Tumour-only sequencing for oncology management: germline-focused analysis and implications

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Abstract

As patients are now routinely having large somatic genomic testing panels undertaken as part of routine management, there is the rising likelihood of uncovering the presence of a germline pathogenic variant. This may be found on testing undertaken on plasma (ctDNA) or tissue. This has led to the need for clear guidelines for oncologists about how to manage such results, including which variants require validation, how this should be undertaken, and what potential problems may arise. This requires an understanding of the limits of testing, and the pitfalls that may be encountered.

In this review, we assess the frequency of detecting germline variants through tumour-only sequencing, the necessary considerations for such information to be analysed and the role of the molecular tumour board in considering results. We assess the additional considerations for

interpretation of the underlying tumour, use of ctDNA or tissue for testing, clonal haematopoiesis and hypermutation.

Introduction

Oncological management has moved into a new genomic era of tumour profiling, targeted treatments and immunotherapy. Alongside this, we have the rise of large genomic panel testing to aid management. Such panels have the benefit of providing mutational analysis for immediately actionable genes, together with those of interest for trial recruitment. Some panels will be additionally able to provide estimates of tumour mutation burden or mutational signatures, of benefit when considering treatments such as immunotherapy or determining tumour of origin. We are also moving forward in undertaking such panels on blood rather than tissue, ensuring those who are unable to have tumour biopsies can still benefit from such technology. These panels bring a wide range of benefits not only in identifying which patients are suitable for each targeted agent, but can

also identify constitutional pathogenic variants in cancer susceptibility genes that have implications for future disease risk for the patient and their family. These technology advances bring with them opportunity for improvement in both immediate management of the current cancer and future cancer prevention. However to ensure avoidance of potential pitfalls around germline findings, careful consideration is required to ensure protocols are established safely, in a patient-centred way, and that the correct interpretation of results is undertaken.

In this review, we will cover analysis for germline variants on large-panel tumour-only testing in cancer patients, along with the emerging issue of whether testing is performed on tissue or circulating tumour DNA derived from plasma.

Informed consent

The first consideration for such testing is that of patient consent. It is currently a legal requirement in most countries for patients to provide explicit, informed consent before undergoing germline testing. In some countries (such as France), this can only be given following appropriate genetic counselling. In direct contrast, it is not standard to gain written consent before somatic testing, which is often undertaken as part of a diagnostic workup on the tumour. It has been suggested that if somatic testing is likely to identify variants that have a high likelihood of being germline in origin (such as tumour BRCA testing in ovarian cancer), patients should be aware that such testing will be undertaken and may have wider consequences. Conversely, such variants can only be defined as germline in origin after sequencing has been undertaken on a constitutional tissue (eg blood), so an alternative would be to undertake specific germline consent only at the stage of obtaining a blood specimen to confirm the mutation. This latter approach would (i) avoid need for detailed consent in the ~90% of patients with no relevant tumour mutations (ii) reduce unnecessary delays in undertaking somatic sequencing and utilising the results but (iii) ensure that patients have the right to choose whether or not to confirm the origin of identified mutations.

European Society of Medical Oncology (ESMO) guidance on germline-focused analysis

In order to ensure a consistent approach to the use and interpretation of such testing, the European Society of Medical Oncology (ESMO) convened a working party to identify key issues for both laboratories and clinicians; undertake analyses of relevant germline and tumour data, and to develop consensus recommendations regarding extent of routine analysis, use of confirmatory germline testing and level of patient information and consent to be utilised. The resulting guidelines were produced by analysing data from the Memorial Sloane Kettering MSK-IMPACT study of matched somatic and germline data (1).

In which genes are the germline findings informative?

As part of the interpretation, consideration was given as to whether pathogenic variants were found in a gene associated with the tumour type ('on-tumour'), or not ('off-tumour'). An example of this would be the identification of a BRCA2 pathogenic variant in a prostate and a cervical tumour. As BRCA2 germline mutations are associated with an increased risk of developing prostate cancer, identification of a pathogenic variant in prostate tumour tissue this would be considered an on-tumour association. Finding the same pathogenic variant in a cervical tumour would be considered off-tumour, or a "secondary" or "incidental" finding as such variants do not increase the risk of developing cervical cancer.

The next consideration was how such information would be utilised to change outcome, or the clinical actionability. This has two components: i) the potential use of the identified variant in management of the patient, either for their existing tumour or prevention of future malignancy; and ii) the options for, and evidence base for intervention in asymptomatic individuals to reduce cancer risk. This can vary greatly by gene, with identification of germline pathogenic variants in some genes having clear management benefit from targeted screening or chemoprophylaxis (eg aspirin in Lynch Syndrome), whilst others either have no clear benefit from screening, or other suitable risk-reducing interventions (2). To aid decision-making in which genes should be considered for return of potential germline

findings, the American College of Medical Genetics (ACMG) have issued recommendations on the set of genes that should be used to return findings. This currently constitutes a set of 25 genes, that were used in the ESMO guidelines (**Table 1)**.

Variant Allele Frequency (VAF)

The variant allele frequency (VAF) on of the variant on tumour-sequencing is not categorical but can give a strong identification as to the likelihood of it being germline in origin. The VAF identifies the proportion of reads in which the mutation was identified ie a VAF of 0.07 indicates the variant was present in 7% of reads. The majority of cancer susceptibility genes are autosomal dominant, in which individuals will have one wild type allele and one altered allele. Hence, in any population of cells, variants of a heterozygous mutation of germline origin would be expected to be present in 50% of reads (VAF of 0.50): thus contamination of tumour cells with normal cells would not influence this proportion. Conversely, somatic (acquired) heterozygous mutations would only be present at VAF of 0.5 in a pure populations of tumour cells: contamination with non-tumour cells and clonal heterogeneity mean that typically heterozygous somatic mutations have VAF or much less than 0.5. However, tumour polyploidy and aneuploidy, as well as PCR artefacts of DNA analysis, can impact on VAF estimate for both somatic and constitutional mutations, so caution is required in inferences from VAF.

Tumour/germline paired analysis: findings from MSK-IMPACT dataset

The MSK-IMPACT dataset comprised 17152 tumour-germline pairs, with which we undertook a quasi-"tumour-only" analysis, using the germline sequencing to validate our inferences. D=Tumour-identified variants for 64 genes associated with cancer susceptibility were filtered: we then retained only variants defined as pathogenic or likely pathogenic variants of tumour VAF >0.2 (for small indels) and >0.3 (for single nucleotide variants). This left a total of 9222 tumour-detected pathogenic variants

(of which 1442 true germline pathogenic variants). We then only retained genes of clear clinical utility in which there was a> 10% likelihood of an identified variant being of germline origin. This was selected, as in many countries, a threshold of a 10% chance of an individual carrying a pathogenic variant in a cancer susceptibility gene is a widely accepted threshold for undertaking germline testing. This resulted in exclusion of the vast majority of variants, leaving only 1,042 variants in 17,152 tumours that would require "germline confirmation". Of these, 653 were true germline variants. If we assume the MSK sample is representative of our oncology patients undergoing large somatic panel testing, this suggests that by applying these guidelines, ~6.5% of patients would have a variant requiring germline follow-up, of which ~63% would be of true germline origin. Thus, clinically actionable germline pathogenic variants would be identified in ~4% of patients in whom such panels are undertaken (1).

Detection of TP53 mutations in tumour tissue

TP53 represents a particular consideration as somatic mutations in this gene occur at high frequency across solid tumours . Germline mutations in this gene are rare, but potentially devastating, resulting in Li-Fraumeni syndrome, with susceptibility to a wide range of paediatric and adult-onset tumours. In the MSK-IMPACT analysis, particular consideration was therefore given to the best way to identify the potential germline carriers from the large number of those with somatic pathogenic variants in TP53. Overall, approximately , ~85% of all tumours carry a TP53 mutation with ~17% of tumours in the MSK-IMPACT analysis (2930/ 17,152) carrying a TP53 mutation passing the filters of high VAF and pathogenic/likely. However, only 1% of these proved to be of germline origin (30/2930). From subgroup analysis, the 'germline conversion rate' could be increased to 11.7% by restricting germline assessment to those with non-brain tumours aged <30 years at diagnosis. Although some of the true-positive pathogenic germline variants are missed, it is not feasible to perform germline follow-up on all of the tumour-detected TP53 mutations and the recommendation from the ESMO group was that germline analysis for TP53 be thus age-restricted. Similarly VHL is highly somatically mutated in renal

tumours and APC in gastro-intestinal tumours, meaning paradoxically that tumour-detected mutations in these genes are more likely to be of true germline origin when detected "off-tumour". The full list of recommendations are shown in **Table 2**.

Pitfalls

There are a number of cautions with the use of tumour sequencing to identify germline mutations.

- The first consideration is the template of the panel. The majority of large tumour-based panels will encompass the entirety of a gene, but some will only target part of a gene, or hotspot regions.
- The second consideration is the coverage: there may be low coverage of certain genes or gene regions such that a germline mutation present is missed
- The types of variants that are detectable is also important. In some genes, a significant proportion of germline pathogenic variants will be large duplications/deletions. Such variants cannot be reliably detected using tumour DNA as the fixation process causes DNA fragmentation reducing the fidelity for the detection of such variants. This is of particular consideration with the BRCA1/BRCA2 genes, in which 15-18% of pathogenic germline variants in the UK population are exon-gene level deletions/duplication. Many of these would be missed in tumour-only sequencing.
- Finally, eligibility for germline testing must be considered, especially in those with off-tumour associations. The availability of such tumour-based testing has often predated guideline changes in who can access germline testing. Most countries will have specific criteria for germline testing, based on tumour specifics or family criteria, such as diagnosis with triple negative breast cancer, or a strong family history of multiple cases. It is therefore possible that in some countries, confirmatory germline testing may not be available in a clinical genetics service to individuals. As acceptance of this testing becomes more widespread,

accessibility to such testing will hopefully improve but at present such individuals may not be able to access confirmatory testing.

Genomics Tumour Advisory Board (GTAB)/MDT

The aim of the ESMO recommendations was to provide guidance to enable laboratories to enact automated pipelines for germline-focused analysis of tumour-only sequence findings, to expedite patient pathways. To ensure robust, consistent communication of potential germline findings between molecular diagnostics, oncology and genetics clinicians, automated multidisciplinary channels must be established. With more complex findings or nuanced clinical scenarios, the decisions over which mutations may be clinically actionable may require "germline review" ahead of patient contact: this should be undertaken in a molecular tumour board or GTAB. The suggested core membership for a GTAB is recommended to include: clinical scientists, oncologists +/- haematologists, clinical geneticists and molecular pathologists. Whilst it would not be necessary to review those with straightforward results, for those with a more complex germline mutation, this forum provides the opportunity for case discussion, eligibility review and clinical recommendations.

Plasma-derived Mutations

One of the biggest advances in the diagnostic pathway for patients with disease in sites that is difficult to, or unable to be biopsied has been the development of technology and assays to detect circulating tumour DNA (3, 4). Currently, this has been used to identify targetable mutations in patients with relapsed disease, or to assess for minimum residual disease, to identify those with a higher risk of relapse (5). It is also of potential interest in identifying certain cancers at an earlier stage, or to guide diagnostic workup in patients present with carcinomas of unknown origin. However, there are a number of limitations with the currently available methods that require further consideration.

Tumour behaviour

There is significant variability by tumour and stage, in the likelihood of detecting ctDNA in plasma. There is a higher chance of detecting ctDNA in those patients with advanced metastatic disease compared with early disease in most tumour types, but some tumour types such as CNS tumours, pancreatic cancer and ovarian tumours may not be detectable, even at late stages. Conversely, colorectal, lung and prostate cancer are more likely to have detectable ctDNA.

Clonal Haematopoiesis (CH)

Clonal haematopoiesis occurs when haematopoietic stem cells or progenitor cells acquire somatic mutations, leading to a clonal population of these cells. These are detectable in patients with and without cancer, and are particularly associated with increasing age and the presence of environmental mutagens such as smoking. Such variants have been found in ~1% of population samples aged <50 years, increasing to approximately 5% of those aged 60-69 and 10% in those aged 70 years or older(6-8). The mutations can occur in a wide range of genes, including those usually associated with cancer (such as ASXL1, TET2 and TP53), and therefore can be wrongly attributed as representing tumour cells.

Retrospective analysis of the MSK-IMPACT samples with solid tumours suggested CH-associated mutations were identified in 4628 (26.5%) of patients, of which 912 would have had at least one CH-derived mutation reported as a somatic tumour mutation. Just under half of these mutations (49.7%) were defined as oncogenic or likely oncogenic by Onco-KB, which could have led to inappropriate assumptions about their role in the malignancy and suitability as a target for treatment (9). This was avoided in MSK-IMPACT by undertaking matched white blood cell sequencing, a technique that has been employed by a number of recent studies to allow filtering out of variants present in both to exclude CH, but is not undertaken in routine clinical practice (10, 11). Razavin et al prospectively

assessed cfDNA and matched white cell DNA in 124 patients with metastatic cancer, all with contemporary tissue biopsies; and 47 controls without cancer. They undertook high-intensity sequencing to a depth of >60 000X. They confirmed that the majority of cfDNA mutations identified in sequencing were due to CH (53.2% in those with cancer and 81.6% in controls), emphasising the importance of excluding such mutations to ensure accurate variant interpretation (10). Of note, while many of these may be present at low levels, these can occur at higher levels and without matched testing to exclude CH, there is a real risk that these could cause issues for interpretation in routine clinical practice and should always be considered, particularly in older patients.

Hypermutated tumours

A particular issue arises with tumours that are hypermutated or ultramutated, such as those associated with mutations in the mismatch repair (MMR) or POL-E genes (12, 13). In these patients, panel testing often produces a lengthy list of pathogenic variants, including multiple possible causative germline variants. In these individuals, there are also likely to be multiple variants in potentially druggable genes identified. However, such variants are less likely to be indicative of a response, as the underlying driving mutation will be the MMR or POL mutation responsible for the hypermutation. These patients are potential candidates for immunotherapy, based on the genomic instability of the tumour (14). Hypermutation may also be induced by specific environmental mutagens such as UV light (melanoma) or cigarette smoking (NSCLC) (15).

Aetiologic implication of “associated” germline variants

PARP inhibitors target cells with via synthetic lethality which have deficiency in homologous recombination. The best-recognised mechanism leading to cellular homologous recombination deficiency (HRD) is initial germline mutation in a key HRD gene such as BRCA1 or BRCA2 followed by somatic loss of the second copy via loss or mutation of the normal allele. Thus the best assay to assess PARP sensitivity is one that captures or measures HRD, such as quantitation of a genomic mutational patterns (signatures) characteristic of HRD, Loss of Heterozygosity (LOH) or Telomeric Allelic

Imbalance (TAI). In practice, many laboratories take the presence of a germline BRCA1/BRCA2 mutation as synonymous with causative implication of BRCA-related HRD in oncogenesis in that tumour. A number of groups have examined how this 'etiologic index' varies across tumours in which mutations in BRCA1/BRCA2 are detected (16, 17). For strongly associated tumour times such as serous ovarian cancer, a detected germline BRCA1 mutation has a high etiologic index (ie is highly likely to be driving oncogenesis). For more weakly associated tumour types, such as cervical cancer, a detected germline BRCA1 mutation has a much lower etiologic index (ie is much less likely to be relevant to oncogenesis in that tumour and more likely to be an incidental finding). In quantifying the implication of the germline HRD-gene mutation in oncogenesis, the etiologic index is of course thus also a predictor of likelihood of effect of a PARP inhibitor. This is a pitfall extending beyond the PARP-HRD paradigm: a somatic/germline mutation detected in a non-associated tumour type is much more likely in that tumour to be a passenger/ incidental finding irrelevant to oncogenesis and thus should not be taken as automatically indicative of likely responsiveness of the respective partner drug.

Conclusion

As the systemic management of malignancies moves more toward targeted agents, it is increasingly considered standard of care to undertake large, genomic panels to guide treatment choice which are typically run tumour-only. Such panels also offer opportunity to identify possible germline pathogenic variants in patients, who can then be offered germline testing to confirm the origin. In addition to germline testing driven by personal and family caner history, germline-focused tumour-analysis offers the possibility of identifying an additional sizeable population of individuals carrying an inherited cancer susceptibility pathogenic variant, both as the cause of their malignancy or as an incidental finding. In each case, this could potentially allow future malignancies in the patient and their wider family to be averted. Over time, such testing could also help identify much of the wider population

with an inherited cancer susceptibility in a far more inclusive way than the current limited options for accessing germline testing, much of which is based on family history criteria. However, there are a range of considerations that must be addressed for such testing and interpretation to be widely implemented.

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Table 1: Recommendations for genes to be included for germline-focused analysis and triggering of germline sample laboratory confirmation.

	Any tumour type		**Associated tumour type only**
Tumour arising any age	*BRCA1* *BRCA2* *BRIP1* *MLH1* *MSH2* *MSH6* *PALB2* *PMS2* *VHL**	*RAD51C* *RAD51D* *RET* *SDHA* *SDHAF2* *SDHB* *SDHC* *SDHD* *TSC2* *MUTYH***	*FLCN* *FH* *BAP1* *POLE*
Tumour arising age<30 only	*RB1* *APC*		*TP53**** *NF1*

*renal tumours to be excluded, ** *MUTYH* should be included for germline-focused tumour analysis but reporting and germline follow-up testing should only follow on detection of two pathogenic variants, *** brain tumours to be excluded,

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Table 2: Recommendations from the ESMO Guidelines on Germline-focussed Analysis of Tumour-only Sequencing

1	Germline-focussed tumour analysis should be carried out in all laboratories as part of routine analysis of a large tumour panel
2	Germline-focussed tumour analysis can be delivered via an automated pipeline so as not to add substantial additional manual work, cost or delay to tumour analysis
3	Variants should be flagged which are (i) predicted to result in protein truncation in genes acting through loss-of-function and/or (ii) classified as Pathogenic/Likely Pathogenic via a well-maintained, comprehensive and curated clinical resource (ClinVar is recommended)
4	Germline-focussed tumour analysis can be restricted to variants of VAF >30% (SNVs) or >20% (small insertions/deletions). Local validation will be required to confirm the accuracy of tumour VAF estimates, especially for PCR-based NGS methodologies
5	Samples known or suspected to be hypermutated should be included for germline-focussed tumour analysis
6	Germline-focussed tumour analysis in the off-tumour context should be restricted to 'High Actionability-CSGs'
7	Recessively acting 'High Actionability CSGs' (currently *MUTYH* alone) should be included for germline-focussed tumour analysis but reporting and germline follow-up testing should be undertaken only on detection of two pathogenic variants
8	Germline-focussed tumour analysis of 'standard actionability' CSGs should be restricted to the on-tumour setting
9	'Standard actionability'-CSGs included for germline-focussed tumour analysis can be restricted to genes of high penetrance
10	Germline-focussed tumour analysis can be restricted to gene-scenarios for which the germline conversion rate is >10%. For selected genes, it may therefore be appropriate to restrict germline-focussed tumour analysis to just those tumours arising age <30 yrs
11	Formal variant review and classification should be undertaken by an experienced clinical scientist before initiation of patient re-contact and/or germline testing
12	Before analysis of their germline sample for the pathogenic variant, adequate information should be provided to the patient regarding the implications of germline testing, along with documentation of their consent
13	The tumour-observed pathogenic variant should be analysed in an appropriate germline sample (lymphocytes, salivia/buccal swab, normal tissue) in a laboratory accredited for germline analysis
14	A patient in whom a germline pathogenic variant is detected should be referred to a specialist genetics service for long term follow-up and management of the family
15	A normal/negative tumour sequencing result should not be taken as equivalent to a normal/negative germline result unless robust analysis of dosage has been carried out. This distinction is particularly important for genes such as *BRCA1* and *MSH2*, for which whole exon deletion/duplications constitute a substantial proportion of pathogenic variants
16	Re-evaluation of this workflow, revised analyses and update of these recommendations should be undertaken at least 2-yearly. Reanalysis should include updated data regarding pathogenicity of variants and penetrance of CSGs, along with review of thresholds for 'germline conversion rates' and VAF cut-offs

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