.
Extended Data Figure 3 | Representative exon-level copy number profiles for genes on chromosome 10 in all 14 metastases collected from the index patient. Exons in PTEN are shown in red.
Extended Data Figure 4 | Loss-of-function mutations in PTEN detected by IMPACT in metastases M06 and M10. Mutations were visualized by the Integrative Genomics Viewer (IGV).
Extended Data Figure 5 | PTEN immunostaining of the 14 metastases collected during the autopsy. Haematoxylin and eosin (H&E) and PTEN expression detected by IHC in 14 metastases collected during the autopsy of the index patient. PTEN staining in PTEN negative samples is only present in stromal cells.
Extended Data Figure 6 | PTEN immunostaining in patients treated with BYL719. PTEN expression detected by IHC in paired samples from six additional patients treated with BYL719. Specimens before starting BYL719 therapy (baseline) and at time of disease progression (post-treatment) are compared.
Extended Data Figure 7 | Inducible loss of PTEN and sensitivity to BYL719 and BKM120.

**a.** Cell viability assay in MCF7 cells with inducible PTEN knockdown treated with increasing concentrations of either BYL719 or BKM120. Error bars, s.e.m.

**b.** Cell viability assay in MDA-MB-453 (MDA453) cells with constitutive PTEN knockdown treated with increasing concentrations of either BYL719 or BKM120. Error bars, s.e.m.

**c.** Quantification of pAKT (S473) and pS6 (S240/4) from Fig. 4d. Student’s t-test was used and P values are indicated.

**d.** Western blot from the PDXs treated as indicated.
Extended Data Figure 8 | Constitutive loss of PTEN and sensitivity to BYL719 and AZD6482. a, Cell viability assay in T47D cells with inducible PTEN knockdown (no. 2) treated with increasing concentrations of either BYL719 or AZD6482 in the presence of doxycycline 1 \( \mu \text{g ml}^{-1} \). Error bars, error bars. b, Quantification of pAKT (S473) and pS6 (S240/4) from Fig. 4g. Student’s t-test was used and \( P \) values are indicated. Error bars, s.e.m. c, Western blot from the PDXs treated as indicated.
## Extended Data Table 1 | Samples analysed from the index patient

| Lesion       | Location              | Cellularity | CT scan | Response | WGS | WES | IMPACT |
|--------------|-----------------------|-------------|---------|----------|-----|-----|--------|
| Primary      | Breast                | Unknown     | Y       | Y        | Y   | N   | Y      |
| M01          | Ovary                 | 75%         | N       | Y        | N   | N   | Y      |
| M02          | Periaortic lymph node | 50%         | Y       | Y        | Y   | Y   | Y      |
| M03          | Liver (posterior)     | 65%         | N       | Y        | N   | N   | Y      |
| M04          | Left Lung (lower lobe)| 75%         | Y       | Y        | N   | N   | Y      |
| M05          | Thoracic spine        | 65%         | N       | Y        | N   | N   | Y      |
| M06          | Right Lung (upper lobe)| 55%       | Y       | Y        | N   | N   | Y      |
| M07          | Liver (dome)          | 70%         | N       | Y        | N   | N   | Y      |
| M08          | Uterus                | 55%         | N       | Y        | N   | N   | Y      |
| M09          | Left Lung (upper lobe)| 75%         | Y       | Y        | N   | N   | Y      |
| M10          | Carina (lymph node)   | 70%         | Y       | Y        | N   | N   | Y      |
| M11          | Right Lung (lower lobe)| 65%       | Y       | Y        | N   | N   | Y      |
| M12          | Liver (left lower lobe)| 50%       | Y       | Y        | N   | N   | Y      |
| M13          | Liver (left lobe)     | 70%         | N       | Y        | N   | N   | Y      |
| M15          | Adrenal gland         | 40%         | N       | Y        | N   | N   | Y      |

Summary of the lesions collected during the autopsy of the index patient, cellularity assessment, imaging, clinical outcome, and sequencing techniques used. N, no; Y, yes.