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Comparative Lesions Analysis Through a Targeted Sequencing Approach

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Corresponding Author:	Vincenzo Corbo, Ph.D. University of Verona Verona, Veneto ITALY
Corresponding Author's Institution:	University of Verona
Corresponding Author E-Mail:	vincenzo.corbo@univr.it
Order of Authors:	Caterina Vicentini
	Andrea Mafficini
	Michele Simbolo
	Matteo Fassan
	Pietro Delfino
	Rita Teresa Lawlor
	Borislav Rusev
	Aldo Scarpa
	Vincenzo Corbo, Ph.D.
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UNIVERSITÀ di VERONA
Dipartimento di DIAGNOSTICA E SANITÀ PUBBLICA

To Anna Justis, Ph.D

Science Editor

JoVE

May 28th, 2019

Dear Editor,

Enclosed please find our revised manuscript entitled "*Comparative lesions analysis through a targeted sequencing approach*".

We have addressed all editorial comments including:

i) providing a link to the editorial policy of Journal of Pathology, where it is indicated that prior permission is not required for reuse of the content of our own publication. A sentence has been added to figure legends to acknowledge the source of original content
ii) removing commercial language from the manuscript and referencing commercial products in the Table of Materials
iii) including more details to our protocol steps

We thank the reviewers for their positive and supportive comments and their informative critique. We are confident that we have addressed the issues raised and feel that this has markedly improved the quality of the manuscript.

We have addressed the editorial and reviewers' comments in the revised version of the manuscript and uploaded the point-by-point response. A copy of the manuscript where changes are indicated in blue coloured font (added text) and as grey-crossed out (eliminated text) has been uploaded as Supplemental File for Review but NOT for Publication.

We also provide a point-by-point response to editorial comments as Supplemental File.

We hope you will find our revised manuscript suitable for publication and look forward to hearing from you.

Sincerely yours

Vincenzo Corbo, PhD
Assistant Professor of Molecular Oncology
University of Verona, 37134, Verona, Italy.
Phone: +39 0458124830
Fax: +39 0458127432

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TITLE:

Comparative Lesions Analysis Through a Targeted Sequencing Approach

AUTHORS AND AFFILIATIONS:

Caterina Vicentini[1], Andrea Mafficini[1], Michele Simbolo[2], Matteo Fassan[3], Pietro Delfino[2], Rita Teresa Lawlor[1], Borislav Rusev[1], Aldo Scarpa[1,2], Vincenzo Corbo[1,2]

[1]ARC-Net Research Centre, University and Hospital Trust of Verona, Verona, Italy
[2]Department of Diagnostics and Public Health, Section of Pathology, University and Hospital Trust of Verona, Verona, Italy
[3]Department of Medicine (DIMED), Surgical Pathology and Cytopathology Unit, University of Padua, Padua, Italy

Email addresses of co-authors:

Caterina Vicentini (caterinavicentini@hotmail.it)
Andrea Mafficini (andrea.mafficini@univr.it)
Michele Simbolo (simbolo.michele@hotmail.it)
Matteo Fassan (matteo.fassan@unipd.it)
Pietro Delfino (pietro.delfino@univr.it)
Rita Teresa Lawlor (ritateresa.lawlor@univr.it)
Borislav Rusev (borislavchavdarov.rusev@univr.it)
Aldo Scarpa (aldo.scarpa@univr.it)

Corresponding author:

Vincenzo Corbo (vincenzo.corbo@univr.it)

KEYWORDS:

intra-tumor heterogeneity, metastases, biomarkers, clonal, targeted sequencing, immunohistochemistry, “driver” alterations, subclonal

SUMMARY:

This article describes a method to identify clonal and subclonal alterations among different specimens from a given patient. Although the experiments described here focus on a specific tumor type, the approach is broadly applicable to other solid tumors.

ABSTRACT:

Assessing intra-tumoral heterogeneity (ITH) is of paramount importance to anticipate failure of targeted therapies and design accordingly effective anti-tumor strategies. Although concerns are frequently raised due to differences in sample processing and depth of coverage, next-generation sequencing of solid tumors have unraveled a highly variable degree of ITH across tumor types. Capturing the genetic relatedness between primary and metastatic lesions through the identification of clonal and subclonal populations is critical to the design of therapies for advance-stage diseases. Here, we report a method for comparative lesions analysis that allows for the identification of clonal and subclonal populations among different specimens from the same

patient. The experimental approach described here integrates three well-established approaches: histological analysis, high-coverage multi-lesion sequencing, and immunophenotypic analyses. In order to minimize the effects on the detection of subclonal events by inappropriate sample processing, we subjected tissues to careful pathological examination and neoplastic cell enrichment. Quality controlled DNA from neoplastic lesions and normal tissues was then subjected to high coverage sequencing, targeting the coding regions of 409 relevant cancer genes. While only looking at a limited genomic space, our approach enables evaluating the extent of heterogeneity among somatic alterations (single-nucleotide mutations and copy-number variations) in distinct lesions from a given patient. Through comparative analysis of sequencing data, we were able to distinguish clonal vs. subclonal alterations. The majority of ITH is often ascribed to passenger mutations; therefore, we also used immunohistochemistry to predict functional consequences of mutations. While this protocol has been applied to a specific tumor type, we anticipate that the methodology described here is broadly applicable to other solid tumor types.

INTRODUCTION:

The advent of next generation sequencing (NGS) has revolutionized the way cancers are diagnosed and treated[1]. NGS coupled to multiregional sequencing have exposed a high degree of intra-tumoral heterogeneity (ITH) in solid tumors[2], which explains in part the failure of targeted therapy due to the presence of subclones with differing drug sensitivity[2]. An important challenge posed by genome-wide sequencing studies is the necessity to distinguish between *passenger* (i.e., neutral) and *driver* mutations in individual cancers[3]. Several studies have indeed shown that, in certain tumors, passenger mutations account for the majority of ITH, while driver alterations tend to be conserved among lesions of the same individual[4]. It is also important to note that large mutational burden (as seen in lung cancers and melanoma) does not necessarily imply a large subclonal mutational burden[2]. Therefore, a high degree of ITH can be found in tumors with low mutational burden.

Metastases are responsible for more than 90% of cancer-related death worldwide[5]; therefore, capturing the mutational heterogeneity of *driver* genes among primary and metastatic lesions is critical to the design of effective therapies for advanced-stage diseases. Clinical sequencing is generally performed on nucleic acids from fixed tissues, which renders genome-wide exploration difficult because of poor DNA quality. On the other hand, the intent of clinical sequencing is to identify actionable mutations and/or mutations that might predict responsiveness/unresponsiveness to a given therapeutic regimen. As it stands, sequencing can be restricted to a smaller fraction of the genome for timely extraction of clinically relevant information. The transition from low-throughput DNA profiling (e.g., Sanger Sequencing) to NGS has rendered it possible to analyze hundreds of cancer-relevant genes at a high depth of coverage, which allows for the detection of subclonal events. Here, we report a method for comparative lesions analysis that allows for the identification of clonal and subclonal populations among different specimens from the same individual. The method described here integrates three well-established approaches (histological analysis, high-coverage multi-lesion sequencing, and immunophenotypic analyses) to predict functional consequences of the variations identified. The approach is schematically described in **Figure 1** and has been applied to the study of 5

metastatic cases of solid pseudopapillary neoplasms (SPNs) of the pancreas. While we describe processing and analysis of formalin-fixed paraffin-embedded (FFPE) tissue specimens, the same procedure can be applied to genetic material from fresh-frozen tissue.

PROTOCOL:

The material used in the study was collected under a specific protocol, which was approved by the local ethics committee. Written informed consent from all patients was available.

1. Histological and immunophenotypical revision of tissue specimens

NOTE: An expert pathologist is responsible for activities described hereafter.

1.1. Histopathological revision of selected cases according to well-established diagnostic criteria.

1.1.1. Use the microtome to cut 4−5 µm thick tissue sections from representative FFPE tissue blocks and mount the sections on standard histology slides.

1.1.2. Perform hematoxylin and eosin staining for each slide using an automated tissue slide stainer (**Table of Materials**).

1.1.3. Review the histopathological diagnosis of the selected tumor cases according to WHO diagnostic criteria.

NOTE: At this stage, the pathologist might identify morphologically distinct areas of the tumor within the same tissue section. It is possible to harvest those areas separately (see section 2). Histological resemblance of tumor and normal cells for SPNs is provided in **Figure 2**.

1.1.4. Perform immunohistochemical staining for established markers to complement histological analysis and estimate immunophenotypic heterogeneity.

NOTE: The pathologist might identify immunophenotypically distinct areas of the tumor within the same tissue section. It is possible to harvest those areas separately (see section 2).

1.2. Evaluate neoplastic cellularity of the tumor tissue section and plan manual microdissection accordingly.

NOTE: This is a pathologist-generated estimate of tumor cellularity.

1.2.1. If neoplastic cell content of the tissue section is higher than 70%, manual microdissection is not mandatory; move directly to step 3.1.

NOTE: Target 70% of neoplastic cell content in order to (i) ensure adequate sensitivity for mutant allele frequency estimates and (ii) to possibly validate clonal mutations by less sensitive

methodologies (e.g., capillary sequencing).

1.2.2. If neoplastic cell content of the tissue section is lower than 70%, microdissection is necessary and move to step 2.

1.3. Evaluate tissue sections from the FFPE block where non-neoplastic tissue has been sampled. This tissue is used as a source of germline DNA.

NOTE: Blood is an alternative source of germline DNA. In this case, proceed with DNA extraction as recommend in the note of step 4.1.

1.3.1. If only non-neoplastic tissue is visible in the tissue section (**Figure 2D**), manual microdissection is not needed; move directly to step 3.1.

1.3.2. If substantial contamination from neoplastic cells is present in the tissue section, then manual microdissection is necessary; move to section 2.

2. Manual microdissection

NOTE: This method is applicable to various solid tumor types, and it is intended to increase neoplastic cells content of tissue specimens. Alternatively, this method can be used to harvest morphologically and/or immunophenotypically distinct areas within the same tissue section.

2.1. Using a microtome, cut up to ten 4−6 µm thick FFPE tumor tissue sections and mount them on uncharged slides.

NOTE: If only one 1 mm^2 nest of cancers cells is visible in the specimen, 10 tissue slides should be cut and subjected to manual microdissection in order to obtain the targeted amount of DNA (40 ng); this assuming that 1 mm^2 cluster contains between 700 and 1000 tumor nuclei.

2.2. Incubate slides at 60 °C for 10 min.

2.3. Place the slides in a rack, and perform the following washes: xylene for 20 min, 100% ethanol for 10 min, 80% ethanol for 10 min, 70% ethanol for 10 min, distilled water for 1 min, hematoxylin counterstain for 10 s, tap water for 1 min.

2.4. On a standard inverted microscope, use a 27 G needle on a syringe as the microdissection tool and collect representative clusters of tumor cells. Harvest a minimum of ten 1 mm^2 clusters of cells to ensure the required amount of DNA for sequencing.

NOTE: If morphologically distinct areas are intended to be analyzed as different entities, then use the microdissection tool to harvest those areas separately.

2.5. Place harvested clusters in 1.5 mL tubes (**Table of Materials**) previously filled with 20 µL of protease K and 180 µL of lysis buffer (**Table of Materials**, both included in the DNA extraction kit) and mix by vortexing.

3. Processing of tissues without prior microdissection

NOTE: This procedure is used for tissue blocks that contain only non-neoplastic cells (source of germline DNA) or contain at least 70% of morphologically homogenous cancer cells.

3.1. Using a microtome cut up to six 4−5 µm thick tissue sections from selected FFPE tissue blocks. Place tissue scrolls in 1.5 mL tubes as described in step 2.5.

4. DNA extraction from normal and neoplastic cells

4.1. Purify DNA from normal and neoplastic tissue using the DNA FFPE tissue extraction kit (**Table of Materials**) according to manufacturer's instructions.

NOTE: When blood is used as a source of germline nucleic acid, purify DNA using a DNA blood extraction kit (**Table of Materials**).

4.2. DNA quantification and quality check

4.2.1. To quantify the amount of double-stranded (dsDNA), add an aliquot of the DNA sample to a solution containing a fluorescent nucleic acid stain (**Table of Materials**) and measure emitted fluorescence using a benchtop fluorometer (**Table of Materials**).

NOTE: The assay measures the intensity of the fluorescent signal emitted from fluorescent dyes attached to dsDNA and determines the amount of DNA using a standard curve.

4.2.2. Use a microvolume spectrophotometer (**Table of Materials**) to measure the 260/280 and 260/230 ratios of the sample in order to qualify DNA. "Pure" DNA should have a 260/280 of 1.8 and a 260/230 in the range of 1.8−2.2.

NOTE: Spectrophotometric evaluation of the sample is intended to evidence the presence of chemical contaminants (e.g., phenol) that impact downstream reactions. In the event of contamination due to chemical reagents, perform a cleanup step using a column-based kit.

5. Library preparation and quantification

NOTE: The schematic flowchart of library preparation and quantification steps is reported in **Figure 3**.

5.1. DNA library preparation (4 primer pools set up for each sample)

NOTE: The 4 primer pools belong to the cancer panel reported in **Table of Materials**. Each pool contains primer pairs that are designed for a multiplexed target selection of 409 genes. A link to the list of genes composing the NGS panel is provided in **Table of Materials**.

5.1.1. Prepare four DNA target amplification reactions for each sample, one per primer pool. For each primer pool (**Table of Materials**), add in a 0.2 mL tube (**Table of Materials**): 4 µL of master mix (**Table of Materials**, included in the library kit), 10 µL of high fidelity primer pool mix (**Table of Materials**, included in the cancer panel library), and 6 µL of DNA (10 ng total).

5.1.2. Amplify target regions of each primer pool separately in four 1.5 mL tubes running the following program: hold at 99 °C for 2 min (activation of the hot-start polymerase), 16 cycles (denature at 99 °C for 15 s, anneal and extend at 60 °C for 8 min), hold at 4 °C.

NOTE: Stopping point. Store target amplification reactions at 4 °C overnight or at -20 °C for longer time.

5.1.3. Partial digestion of amplicons ends

NOTE: The manual provided by the NGS manufacturer kit foresees a step in which the different amplification reactions from each sample are combined before partial digestion. Avoid that step and keep processing each amplification digestion separately.

5.1.3.1. Add 2 µL of specific digestion mix (**Table of Materials**, included in the library kit) to each amplified pool of each sample (the total volume of each 0.2 mL tube is 22 µL). Vortex thoroughly and centrifuge each tube to collect droplets.

5.1.3.2. Load the tubes into the thermal cycler and run the following program: 50 °C for 10 min, 55 °C for 10 min, 60 °C for 20 min, 10 °C hold (for up to 1 h).

NOTE: Stopping point. Store plate at -20 °C for longer periods.

5.1.4. Ligate adapters to amplicons and purify.

NOTE: Use a different barcode adapter for each sample when sequencing multiple libraries on a single chip. All four amplification reactions from the same sample must receive the same barcode.

5.1.4.1. Prepare adapters (**Table of Materials**, barcodes adapters kit). For each barcode X, prepare a mix of P1 adapter and unique barcode adapters (**Table of Materials**) at a final dilution of 1:4. Mix 4 µL of water with 2 µL of P1 adapter and 2 µL of unique barcode adapters. Use 2 µL of this barcode adapter mix for downstream passages.

5.1.4.2. Perform the ligation reaction by adding to each amplified pool of each sample in this order: 4 µL of switch solution (**Table of Materials**, included in the library kit), 2 µL of barcode

adapter mix and 2 µL of DNA ligase (**Table of Materials**, included in the library kit) (total volume = 30 µL).

5.1.4.3. Vortex thoroughly and briefly centrifuge to collect droplets.

5.1.4.4. Load each tube into the thermal cycler and run the following program: 22 °C for 30 min, 68 °C for 5 min, 72 °C for 5 min, 4 °C hold (for up to 24 h).

NOTE: Stopping point. Store plate at -20 °C for longer periods.

5.1.5. Library purification and amplification

5.1.5.1. Centrifuge each tube and then transfer each barcoded sample in a 1.5 mL tube. Add 45 µL of beads-based purification reagent (**Table of Materials**) to each pool of each sample. Pipet up and down 5x to mix the bead suspension with the DNA.

5.1.5.2. Incubate the mixture for 5 min at room temperature (RT), then place each tube in a magnetic rack and incubate for 2 min. Carefully remove and discard supernatant without disturbing the pellet.

5.1.5.3. Add in each tube 150 µL of fresh 70% ethanol. Wash the beads moving each tube side-to-side of the two positions of the magnet. Discard the supernatant and pay attention not to disturb the pellet.

5.1.5.4. Repeat procedures described in step 5.1.5.3 and then keep the tubes in the magnet and air-dry the beads at RT for 5 min.

5.1.5.5. Remove tubes with purified libraries of each primer pool from the magnet and add 50 µL of high-fidelity PCR Mix (**Table of Materials**) and 2 µL of library amplification primer mix (**Table of Materials**, included in the library kit) to the beads pellet of each tube.

5.1.5.6. Vortex each 1.5 mL tube and briefly centrifuge to collect droplets.

5.1.5.7. Place 1.5 mL tubes in the magnet for 2 min and carefully transfer the supernatant (~50 µL) from each tube to a new 0.2 mL tube without disturbing the pellet.

5.1.5.8. Amplify libraries of each primer pool running the following program: hold at 98 °C for 2 min to activate the enzyme, 5 cycles (denature at 98 °C for 15 s, anneal and extend at 64 °C 1 min), hold at 4 °C. Store samples at -20 °C.

5.1.6. Purification of amplified library

5.1.6.1. Centrifuge each tube to collect the contents at the bottom and transfer each amplified library sample to a 1.5 mL tube.

5.1.6.2. Add 25 µL of beads-based purification reagent. Pipet up and down 5x to mix the bead suspension with the DNA.

5.1.6.3. Incubate the mixture for 5 min at RT, and then place tubes into the magnet for 5 min.

5.1.6.4. Carefully transfer the supernatant, which contains the amplicons, from each tube to new tubes without disturbing the pellet.

5.1.6.5. Add 60 µL of beads-based purification reagent to the supernatant of each tube and pipet up and down in order to mix bead suspension and DNA.

5.1.6.6. Incubate the mixture for 5 min at RT and then place the tubes in the magnet for 3 min.

5.1.6.7. Discard the supernatant from each tube and pay attention not to disturb the pellet.

NOTE: The amplicons are bound to the beads.

5.1.6.8. Add in each tube 150 µL of fresh 70% ethanol. Wash the beads moving the tubes side-to-side in the two positions of the magnet. Discard the supernatant and pay attention not to disturb the pellet.

5.1.6.9. Repeat the procedure in step 5.1.6.8 and air dry the beads at RT for 3 min. Avoid excessive drying of the beads.

5.1.6.10. Remove the tubes from the magnet and add 50 µL of low Tris EDTA (TE) (**Table of Materials**, included in the library kit) to the pellet to disperse the beads.

5.1.6.11. Pipet the mixture up and down several times and incubate at RT for 2 min.

5.1.6.12. Place the tubes in the magnet for at least 2 min and carefully transfer the supernatant, which contains the amplicons, to new tubes without disturbing the beads.

5.2. Library quantification

5.2.1. Use a fragment analysis instrument (**Table of Materials**) to run a DNA chip according to high sensitivity DNA kit protocol.

6. Libraries pooling and sequencing run

6.1. Dilute each library to 100 pM with nuclease-free water. Combine 10 µL of each 100 pM libraries for a single run in a single tube. Mix the combined libraries by pipetting and proceed to templating and sequencing.

6.2. Planned run creation

NOTE: A typical run includes four samples that can be loaded on two different types of semiconductor chip (Ion PI chip or Ion 540 chip) based on the sequencer available in the laboratory (Ion Proton System or GeneStudio S5 System, **Table of Materials**). These systems typically produce 80 million reads per run.

6.2.1. Open the Torrent Browser on a computer connected to the sequencing system (e.g., Ion Chef System) and plan a new run using the generic template for the selected application (AmpliSeqDNA).

6.2.2. Switch to the **Kits** tab of the plan. Select **Ion Proton System** or **Ion GeneStudio S5 System** from the instrument dropdown list based on the sequencing system being used.

6.2.3. Depending on the sequencing system, select the appropriate chip (PI chip for Ion Proton Systems; 540 chip for Ion GeneStudio S5 Systems) from the **Chip Type** dropdown list.

6.2.4. Select **Ion AmpliSeq 2.0 Library Kit** from the **Library Kit** dropdown list.

6.2.5. Select the **Ion Chef** button for Template Kit, then select the appropriate **Chef Kit** (Ion PI Hi-Q Chef Kit for PI chip or Ion 540 Kit-Chef for 540 chip) from the **Template Kit** dropdown list.

6.2.6. Select the appropriate sequencing kit (Ion PI Hi-Q Sequencing 200 Kit for PI chip or Ion S5 Sequencing Kit for 540 chip) from the **Sequencing Kit** dropdown list, then select **IonXpress** from the Barcode Set dropdown list.

6.2.7. Switch to the **Plugin** tab and select the **Coverage Analysis** plugin.

6.2.8. Switch to the Plan tab. Select the GRCh37/hg19 genome from the Reference Library dropdown list. From the **Target Regions** dropdown list, select the appropriate panel to be sequenced.

6.2.9. Set the number of barcodes to be sequenced and the label of the library sample tubes into the appropriate fields.

6.2.10. Set the barcode name and sample name for each library.

6.3. Sample libraries dilution and loading.

6.3.1. Dilute the stock library with nuclease-free water to 25 pM for PI chip or 32 pM for 540 chip.

6.3.2. Pipet 50 µL of each diluted library to the bottom of the appropriate library sample tube.

6.3.3. Turn on the sequencing system and follow the on-screen instructions to load all required

reagents and to import the run plan.

6.3.4. Load the library sample tube containing the pooled libraries and start clonal amplification program.

NOTE: The Ion Chef System requires two chips to be prepared per run.

6.4. Sequencing on Ion Proton.

6.4.1. Turn on the sequencing system and follow the on-screen instructions to initialize the sequencing and to import the run plan.

6.4.2. Load the sequencing chip after removing it from the sequencing system .

NOTE: Be grounded before picking up a chip in order to avoid chip damage due to electrostatic discharge. Chip handling/positioning along with examples of successful/unsuccessful loading are reported in **Figure 4**.

6.4.3. Close the chip compartment lid and wait until the **Chip Status** icon indicates "Ready".

6.4.4. Empty the waste container when the first run is complete, then sequence the remaining chip as soon as possible.

7. **Mutations and copy-number variations (CNVs) analysis**

NOTE: Alignment of sequencing data to the GRCh37/hg19 human reference genome is automatically performed once set in the Plan (step 6.2.8).

7.1. Use the Coverage Analysis plugin (**Table of Materials**) output to verify depth of coverage and uniformity.

7.2. Perform the variant calling using the Torrent variant caller plugin (**Table of Materials**), selecting the **germline** or **somatic** workflow as appropriate.

7.3. Download filtered variants Variant Call Format (VCF) files. Annotate variants using the Variant Effect Predictor (VEP) software[6] and NCBI RefSeq database.

7.4. Load sequencing data on the Ion Reporter Software using the Ion Reporter uploader plugin and use the **Comprehensive Cancer Panel tumor-normal pair** workflow to analyze the data in order to estimate CNVs.

7.5. Manually filter mutations and CNVs based on scores assigned by the software and verify them visually with the Integrative Genomics Viewer (IGV)[7].

7.5.1. Visually inspect the alignment for unusual reads (due to, for example, mispriming, overamplification or reads soft-clipping) that may generate artefactual calls.

7.5.2. Verify CNVs by inspecting the normalized coverage for all amplicons across a given gene against the normalized coverage of the baseline.

NOTE: This helps correcting false calls due to the segmentation software, which sometimes calls a CNV based on symmetrically abnormal coverage of few amplicons at the end of one gene and at the beginning of the successive gene.

7.6. Indexing of somatic mutations

7.6.1. For each case, index mutation calls from sequencing of normal tissue/blood, primary tumor and metastases.

7.6.2. Flag as germline and discard the calls that are also evident from sequencing data of germline DNA.

7.6.3. Flag as clonal/founder the mutations that are shared among all lesions of a given patient.

7.6.4. Flag as subclonal/progressor the mutations that are detected in some but not all lesions of a given patient.

8. Immunophenotypic analysis: immunohistochemistry for relevant protein expression

NOTE: Immunohistochemistry was used to validate the functional consequences of inactivating mutations in tumor suppressor genes.

8.1. Using a microtome, cut 3−4 µm thick FFPE tissue sections and mount them on charged slides and incubate slides at 60 °C for 10 min.

8.2. Place the slides in a rack, and perform the following steps: xylene for 10 min, xylene for 10 min, 95% ethanol for 4 min, 95% ethanol for 4 min, 70% ethanol for 4 min, 70% ethanol for 4 min, distilled water for 4 min.

8.3. Perform antigen retrieval based on indication of antibody's manufacturers (**Table of Materials**).

8.3.1. Place the slides in a rack with the specific antigen retrieval solution and submerge in a water bath at sub-boiling temperatures.

8.3.2. Remove the slides from the bath and run tap water to cool down for 10 min.

8.4. Place slides in a new rack with 3% H_2O_2 in 1x Tris-buffered saline (TBS) for 20 min at RT in

order to inactivate endogenous peroxidases.

8.5. Place slides in a new rack and wash 3x with TBS and 0.1% of Tween 20 (TBST).

8.6. Use a PAP pen (**Table of Materials**) to draw a hydrophobic circle around slide-mounted tissue.

8.7. Place the slides in a humid chamber and perform blocking for 1 h at RT using 5% bovine serum albumin (BSA) in TBST as blocking solution.

8.8. Incubate slides with primary antibodies (**Table of Materials**) in a humid chamber overnight at 4 °C. Dilute primary antibody with blocking solution as recommended.

8.9. Place slides in a new rack and wash 3x with TBST.

8.10. Incubate slides with specific secondary antibodies (anti-mouse or anti-rabbit) (4−5 drops for 1 slide) for 30 min at RT in a humid chamber.

8.11. Place slides in a new rack and wash 3x with TBST and after in distilled water.

8.12. Prepare 3,3'-diaminobenzidine (DAB) solution diluting 1 drop in 1 mL of dilution buffer (**Table of Materials**). Incubate slides maximum for 5 min checking under the microscope.

8.13. Use a positive control to calculate the incubation time by following the development of the reaction under the microscope.

NOTE: Each antibody needs a specific incubation time.

8.14. Inactive DAB (stop chromogen precipitation) by submerging slides in distilled water.

8.15. Counterstain slides by immerging them in a new rack with hematoxylin for 10 s and then rinse with tap water.

8.16. Place the slides in a rack, and perform the following steps: 70% ethanol for 4 min, 70% ethanol for 4 min, 95% ethanol for 4 min, 95% ethanol for 4 min, xylene for 10 min, xylene for 10 min.

8.17. Seal slides putting a couple of drops of mounting medium (**Table of Materials**) on each tissue slide and cover with coverslip.

8.18. Apply pressure on the coverslip in order to move excess medium and air bubbles away from the tissue and out from the coverslip and air dry.

8.19. Evaluate and score staining results under inverted microscope with an expert pathologist.

NOTE: The scoring system will depend upon the specific antigens, for which information in the literature might already exists. If information is not available in literature, derive a scoring system using expression of the antigen in normal tissue as reference.

REPRESENTATIVE RESULTS:

The study workflow is illustrated in **Figure 1**. Multi-lesions (n = 13) sequencing of 5 SPN cases targeting the coding sequences of 409 cancer related genes identified a total of 27 somatic mutations in 8 genes (*CTNNB1*, *KDM6A*, *BAP1*, *TET1*, *SMAD4*, *TP53*, *FLT1*, and *FGFR3*). Mutations were defined as founder/clonal when shared among all lesions of a given patient, and progressor/subclonal when detected in some but not all lesions of a given patient (**Figure 5A,B**). Overall, the majority of point mutations identified across the cohort were clonal events, which included mutations of *CTNNB1*, *KDM6A*, *TET1,* and *FLT1*. Consistently, immunohistochemical staining for β-catenin (protein product of *CTNNB1*) and KDM6A was homogenous among the diverse lesions of cases with mutations of the corresponding genes (**Figure 6A,B**). The moderate staining for KDM6A in mutated specimens suggested that genetic alteration was likely to alter function rather than protein expression. KDM6A loss of function in pancreatic tumors is associated to upregulation of the hypoxia marker GLUT1[8], and accordingly GLUT1 was overexpressed in cases bearing KDM6A mutations (**Figure 6C**). Subclonal mutations were found to affect *BAP1*, *SMAD4, TP53,* and *FGFR3.* Immunohistochemistry for BAP1 and TP53 confirmed that mutations in those genes were subclonal (**Figure 6D,E**). Copy-number variation (CNV) analysis was performed using sequencing data and revealed alterations in all the specimens analyzed as shown in **Figure 7A**. Differently from point mutations, majority of CNV alterations were subclonal (**Figure 7B**).

FIGURE LEGENDS:

Figure 1: Flow chart of the analysis conducted on metastatic lesions.

Figure 2: Representative histology of normal and tumor tissues. (**A, B**) Tumor tissue (T) adjacent to normal tissue (N). In these two tissue sections, the tumor and normal tissues are identifiable as separate and well-confined areas. (**C**) Clusters of normal pancreatic cells (N*) can be seen as embedded within the tumor tissue (T). (**D**) Morphology of normal pancreatic tissue. Scale bar represent 1 mm.

Figure 3: Schematic flow chart of the library preparation and quantification protocol step.

Figure 4: Ion proton chip loading and running. (**A**) Chip direction and placement in the chip clamp (left). Metal tab back replacement (right). (**B**) Heatmaps displaying the density of libraries in two different chip loadings. Example of a good loading density (top) due to a successful clonal amplification of libraries, resulting in 94% loading of the chip surface with sequencing particles (139 million reads, final output 90 million reads after automatic quality filtering). Example of a poor loading (bottom) due to an inefficient clonal amplification of libraries, resulting in 40%

loading of the chip surface with sequencing particles (59 million reads, final output 12 million reads after automatic quality filtering).

Figure 5: Somatic alterations in metastatic lesions. (**A**) Somatic mutations identified in matched primary/metastatic lesions. (**B**) Total somatic mutations are displayed per case, including alterations shared among all lesions (founder/clonal) and those detected in one or more but not all of the specimens for a given case (progressor/subclonal). The number of individual metastatic lesion (m) sequenced per case is indicated. This figure has been republished from Amato et al.[8].

Figure 6: Immunostaining for β-catenin, **KDM6A, GLUT1, BAP1 and TP53 in primary and metastatic lesions.** (**A**) Representative immunohistochemical images showing nuclear accumulation of β-catenin in all specimens (primary and metastatic) from a SPN bearing mutation of *CTNNB1.* (**B**) Immunohistochemical staining of lesions from a metastatic SPN bearing clonal mutation of KDM6A. (**C**) Overexpression of GLUT1 in one SPN bearing KDM6A mutation, whereas no immunoreactivity was observed in wild type tissue. (**D, E**) BAP1 and TP53 expression data denotes that the mutations in these two genes are subclonal. Scale bars represent 100 μm and inset magnification is 600X. This figure has been modified from Amato et al.[8].

Figure 7: Somatic copy-number changes in metastatic lesions. (**A**) The virtual karyotype view shows the location, proximity and copy number status of altered genes in a representative case. The coloring scheme of chromosomal bands is the following: black and gray = Giemsa positive, light red = centromere, purple = variable region. Alterations are annotated according to the color codes presented in figure. Abbreviations: CNV, copy number variation; P, primary SPN; L(a-c), liver metastases. (**B**) Total somatic alterations (genes affected by CNV) are displayed per case, including alterations shared among all lesions (founder/clonal) and those detected in one or more (but not all) of the specimens for a given case (progressor/subclonal). The number of individual metastatic lesion (m) sequenced per case is indicated. This figure has been republished from Amato et al.[8].

DISCUSSION:

Our method enables the identification of molecular alterations involved in progression of solid tumors through integration of vertical data (i.e., morphology, DNA sequencing, and immunohistochemistry) from distinct lesions of a given patient. We demonstrated the capability of our method to detect clonal and subclonal events in a mutational silent tumor type (i.e., SPN, solid-pseudopapillary neoplasm of the pancreas) by interrogating the coding sequences of 409 cancer relevant genes[8]. An advantage of the amplicon-based targeted sequencing approach used here is the uniformity of coverage (90% target bases are covered 100x, 95% are covered 20x) across interrogated regions (15,992) at a typical mean coverage depth of 1000x. High depth-of-coverage coupled to neoplastic cell enrichment through microdissection guarantees high sensitivity for the detection of low allele frequency events. As we have previously shown[9], the targeted sequencing approach allows the detection of mutations down to a 2% allele frequency on DNA samples from FFPE tissue. As an example, in the present work we were able to identify a 4% allele frequency TP53 missense mutation as a subclonal event in a metastatic specimen (**Figure 5**) and validated this occurrence by immunohistochemistry (**Figure 6**). Our protocol

envisages the sequencing of matched tumor and germline DNA in order to identify somatic events and accordingly reduce the false detection rate of subclonal mutations of cancers-only pipeline[10]. When matched germline DNA is not available, one might consider adopting more conservative parameters in the analysis of sequencing data, including stringent filters based on minimum depth of coverage as well as limiting variants calling to "hot-spots mutations" and mutations extensively annotated in available databases. Sequencing germline DNA alongside matched tumors has also the advantage of enabling accurate detection of copy-number variations (CNV). Alternatively, pools of gender-matched diploid genomes might be used to reduce noise from sequencing data and facilitate detection of CNV. In addition to the inclusion of germline DNA, we modified the library protocol to reduce primer pool imbalance and improve CNV calling. According to the original protocol, the four amplicon pools produced from each DNA sample after the multiplex PCR should be mixed together and the remaining steps would be performed in one tube per sample. This however causes fluctuations in per-pool mean coverage depth due to the fact that multiplex PCR may have different efficiency across different tubes. There was no pool quantification/normalization step to account for this effect in the original protocol. To avoid the above described fluctuations, we decided to keep each of the four amplicon pools separated throughout the whole library production protocol, until they could be quantified. Upon quantification, the same amount of each of the four pools for each DNA sample could be added to the final library pool, ensuring that the final average coverage was as uniform as possible.

The assessment of intra-tumor heterogeneity (ITH) at genetic level has important clinical implications but similarly poses new challenges[2]. A major challenge is indeed the necessity of distinguishing between *driver* mutations and stochastic events (i.e., *passenger* mutations). The distinction between *driver* and *passenger* mutations is often accomplished computationally, but not without biases. While systematic functionalization of detected variants is costly and time-consuming, functional consequences of genetic variants might be evaluated, at least for a subset of genes, by immunohistochemical analysis of the corresponding protein or, indirectly, by measuring expression of surrogate markers of protein dysfunction. Our protocol has been applied to FFPE tissues, which represents the major source of materials in the clinical setting yet posing challenges for sequencing; quality of isolated nucleic acids should always be evaluated prior to sequencing[11]. Although targeted sequencing has the major advantage of being cost-effective and not highly demanding in terms of computational requirements, it has the major disadvantage of interrogating only a limited portion of the genome, which likely leads to underestimate intra-tumor heterogeneity. Moreover, this approach is not considering relevant epigenetic and transcriptomic differences between metastasis and primary tumors that have been recently shown to outweigh genetic differences in certain tumor types[4,12,13]. However, one would envisage that technological advancements will soon enable integration of a richer vertical data ensemble for a better assessment of ITH. Our approach prefers depth to physical coverage of the genome, which limits our ability of building proper SNV-based phylogenies. Yet, our method provides the opportunity of exploring genetic relatedness in clinical specimens with appropriate sensitivity and specificity due to integration of molecular and histopathological analyses. We have successfully applied this protocol on a specific tumor type (e.g., SPN) and predict that the method will similarly work on other solid tumor types.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1_R1

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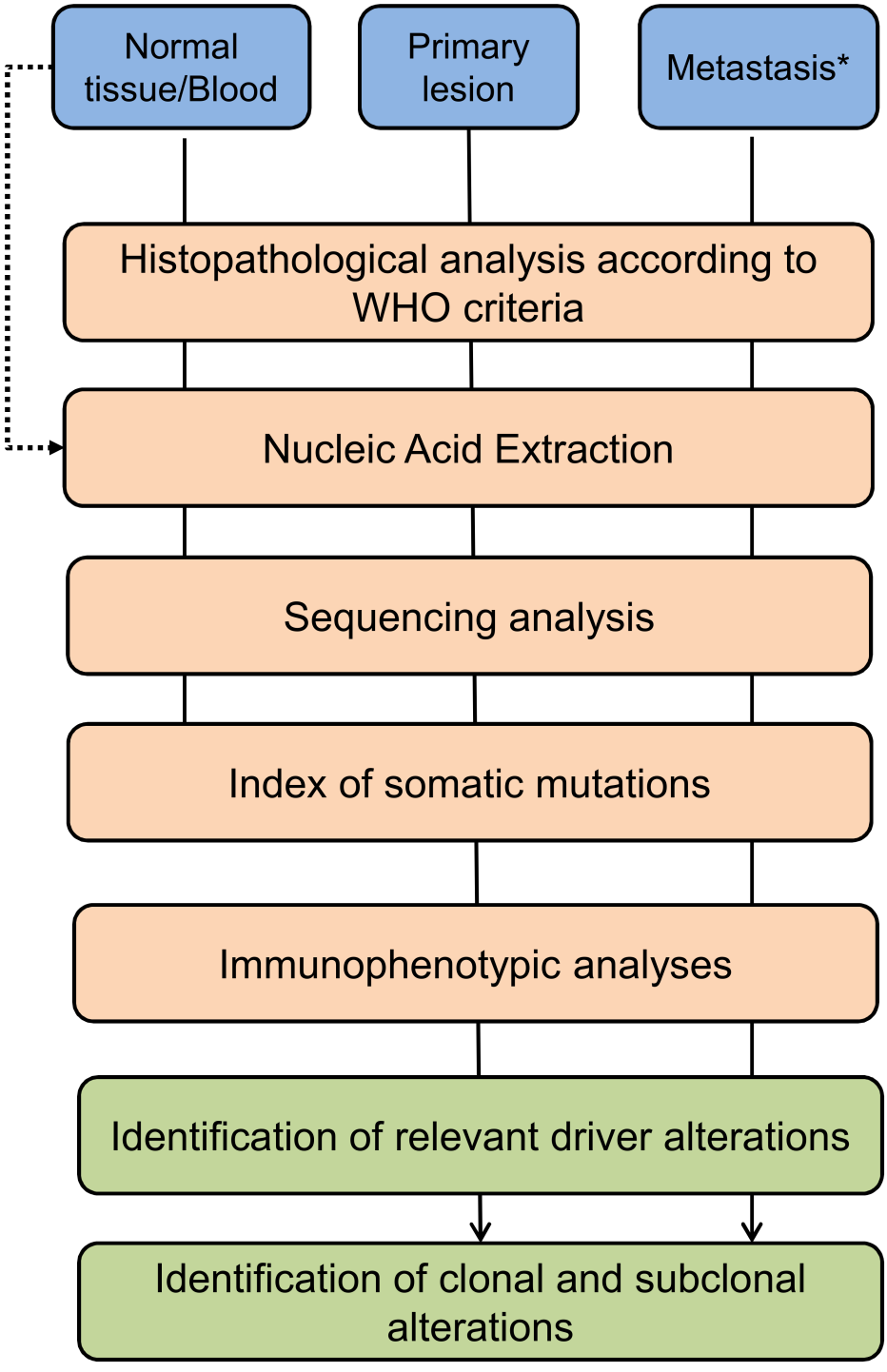

* Matched normal tissue, primary lesion and metastasis.

Figure 2_R1

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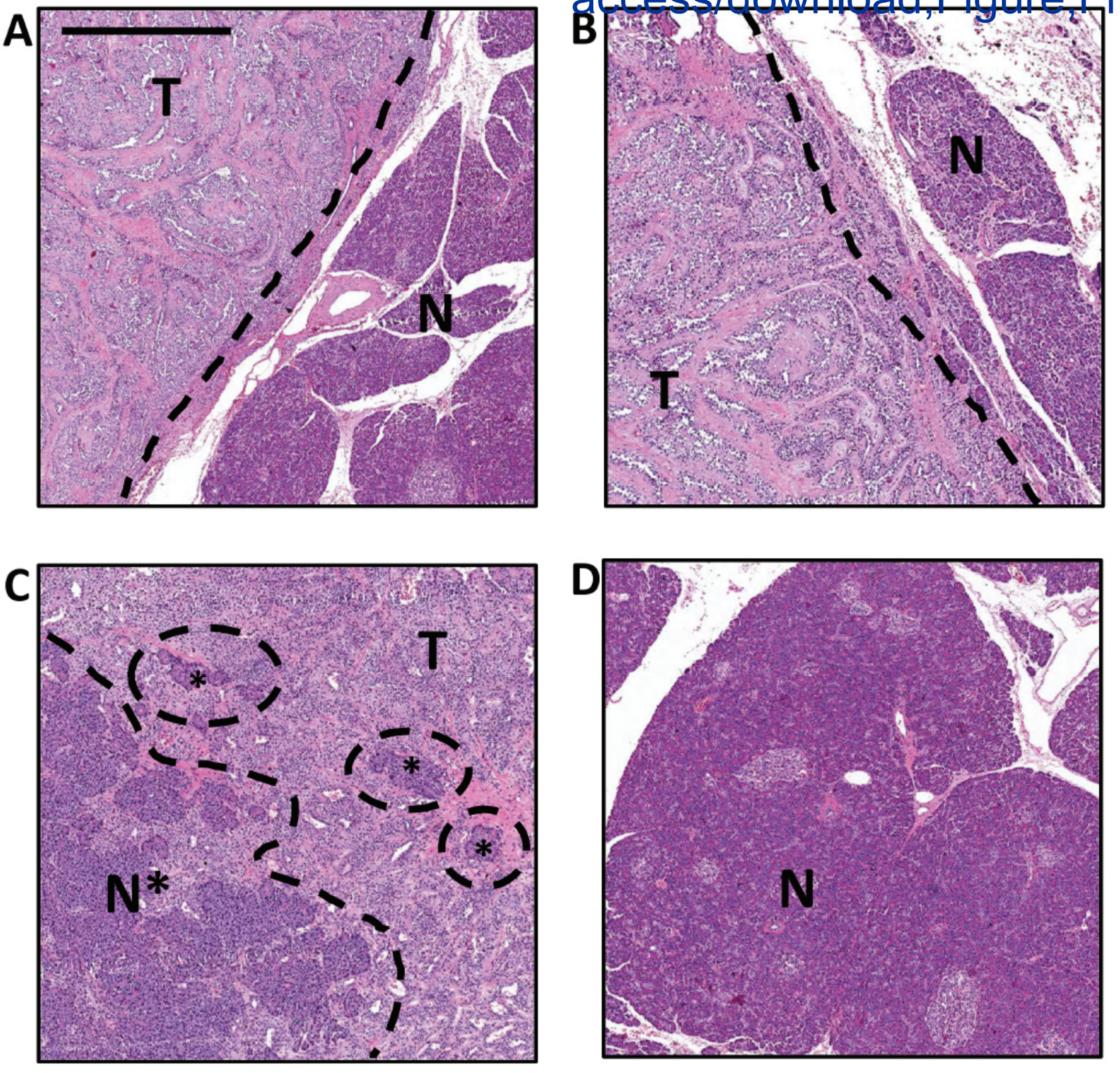

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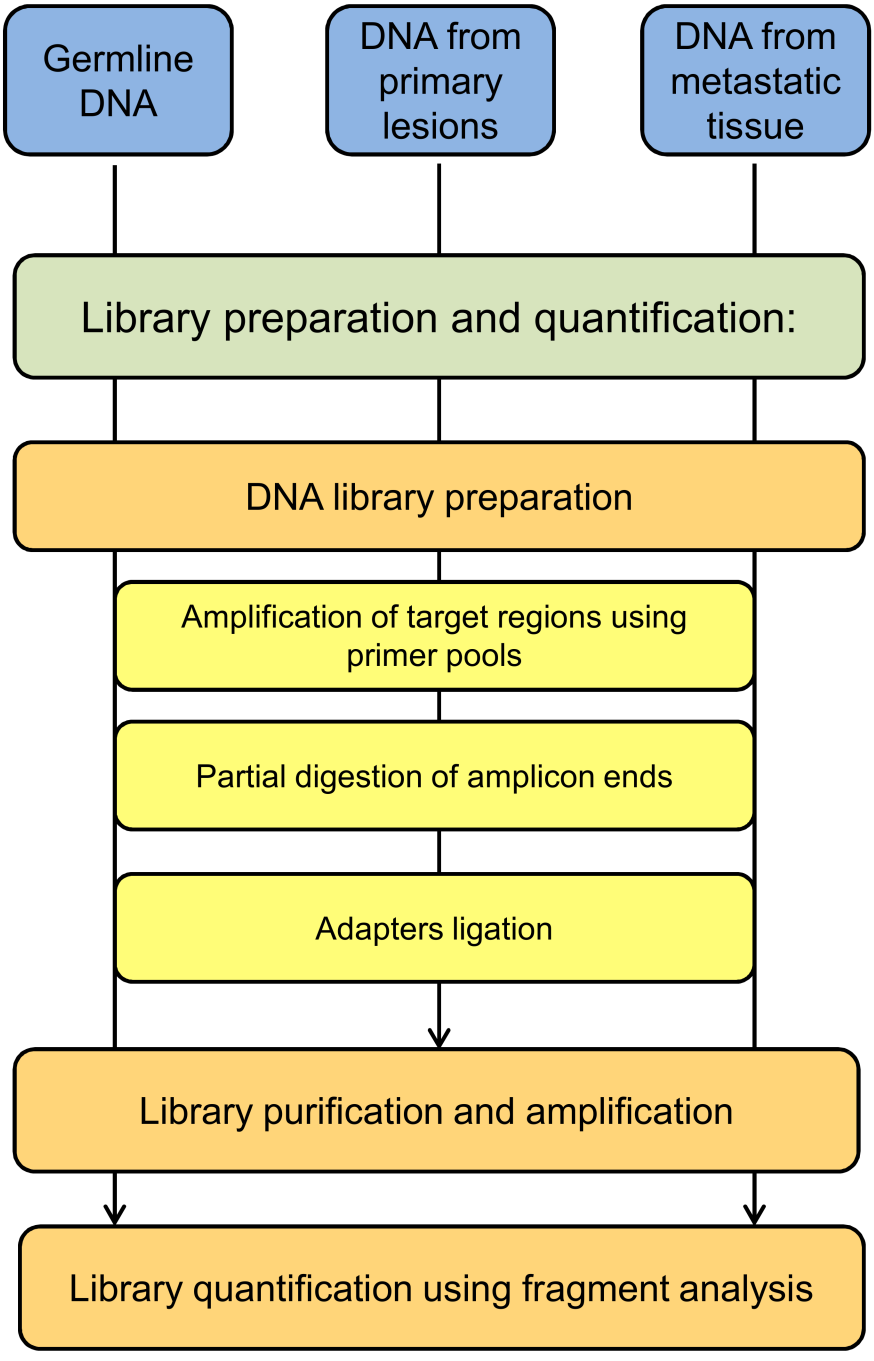

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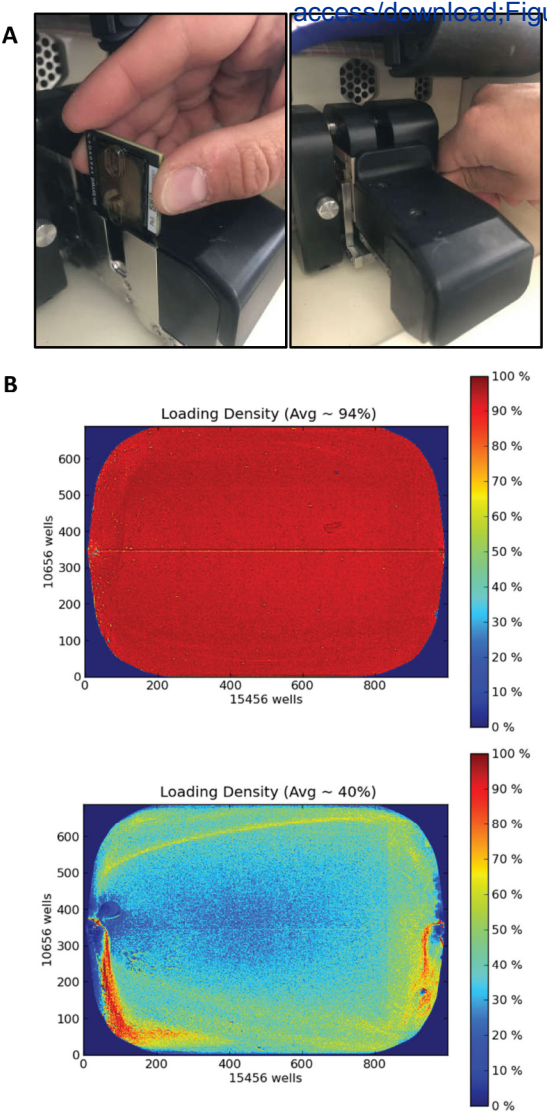

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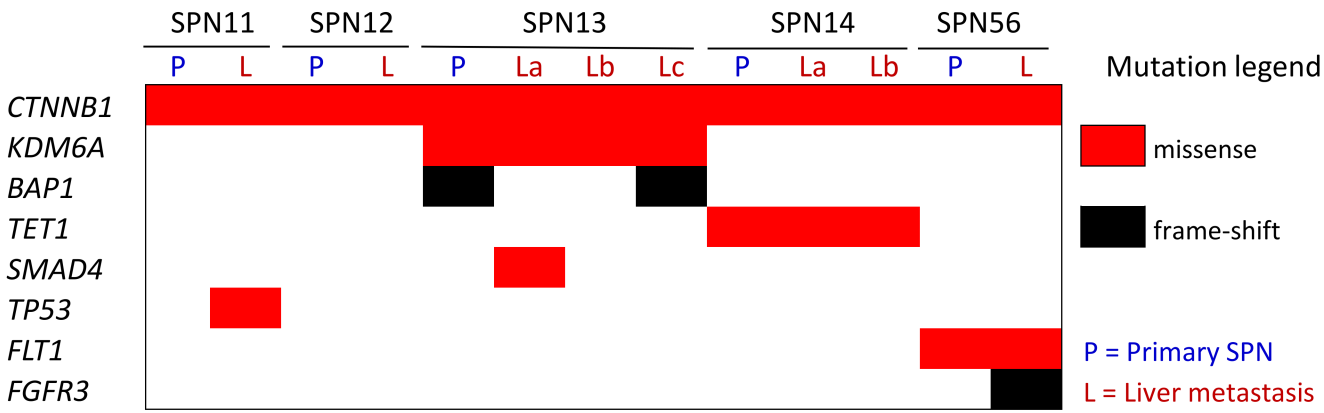

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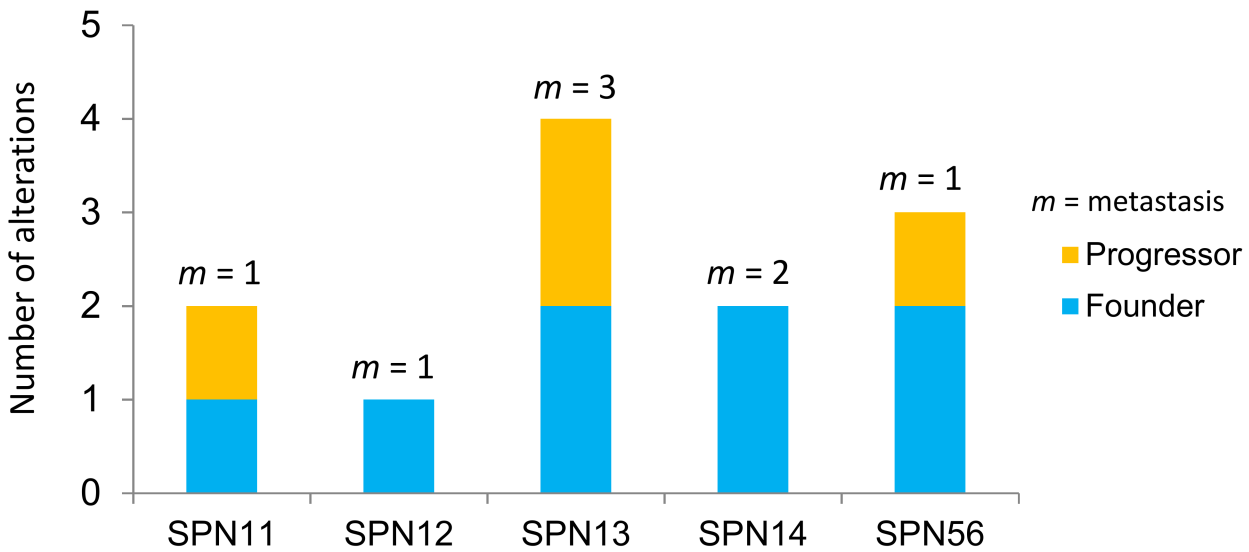

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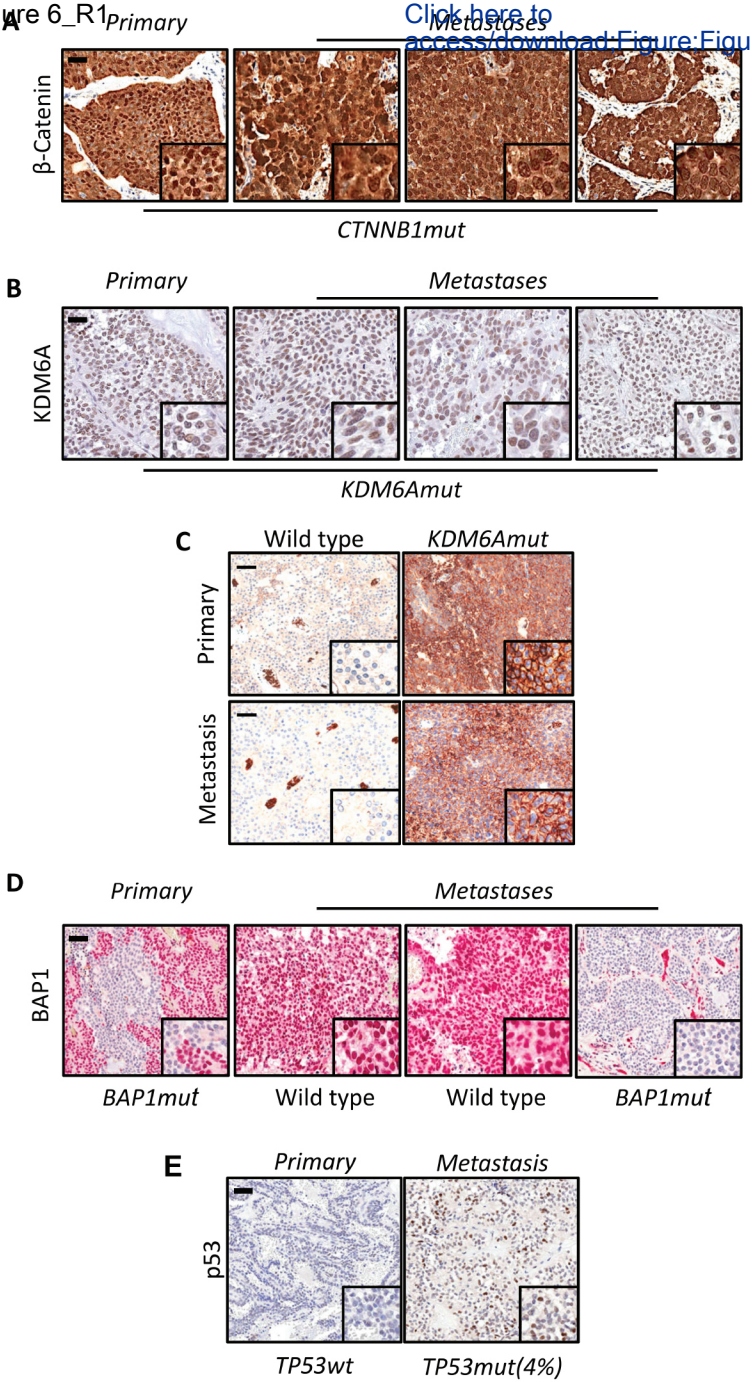

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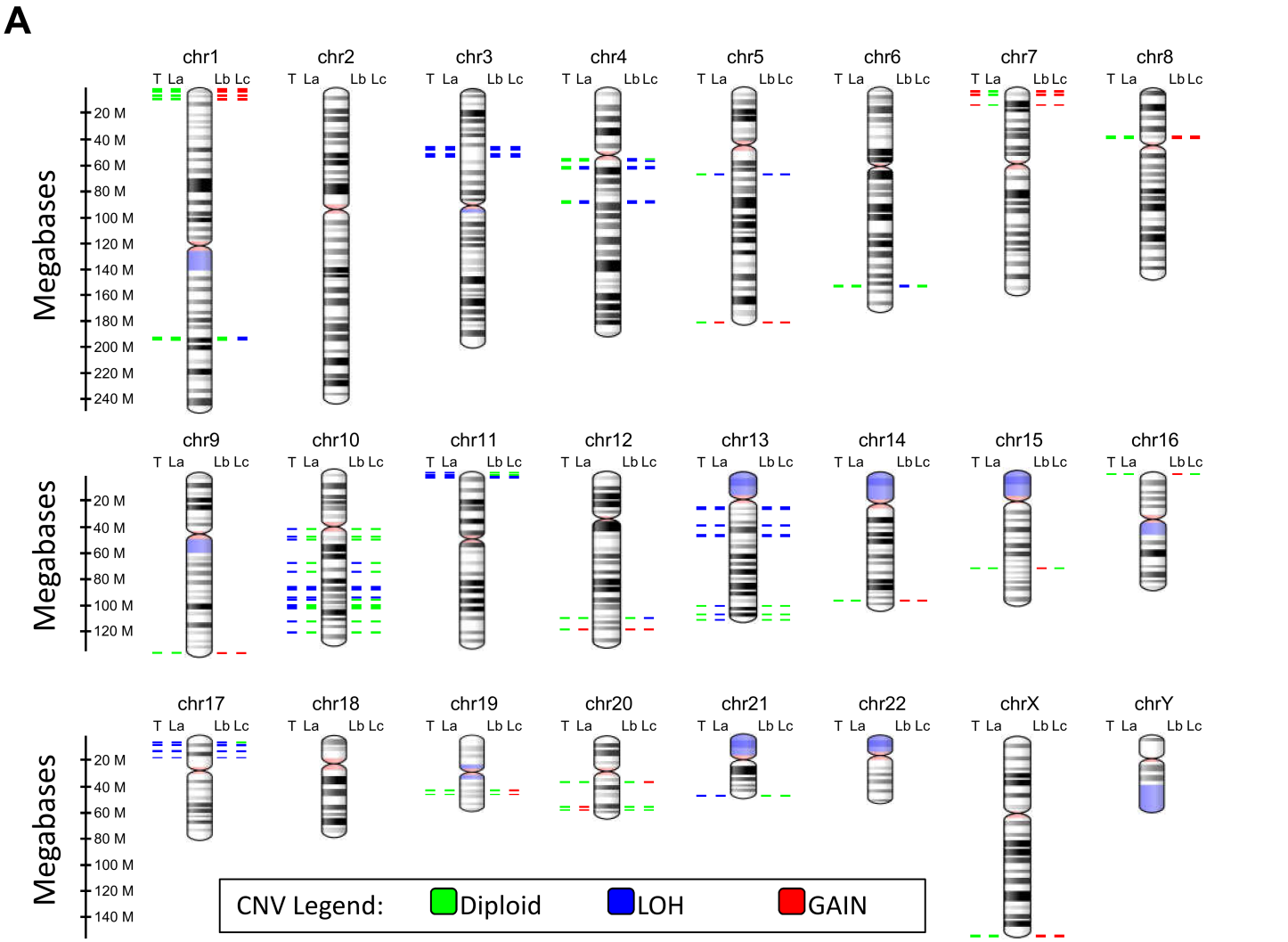

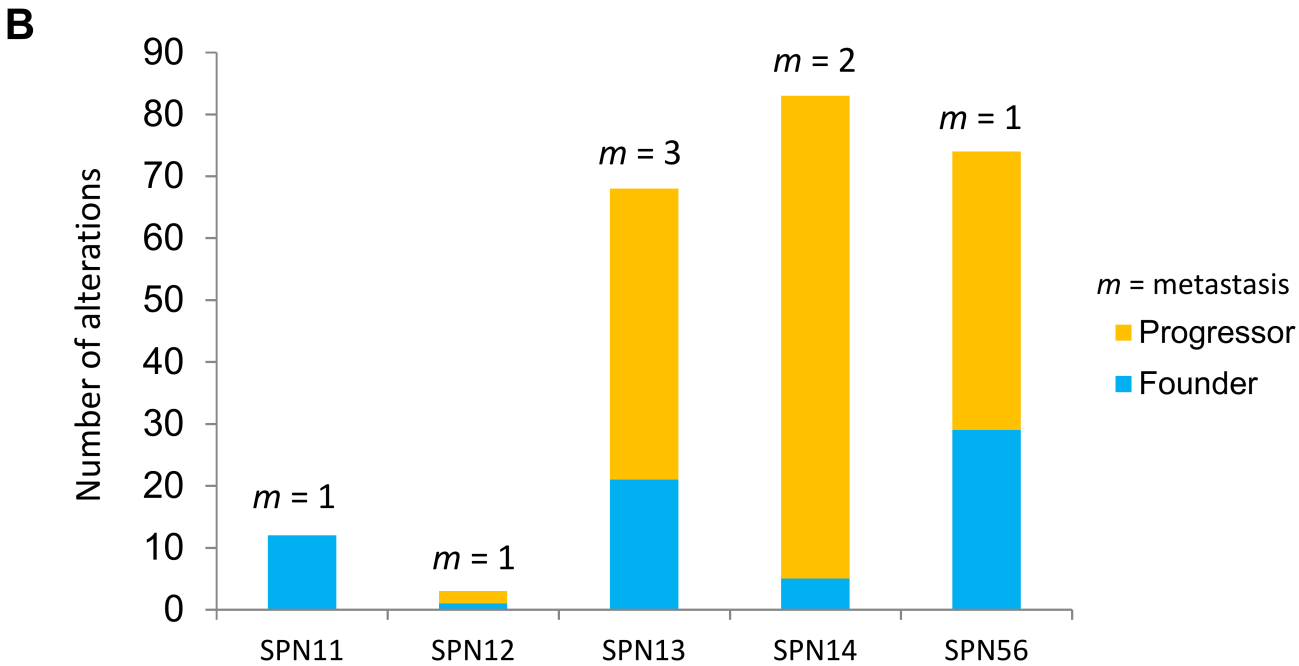

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Name of Material/ Equipment	Company
2100 Bioanalyzer Instrument	Agilent Technologies
Agencourt AMPure XP Kit	Fisher Scientific
Agilent High Sensitivity DNA Kit	Agilent Technologies
Anti-BAP1	Santa Cruz Biotechnology
Anti-GLUT1	Thermo Scientific
Anti-KDM6A	Cell Signaling
Anti-p53	Novocastra
Anti-βcatenin	Sigma-Aldrich
Blocking Solution	home made
Endogenous peroxidases inactivation solution	home made
Leica CV ultra	Leica
Epitope Retrieval Solution 1	Leica Biosystems
Epitope Retrieval Solution 2	Leica Biosystems
Eppendorf 0.2 ml PCR Tubes, clear	Eppendorf
Eppendorf DNA LoBind Tubes, 1.5 mL	Eppendorf
Ethanol	DIAPATH
ImmEdge Pen Hydrophobic Barrier Pen	Vector Laboratories
ImmPACT DAB Peroxidase	Vector Laboratories
ImmPRESS Anti-Rabbit Ig Reagent Peroxidase	Vector Laboratories
ImmPRESS Anti-Mouse Ig Reagent Peroxidase	Vector Laboratories
Integrative Genomics Viewer (IGV)	Broad Institute
Ion AmpliSeq Comprehensive Cancer Panel	Thermofisher Scientific
Ion AmpliSeq Library Kit 2.0	Thermofisher Scientific
Ion Chef Instrument	Thermofisher Scientific
Ion PI Chip Kit v3 or Ion 540 Chip	Thermofisher Scientific
Ion PI Hi-Q Chef Kit or Ion 540 Kit-Chef	Thermofisher Scientific
Ion PI Hi-Q Sequencing 200 Kit or Ion S5 Sequencing Kit	Thermofisher Scientific
Ion Proton or Ion GeneStudio S5 System	Thermofisher Scientific
Ion Reporter Software - AmpliSeq Comprehensive Cancer Panel tumour-normal pair	Thermofisher Scientific
Ion Reporter Software - uploader plugin	Thermofisher Scientific

Ion Torrent Suite Software - Coverege Analysis plugin	Thermofisher Scientific
Ion Torrent Suite Software - Variant Caller plugin	Thermofisher Scientific
Ion Xpress Barcode Adapters 1-96 Kit	Thermofisher Scientific
NanoDrop 2000/2000c Spectrophotometers	Thermofisher Scientific
NCBI reference sequence (RefSeq) database	NCBI
Platinum PCR SuperMix High Fidelity	Fisher Scientific
QIAamp DNA Blood Mini Kit	Quiagen
QIAamp DNA FFPE Tissue	Quiagen
Qubit 2.0 Fluorometer	Thermofisher Scientific
Qubit dsDNA BR Assay Kit	Thermofisher Scientific
TBST	home made
Tissue-Tek Prisma Plus & Tissue-Tek Film	Sakura Europe
Variant Effect Predictor (VEP) software	EMBI-EBI
Xilene, mix of isomeres	Carlo Erba

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492306

Comments/Description
Automated electrophoresis tool
Beads technology for the purification of PCR products; beads-based purification reagent
Library quantification
Antibody
Antibody
Antibody
Antibody
Antibody
5 % Bovine serum albumin (BSA) in TBST
3% H_2O_2 in Tris-buffered saline (TBS) 1x
Entellan mountin media
Citrate based pH 6.0 epitope retrieval solution
EDTA based pH 9.0 epitope retrieval solution
Tubes
Tubes
IHC deparaffinization reagent
Hydrophobic Pen
HRP substrate
Secondary antibody
Secondary antibody
https://software.broadinstitute.org/software/igv/home
Multiplexed target selection of 409 cancer related gene. https://assets.thermofisher.com/TFS-Assets/CSD/Reference-Materials/ion-ampliseq
Preparation of amplicon libraries using Ion AmpliSeq panels
Automated library preparation, template preparation and chip loading
Barcoded chips for sequencing
Template preparation
Sequencing
Sequencing system
Workflow
Data analysis tool

Plugin that describe the level of sequance coverage produced
Plugin able to identify single-nucleotide polymorphisms (SNPs), insertions and deletions in a sample across a reference
Unique barcode adapters
DNA purity detection
https://www.ncbi.nlm.nih.gov/refseq/
SuperMix for PCR amplification; high-fidelity PCR mix
DNA blood extraction kit
DNA FFPE tissue extraction kit
DNA quantification
Kit for DNA quantification on Qubit 2.0 Fluorometer
Tris-buffered saline (TBS) and 0.1% of Tween 20
Automated tissue slide stainer instrument
http://grch37.ensembl.org/Homo_sapiens /Tools/VEP
IHC deparaffinization reagent

-cancer-panel-gene-list.pdf

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CORRESPONDING AUTHOR

Name:	VINCENZO CORBO
Department:	DEPARTMENT OF DIAGNOSTIC AND PUBLIC HEALTH
Institution:	UNIVERSITY OF VERONA
Title:	ASSISTANT PROFESSOR
Signature:	
Date:	13-02-2019

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Reviewer 1 (Major Concerns):

1. According to the abstract aim of the protocol is to sequence normal and tumor tissue. Yet, processing of normal tissue is not described in the protocol.
We thank the reviewer for the informative critique. We have now incorporated the description of normal tissue processing in our protocol. The aim of the protocol is the indexing of somatic mutations, which can be used to evaluate the genetic relatedness of lesions of an individual patient. Therefore, any source of germline DNA (blood or tissue) can be incorporated in our protocol. In our work, we have isolated DNA from uninvolved normal pancreatic tissue, which is tissue located at least 2 cm from the resected neoplastic lesion and sampled on a different paraffin block. In this case, microdissection is not necessary, yet DNA extraction from scrolls obtained from normal tissue will occur using the very same procedure as per microdissected tumor tissue. We have added text to the dedicated section to clarify this point (see Step 1.3 and Step 3 of the revised version of the manuscript; see also modified Figure 1 and the new Figure 2).

2. The authors wrote that capturing the phylogenetic relationship between lesions was important. Which method do the authors use for prediction? In my opinion that should also be described.
We agree with the reviewer about the necessity of a better description of the approach used to predict relationship among lesions from individual patients. Our classification is based on the assumption that, during progression of the disease, disseminated tumor cells at various stages of the disease seed metastases, which are therefore clonally related. The implication is that metastases inherit genomic alterations, albeit at varying fraction, from the parental clone (defined as clonal mutations). Ongoing and parallel tumor evolution at each site (primary and metastatic) leads to the accumulation of private alterations (progressor) that represent subclonal events beyond the parental clone (defined as sublclonal mutations). We did not provide a graphical display of the phylogenetic relationship among lesions as the number of detected somatic mutations per patient, which makes difficult to temporally resolve branches, limited us. Thanks to this comment from the reviewer, we realized that our approach is limited with the regard to building precise SNV-based phylogenies, and therefore we have made changes to the text in order to reduce the prominence of our initial statement and clarify that the method can be rather used to identify genetic relatedness among lesions. However, one might envision that fully clonal alterations (i.e., genetic alterations shared by all samples from a given patient although at varying allele frequencies) represent the trunk of a phylogenetic tree; somatic variants that are unique or shared by some, but not all, of the lesions under investigation are considered as “progressor” and therefore constitute the branches of the tree. This classification approach implies indexing somatic mutations with high confidence to avoid false positive (due to sequencing artefacts) as well as to accurately estimate variant allele frequencies. For this reason, we used high-coverage deep sequencing (mean coverage 1000x) on DNA from samples with high-neoplastic cellularity. The list of high confidence SNV variants for each patient is then used to infer relatedness among lesions as described above. We have introduced a new paragraph to describe the procedure for identification of clonal and subclonal mutations (see Step 7.6 of the revised version of the manuscript) and made modification to the Figure 1, which now better describes the flow of analyses being conducted in this protocol.

3. What does "tissue specimens were reviewed for morphological and immunophenotypic characteristics" mean? According to Figure 1 that step is important, but it is not described at all.
Due to this comment from the reviewer, we realized that an adequate description of the revision of tissues before processing was not provided in the original version of the manuscript. We now added text to the dedicated section (Step 1) to describe that each tissue specimen has been

subjected to careful histopathological and immunophenotypical revision by an expert pathologist according to the criteria described in the blue book of the WHO (World Health Organization) for SPNs. The morphological revision has different purposes, including: (i) the evaluation of the neoplastic cellularity in the FFPE tissue, which is important to define suitability of the tissue for downstream analysis; (ii) the potential identification of morphologically distinct areas that might be processed separately; (iii) the evaluation of immunophenotypic features of the tissue, which might reveal heterogeneity. Although we focused this work on SPNs, the approach is broadly applicable to other solid malignancies by referring to established histopathological criteria for a given disease. The purposes of the histopathological revision of tissues along with related activities are now detailed in the Step 1 of the revised version of the manuscript.

4. Step 1.1.4: How many tumor cells/clusters should be scraped out? If cluster morphology indicates clonal differences: should clusters be processed separately? What about normal tissue?

We thank the reviewer for his/her comment. As clarified in response to comment #1, normal tissue has not been subjected to microdissection as it is only used as source of germline DNA. We agree with the reviewer that a more detailed description of the microdissection process needed to be provided. As neoplastic cell content will influence the detection of low allele frequency mutations, microdissection is a critical step in our protocol but needs to be performed only when the tissue under investigation contains a significant fraction (> 30%) of contaminating non-neoplastic cells.
We now clarify that the entire process, from evaluation of neoplastic cellularity to the manual microdissection, is driven by an expert pathology.
The way microdissection is performed is also influenced by the spatial distribution of cancer cells within the tissue. If sparse clusters of cells are embedded within tumor stroma, then the individual clusters need to be scraped out. However, when contaminating non-neoplastic cells are identifiable as a distinct and confined region within the tissue section, then neoplastic cell content can be increased by simply removing the non-neoplastic region and the rest is harvested for nucleic-acid extraction. This is the case for SPNs, which we analysed in our original publication.
However, we now clarify that the number of tissue slides to be cut for manual microdissection depends on the number of identifiable 1 mm^2 nests of tumor cells and that, if only one 1mm^2 nest of cancer cells is visible in the tissue specimen, then 10 tissue slides should be cut and subjected to manual microdissection in order to obtain the targeted amount of DNA (40ng). Based on our experience, 1mm^2 nest contains between 700 and 1000 tumor nuclei.
As also explained in response to comment #2, morphological and immunophenotypical analyses are also conducted to identify heterogeneity within individual tissue section. We agree that it would be recommended to process separately clusters of cells that display phenotypic differences; as this was not our case, we did not process clusters of cells separately but now incorporate a note to describe this possibility. A better description of the microdissection process is now provided in Step 2 of the revised version of the manuscript.

5. The authors should check whether the Table of materials is complete. For example, what about the Agencourt AMPure XP Reagent or different buffers such as TBSt?

We apologize for the mistake and amended the Table of materials to include missing information.

6. Step 3.1.4: How do the authors verify aberrations using IGV?

A detailed description of the procedure used to verify alterations using IGV has been now provided in the text. Please, refer to Step 7.5 of the revised version of the manuscript.

7. The authors conclude that they demonstrated sensitivity and specificity in detecting clonal and subclonal events in a defined genomic space using clinical specimens. What is the sensitivity of this approach? How many specimens should be analyzed to confirm

clonality? What about another method for detection/confirmation of low frequency mutations, such as Droplet-PCR?

We thank the reviewer for his/her comment and agree that there has been missing information about performance of our approach in the original version of the manuscript. As we have previously showed (Mafficini, Amato, Fassan *et al.* PLoS One 2014), the targeted sequencing approach allows the detection of mutations down to a 2% allele frequency on DNA samples from FFPE tissue. As an example, we were able to identify a 4% allele frequency *TP53* missense mutation as a subclonal event in a metastatic specimen (new Figure 5A) and validated this occurrence by immunohistochemistry (new Figure 6E).

Older tissue samples (>5 years) may undergo biochemical degradation (Deamination), resulting in sequencing artefacts, which cause a higher frequency of false positive calls. Therefore, the actual sensitivity depends on the quality of the starting material. As per our definition (see also response to comment #1), clonal mutations are those detected in all analysed samples from a given tumor/metastasis set. Subclonal mutations, on the other hand, are those detected only in some of the analysed samples, which means they have accumulated beyond the parental clone and as such are progressor mutations. While the classification of clonal/subclonal events gets the more accurate the more samples are analysed from the same case, even cases with only two different samples may give interesting information. The confirmation of mutation calls was performed on a selection of all detected variants to verify that the assay was capable of detecting mutations with good sensitivity and specificity. This is indicated in our original publication (Amato et al. Journal of Pathology. 2018) but not reported here. This confirmation has been performed several times and at the present time the confidence of variant calls from targeted NGS is even superior to that from Sanger sequencing. The point of these assays is obtaining large wealth information from a limited amount of available sample. From this standpoint, it would be impossible to confirm all mutations. As for the use of ddPCR, it could have been used to confirm low frequency mutations but, in our hands, its sensitivity constitutes both its main strength and its weakness, leading to false positives with FFPE DNA. We have now included information about sensitivity in the discussion section of the revised version of the manuscript.

Minor Concerns:

1. What does "the number of sections depends on the amount of tumor cells in the section" mean? Any recommendation?

Following reviewer comment, we have added text to provide a more detailed description of the microdissection procedure, including recommendations about the minimum number of cells needed for DNA isolation (see Step 2 of the revised version of the manuscript). Please, see also responses to comments # 2 and # 4.

2. Step 1.4.1: The authors need to describe how spectrophotometer data should be interpreted or at least which values indicate a good DNA sample. Any recommendation, if values indicate contamination?

Following reviewer comment, we have added in the protocol the values of the 260/280 and 260/230 ratios that indicate a good DNA sample. In the event of contamination due to chemical reagents (e.g., phenols), we suggest performing a cleanup step using column based-kit. Please see Step 4.2.2 of the revised version of the manuscript.

3. Step 2.1: What does primer pool mean? Does the kit contain 4 different pools? For each step, the authors need to clearly indicate the kit that contains the used components.

Due to this comment from the reviewer, we realized that this step is not properly described and amended the text accordingly. In our work, we used the Ion AmpliSeq Comprehensive Cancer

Panel (as reported in the Table of Materials), which contains 4 individual tubes each comprising a pool of primer pairs needed to simultaneously amplify distinct regions of the 409-targeted genes. Different pools contain primer-pairs that target overlapping regions. To avoid preferential amplification of DNA regions, which will affect accuracy of copy-number variation calling, we process each primer' pool amplification separately and mix the resulting libraries before sequencing run. This is different from manufacturers' recommendations and was described in original version of the manuscript. We have now made changes to the text to better describe the content of the kit used for multiplex targeting of the 409 genes (see the following link that have been added to Table of Materials: https://assets.thermofisher.com/TFS-Assets/CSD/Reference-Materials/ion-ampliseq-cancer-panel-gene-list.pdf)

4. Is it possible to perform this protocol using Illumina instruments and kits? We thank the reviewer for his/her comment, and we think this is a relevant question. Indeed, it is possible to use this procedure with an Illumina sequencer, yet with major modifications. The AmpliSeq Comprehensive Cancer Panel used here is available also for sequencing on Illumina instruments, and therefore the first part of the protocol is virtually identical until library quantification. Following libraries quantification, the preparation of libraries for sequencing on Illumina Instruments is different due to the different principle used for clonal enrichment/sequencing. Similarly, since the data produced on Illumina are different, different software is needed to perform alignment and variant/CNV calling, as the IonReporter and Torrent Suite are built specifically to deal with ion flow sequencing data. Therefore, the authors advise against relying on the present protocol for sequencing with Illumina, although it may be used as a general guide for that purpose.

Reviewer 2 (Manuscript Summary)

Overall manuscript is well written about how multiple lesions can be used from different cases and study the intratumoral heterogeneity. This will definitely increase the impact to the field especially the readers of Journal JOVE.
We thank the reviewer for his/her supportive comment.

Major Concerns:

1. How were the cancer cells detected under the microscope? It might be easy for the authors as it is their expertise, but mentioning specific details are important for readers to follow the method. Maybe including a figure to show the morphology of cells would be helpful.
We thank the reviewer for the informative comment and agree with him/her that manual microdissection of tumor cells is not an easy task. The identification of cancer cells in tissue slides is performed by an expert pathologist, who is able to distinguish between normal and neoplastic cells solely based on morphological features. We made modification to the text to highlight that this critical step of the protocol should be performed or assisted by a pathologist. As requested by this reviewer, we have added a new figure to show the morphology of normal and neoplastic cells in SPNs (see new Figure 2).

2. Include another schematic showing step wise protocol for step 2.
As suggested by the reviewer, we have now added a new figure (Figure 3) that describes the major steps for Step 5 (originally Step 2) of the revised version of the manuscript.

Minor Concerns:
1. It will be helpful to include ion proton chip image with the direction the chip should be loaded.
As suggested, we have added a new figure (Figure 4 of the revised version of the manuscript) displaying both ion proton chip positioning/loading and two examples of different loading densities, the latter referring to either successful or unsuccessful clonal amplification of the libraries.

2. Authors should comment on how their approach is advantageous over currently available methods.
We thank the reviewer for the comment. We have now added more text to discuss the advantages as well as the limitations of our approach in the pertinent discussion section. We think that our method offers some advantages over methods commonly described in the literature, and this is not due to specific technological advances but rather relies on a multidimensional approach that incorporates molecular and pathological analyses. The main purpose of the method is the high confidence indexing of somatic mutations to be used to infer relatedness among lesions from the same patient. We believe that specific strengths of our approach are: (i) the inclusion of germline DNA for detection of somatic events, which substantially reduce the false positive rate (specificity) that can be observed with cancer-only pipeline; (ii) the use of high-coverage deep sequencing (mean coverage 1000X) combined with high neoplastic cellularity (above 70%) of the specimens under investigation, which increases the likelihood of detecting low-allele frequency mutations (sensibility); (iii) the careful morphological and immunophenotypic evaluation of the tissues in order to include/exclude phenotypically different areas of the tumors from the analysis; (iv) where possible, confirming the effect of somatic mutations in order to distinguish between stochastic and

“driver” events; (v) the possibility of analysing hundreds of genes from low input DNA, which enable interrogation of archival tissues and provide enough flexibility to permit multiple sampling from the same tissue block if needed. Our approach is not without limitations. First, as explained in response to comment 7 from reviewer # 1, sequencing analysis from FFPE tissue can be difficult when samples are old since deamination process will negatively impact false discovery rate. Then, our approach prefers depth to physical coverage of the genome, which limits our ability of building proper SNV-based phylogenies. However, as this approach is applicable on clinical samples, we believe that advantages outweigh disadvantages.

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We would like to thank the editor for the informative critiques and valuable comments. We have addressed the editorial comments in the revised version of the manuscript. Below, please find the point-by-point response.

A copy of the manuscript where changes are indicated in blue coloured font (added text) and as grey-crossed out (eliminated text) has been uploaded.

Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.
We thank the editor for formatting our manuscript. Accordingly, we have made the required amendments to this version of the manuscript. Changes are indicated in blue colored font (added text) and as grey-crossed out (eliminated text)

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We apologize for the mistake and removed commercial language from the manuscript. All commercial products have been referenced in the revised version of the Table of Materials. To ensure accuracy, some commercial terms have been retained as they appear in menu/list selection and software commands.

3. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.
We apologized for the mistake. We have modified the text according to editor's suggestion.

4. Please be as specific as you can with respect to your experiment providing all necessary details.
As suggested, we have revised the protocol by adding more details. Accordingly, descriptive parts of the protocol have been shortened to adhere to the 10 pages limit of the protocol section.

5. Please address specific comments marked in the attached manuscript.
We thank the editor for the informative critiques. We have carefully revised the protocol addressing all the specific comments made by the editor.

6. Once formatted please ensure that the protocol is no more than 10 pages long and the highlighted content is no more than 2.75 pages long including heading and spacings. Note that the highlighted content should contain essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next.

As suggested, we have highlighted the essential steps of the protocol paying attention not to exceed the required number of pages. The protocol now spans 10 pages and the highlighted text is 2.75 pages long. The steps describing manual microdissection and analysis of sequencing data drew reviewers' attention, therefore we decided to include those in the highlighted text.