Matthew Evans (matthew.evans7@nhs.net)
06/09/2022

FRCPath Part 2 Histopathology Molecular Pathology OSPE

Contents

OSPE 1: Non-small cell carcinoma

A 56 year old woman underwent left upper lobectomy two years previously for lung adenocarcinoma. She now re-presents with metastatic disease. The oncologists request molecular testing on the previously resected lung cancer to guide systemic treatment. Read the molecular report below.

MOLECULAR PRE-ANALYTIC ASSESSMENT:

Representative tumour block: 1B
Neoplastic cell percentage: 10%
Percentage area necrosis: 0%

PD-L1 IMMUNOHISTOCHEMISTRY:

PD-L1 immunohistochemistry has been performed on new sections from the representative tumour block using the Dako 22C3 PD-L1 PharmDX antibody.

The section contains more than 100 viable tumour cells so is suitable for assessment.

100% of the tumour cells show convincing membranous staining for PD-L1.

PD-L1 tumour proportion score (TPS) is 100%.

The tumour is regarded as being HIGH POSITIVE for PD-L1.

GENOMIC TESTING:

REPORT SUMMARY:

Detection of EGFR variant, c.2239_2248delinsC p.(Leu747_Ala750delinsPro)
No evidence of variants associated with therapeutic or prognostic implications in BRAF, KRAS, MET, ALK, ROS1, RET, NTRK1, NTRK2 and NTRK3 genes

Janet Smith has metastatic lung adenocarcinoma. Molecular testing of the EGFR, BRAF, KRAS, MET, ALK, ROS1, RET, NTRK1, NTRK2 and NTRK3 genes has been requested to facilitate clinical management. Percentage of neoplastic cells, reported by referring centre, is estimated to be 10% in the FFPE sections provided (Block Ref: 22HS04158C 1B).

Molecular analysis detected EGFR gene (NM_005228.5) sequence variant, c.2239_2248delinsC p.(Leu747_Ala750delinsPro).

Molecular analysis has not detected any clinically actionable variants in the BRAF, KRAS, MET (Ex14 skipping and amplification), ALK, ROS1, RET, NTRK1, NTRK2 and NTRK3 genes.

These results are dependent upon the information supplied being correct and complete.

Basis of test:

The Ion Torrent Genexus Next Generation Sequencing (NGS) platform has been used to sequence hotspot regions of genes covered by the pan cancer Oncomine Precision Assay (OPA) GX5 w2.6.0 DNA Panel. Data analysis was performed using the Ion Torrent Genexus Software 6.2.1. This test will detect sequence variants as detailed in attached pdf. Sensitivity of assay is 5% sequence variation in background wildtype DNA for a tumour cell content of 20%.

The NextSeq next generation sequencing (NGS) platform has been used to sequence the genes covered by the Archer FusionPlex® NHS vs.1 solid tumour NGS panel (see attached pdf). Data analysis was performed using the bioinformatics pipeline Archer Analysis 6.2.7. This test will detect fusion partners involving the exons listed within the targeted genes. Analysis requires a minimum RNA quality as assessed on the expression of 4 control genes (CHMP2A, GPI, RAB7A, and VCP).

Please note that tumour only analysis cannot exclude that detected variants may be germline in origin. Variant nomenclature used according to HGVS guidelines vs 20.05. DNA and RNA has been stored.

Answer the following questions.

1. Explain why PD-L1 immunohistochemistry has been undertaken in this case, and describe why PD-L1 is important . *[3 marks]*
2. Explain what is meant by the nomenclature c.2239_2248delinsC p.(Leu747_Ala750delinsPro). You do not need to know the full names of the amino acids. *[2 marks]*
3. Explain the function of the EGFR protein, and how activating *EGFR* variants can drive cancer development. *[4 marks]*
4. Next-generation sequencing (NGS) was used to identify the *EGFR* variant described. State two other techniques which could be used to identify an *EGFR* variant. *[2 marks]*
5. The neoplastic cell percentage was estimated to be 10%. The assays used for testing were developed to be used for samples with a minimum neoplastic cell percentage of 20%. Explain the significance of having a neoplastic cell percentage lower than the minimum required for the assay in general terms, and in this case specifically. *[3 marks]*
6. State one possible way of increasing the neoplastic cell percentage of a sample, and explain how this works. *[2 marks]*
7. Explain why separate DNA and RNA next-generation sequencing (NGS) panels have been used in this case. *[3 marks]*
8. This patient is treated with afatinib (a tyrosine kinase inhibitor, TKI). She responds well, but 12 months later she clinically deteriorates and imaging reveals new metastases. What is the most likely explanation for this change, and explain what would be next course of action. *[4 marks]*

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OSPE 1: Non-small cell carcinoma (answers)

Answer the following questions.

1. Explain why PD-L1 immunohistochemistry has been undertaken in this case, and describe why PD-L1 is important . *[3 marks]*
   PD-L1 expression predicts response to immune checkpoint inhibitors. [1 mark]
   Cancer cells are heavily mutated and so express abnormal proteins called neoantigens. These can be recognised as being foreign by the immune system, which can attack the tumour cells. [1 mark]
   However, tumour cells can express immune checkpoints like PD-L1, which switch off the immune response. Immune checkpoint inhibitors prevent this inhibition, allowing the immune system to attack the tumour cells. [1 mark]
2. Explain what is meant by the nomenclature c.2239_2248delinsC p.(Leu747_Ala750delinsPro). You do not need to know the full names of the amino acids. *[2 marks]*
   The nucleotides between base positions 2239 and 2248 in the coding DNA of the *EGFR* gene have been deleted, and a cytosine residue inserted in their place. [1 mark]
   This has resulted in the deletion of the amino acids between positions 747 (leucine) and 750 (alanine), which have been replaced by a proline. [1 mark]
3. Explain the function of the EGFR protein, and how activating *EGFR* variants can drive cancer development. *[4 marks]*
   EGFR is a receptor tyrosine kinase. [1 mark]
   It binds growth factor, which causes activation of the protein by dimerisation and autophosphorylation. [1 mark]
   This triggers activation of intracellular signalling pathways which ultimately drive cell growth and proliferation. [1 mark]
   Activating variants in the *EGFR* gene result in the expression of a protein which is constitutively active, causing uncontrolled pro-growth and pro-survival signalling even in the absence of growth factor binding. [1 mark]
4. Next-generation sequencing (NGS) was used to identify the *EGFR* variant described. State two other techniques which could be used to identify an *EGFR* variant. *[2 marks]*
   Any of: real-time PCR, direct/Sanger sequencing, pyrosequencing, immunohistochemistry. ISH and reverse transcription PCR would not be appropriate answers.
5. The neoplastic cell percentage was estimated to be 10%. The assays used for testing were developed to be used for samples with a minimum neoplastic cell percentage of 20%. Explain the significance of having a neoplastic cell percentage lower than the minimum required for the assay in general terms, and in this case specifically. *[3 marks]*
   The neoplastic cell percentage is the percentage of nucleated cells in the sample which are neoplastic. If the neoplastic cell percentage is lower than the minimal stipulated for a test, it is possible that a variant which is present could be missed, resulting in a false negative result. [1 mark]
   Here, even though the neoplastic cell percentage is low, the *EGFR* variant detected can be considered genuine. [1 mark]
   It is possible that the negative findings for this case could represent false negatives. However, because driver alterations are usually mutually exclusive, the fact that the *EGFR* variant was detected means that this is highly unlikely. [1 mark]
6. State one possible way of increasing the neoplastic cell percentage of a sample, and explain how this works. *[2 marks]*
   Macrodissection could be used. [1 mark]
   This involves marking on the H&E an area of the tissue which contains the highest neoplastic cell. The lab then uses this as a guide to remove the unmarked tissue for the sections used for testing. This enriches the neoplastic cell percentage by removing non-neoplastic tissue. [1 mark]
7. Explain why separate DNA and RNA next-generation sequencing (NGS) panels have been used in this case. *[3 marks]*

The DNA panel has been used to look for small variants and the RNA panel to look for large-scale rearrangements. *[1 mark]*
Gene rearrangements frequently occur within intron sequences which are often long and repetitive, and therefore difficult to sequence. *[1 mark]*
If DNA is sequenced, it will be necessary to sequence these long intronic sequences, and so there is a risk of missing rearrangements. However, RNA does not contain intronic sequences and so it is easier to detect rearrangements by sequencing RNA. *[1 mark]*

8. This patient is treated with afatinib (a tyrosine kinase inhibitor, TKI). She responds well, but 12 months later she clinically deteriorates and imaging reveals new metastases. What is the most likely explanation for this change, and explain what would be next course of action. *[4 marks]*

   The most common reason for secondary resistance to anti-EGFR TKIs is the acquisition of a secondary *EGFR* T790M variant. [1 mark]
   It is important that the patient receives repeat *EGFR* variant testing to determine whether a T790M variant has been acquired. [1 mark]
   This can be via plasma testing or repeat tumour biopsy. [1 mark]
   If a T790M variant is detected, the patient can be changed to a different TKI (Osimertinib), usually with very good effect. [1 mark]

OSPE 2: Colorectal cancer

Read the histology report below.

SPECIMEN TYPE:
Liver biopsy

CLINICAL DETAILS:
Multiple liver nodules. ? metastases.

MACROSCOPY:
A single core of cream-coloured tissue measuring up to 8 mm.

MICROSCOPY:
Section shows a core of liver parenchyma which is largely effaced by malignant cells. Many contain luminal necro-inflammatory debris.

Immunohistochemistry shows the following:

Cytokeratin 20:	Positive
Cytokeratin 7:	Negative
CDX2:	Positive
PAX8:	Negative
ER:	Negative (Quick score 0/8)

DIAGNOSIS:
Liver biopsy – Adenocarcinoma, consistent with colorectal origin

MOLECULAR PRE-ANALYTIC ASSESSMENT:

Representative tumour block:	1A
Neoplastic cell percentage:	20%
Percentage area necrosis:	5%

MMR IMMUNOHISTOCHEMISTRY:
The tumour cells show loss of nuclear expression of the mismatch repair proteins MLH1 and PMS2. There is preserved nuclear staining for MSH2 and MSH6.

There is immunohistochemical evidence of mismatch repair deficiency.

This is regarded as an MMR-deficient (dMMR) neoplasm which is expected to show microsatellite stability (MSI).

This pattern of mismatch repair deficiency can occur because of sporadic or germline mechanisms. BRAF variant testing is underway to provide further information.

MOLECULAR TESTING:
REPORT SUMMARY:
A colorectal cancer sample was tested for variants in the KRAS, NRAS and BRAF genes:
KRAS: No variants detected
NRAS: No variants detected
BRAF: No variants detected

TECHNICAL DETAILS:

Testing modality: Real-time PCR
Testing platform: Biocartis Idylla™
Assay: Idylla™ KRAS Mutation Test

DETECTABLE VARIANTS:
This assay is capable of detecting the following variants in the *KRAS* gene:
Codon 12 (exon 2): G12C (c.34G>T), G12R (c.34G>C), G12S (c.34G>A), G12A (c.35G>C), G12D (c.35G>A) and G12V (c.35G>T)
Codon 13 (exon 2): G13D (c.38G>A)
Codon 59 (exon 3): A59E (c.176C>A), A59G (c.176C>G) and A59T (c.175G>A)
Codon 61 (exon 3): Q61K (c.181C>A), Q61K (c.180_181TC>AA), Q61L (c.182A>T), Q61R (c.182A>G), Q61H (c.183A>C) and Q61H (c.183A>T)
Codon 117 (exon 4): K117N (c.351A>C) and K117N (c.351A>T)
Codon 146 (exon 4): A146P (c.436G>C), A146T (c.436G>A) and A146V (c.437C>T)

This assay has a limit of detection of 5% or lower for all detectable KRAS variants.

RESULTS:
Real-time PCR has revealed no evidence of a pathogenic variant in exons 2, 3 or 4 of the KRAS gene.

RESULT: NO EVIDENCE OF A PATHOGENIC VARIANT IN EXONS 2, 3 OR 4 OF THE KRAS GENE

(Genbank accession number: NM_004985.3; variant nomenclature according to HGVS guidelines)

TECHNICAL DETAILS:
Testing modality: Real-time PCR
Testing platform: Biocartis Idylla™
Assay: Idylla™ NRAS-BRAF Mutation Test (real-time PCR)

DETECTABLE VARIANTS:
This assay is capable of detecting the following variants in the *NRAS* gene:
Codon 12 (exon 2): G12C (c.34G>T), G12S (c.34G>A), G12A (c.35G>C), G12D (c.35G>A) and G12V (c.35G>T)
Codon 13 (exon 2): G13D (c.38G>A), G13V (c.38G>T) and G13R (c.37G>C)
Codon 59 (exon 3): A59T (c.175G>A)
Codon 61 (exon 3): Q61K (c.181C>A), Q61L (c.182A>T), Q61R (c.182A>G), Q61H (c.183A>C) and Q61H (c.183A>T)
Codon 117 (exon 4): K117N (c.351G>C) and K117N (c.351G>T)
Codon 146 (exon 4): A146T (c.436G>A) and A146V (c.437C>T)

This assay is capable of detecting the following variants in the *BRAF* gene:
V600E: c.1799T>A and c.1799_1800delinsAA
V600D: c.1799_1800delinsAC
V600K: c.1798_1799delinsAA
V600R: c.1798_1799delinsAG

This assay has a limit of detection of 5% or lower for the most prevalent *BRAF* and *NRAS* variants.

NRAS RESULTS:
Real-time PCR has revealed no evidence of a pathogenic variant in exons 2, 3 or 4 of the NRAS gene.

RESULT: NO EVIDENCE OF A PATHOGENIC VARIANT IN EXONS 2, 3 OR 4 OF THE NRAS GENE

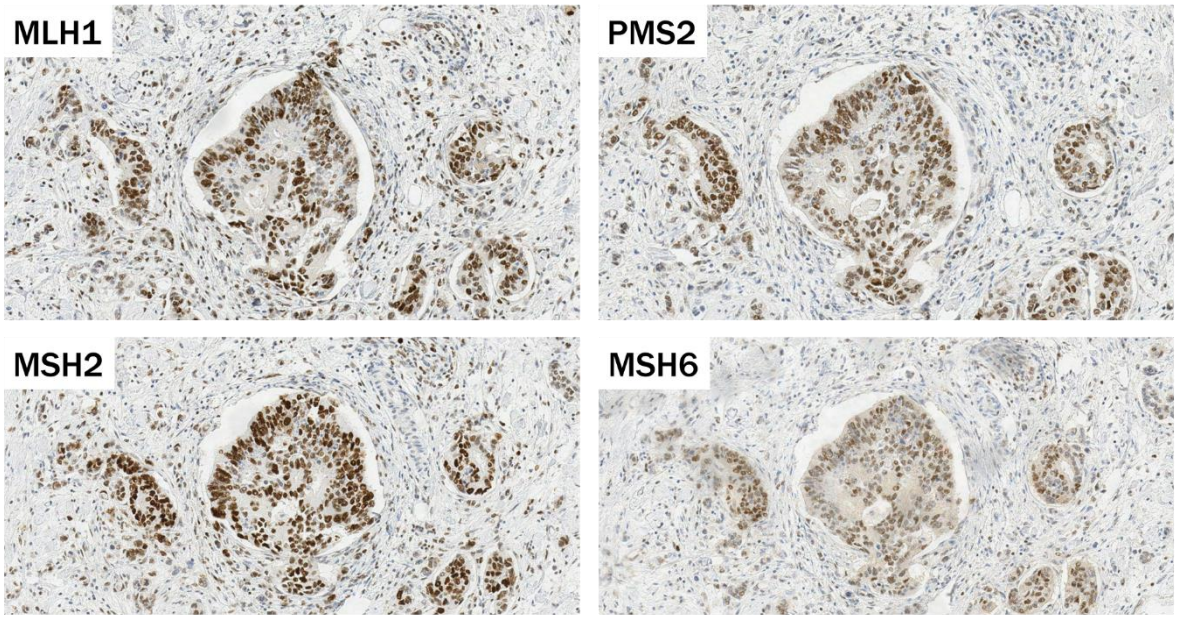
BRAF RESULTS:
Real-time PCR has revealed no evidence of a pathogenic variant in codon 600 of the BRAF gene.

RESULT: NO EVIDENCE OF A PATHOGENIC VARIANT IN CODON 600 OF THE BRAF GENE

(Genbank accession numbers: NM_002524.4 and NM_004333.5; variant nomenclature according to HGVS guidelines)

Answer the following questions.

1. State three reasons for undertaking MMR immunohistochemistry in colorectal cancer. *[3 marks]*
2. Explain what MSI testing is, and explain how it relates to MMR immunohistochemistry. *[4 marks]*
3. Explain the significance of the MMR protein expression pattern seen here with respect to the possibility of Lynch syndrome, in light of the results of molecular testing provided. What further testing is required, and what would the significance of its results? *[5 marks]*
4. Describe the pattern of MMR expression seen in the following (different) case. Explain its significance both in terms of the likelihood of a familial cancer syndrome and in terms of its implications for the patient's treatment. *[7 marks]*



5. This patient is being considered for systemic therapy. How would the results of *KRAS*, *NRAS* and *BRAF* variant testing presented here affect the systemic therapy which the patient could receive? *[2 marks]*
6. Real-time PCR has been used here rather than next-generation sequencing (NGS). State two advantages and two disadvantages of real-time PCR compared to next-generation sequencing (NGS). *[4 marks]*
7. The real-time PCR assays detect only pathogenic variants. Explain what is meant by 'pathogenic' variant. *[1 mark]*
8. Pathogenic variants in *KRAS*, *NRAS* and *BRAF* tend to be mutually exclusive. Explain what this means and why this is the case. *[3 marks]*

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OSPE 2: Colorectal cancer (answers)

Answer the following questions.

1. State three reasons for undertaking MMR immunohistochemistry in colorectal cancer. *[3 marks]*
   Any of:
   To screen for Lynch syndrome
   To determine eligibility for immune checkpoint inhibitors
   To determine the suitability of 5FU-containing chemotherapy
   To determine prognosis (dMMR colorectal cancers are associated with more indolent behaviour)
2. Explain what MSI testing is, and explain how it relates to MMR immunohistochemistry. *[4 marks]*
   Microsatellites are short repetitive DNA sequences found throughout the genome. [1 mark]
   The MMR system helps to keep the length of each microsatellite constant. If the MMR system is not properly functioning, microsatellite can expand or contract, resulting in the two alleles of the microsatellite being of different lengths – this is microsatellite instability. [1 mark]
   MSI testing involves assessing the lengths of a set of microsatellites to infer the presence of an underlying MMR defect. [1 mark]
   In general, MSI testing and MMR IHC are highly concordant and can be used interchangeably in colon cancer. [1 mark]
3. Explain the significance of the MMR protein expression pattern seen here with respect to the possibility of Lynch syndrome, in light of the results of molecular testing provided. What further testing is required, and what would the significance of its results? *[5 marks]*
   There is loss of MLH1 and PMS2 expression with preserved MSH2 and MSH6 expression. This could represent either a germline MMR defect (i.e. Lynch syndrome) or a sporadic/somatic mechanism of MMR deficiency. [1 mark]
   Somatic MMR deficiency is most often caused by hypermethylation of the promoter region of the *MLH1* gene. [1 mark]
   In colorectal cancer, it is known the presence of *BRAF* V600E variants is associated with the presence of *MLH1* promoter hypermethylation. [1 mark]
   No *BRAF* V600E variant has been detected here and so it is still not clear whether the MMR defect is germline or somatic. [1 mark]
   MLH1 promoter methylation testing will be required as a next step. [1 mark]
4. Describe the pattern of MMR expression seen in the following (different) case. Explain its significance both in terms of the likelihood of a familial cancer syndrome and in terms of its implications for the patient's treatment. *[7 marks]*
   The tumour cells show preserved nuclear staining for MLH1, PMS2, MSH2 and MSH6. [4 marks]
   This is therefore an MMR proficient tumour. [1 mark]
   The patient is unlikely to have underlying Lynch syndrome, although the possibility of a different familial cancer syndrome cannot be excluded. [1 mark]
   The patient is unlikely to benefit from immune checkpoint inhibitors. [1 mark]
9. This patient is being considered for systemic therapy. How would the results of *KRAS*, *NRAS* and *BRAF* variant testing presented here affect the systemic therapy which the patient could receive? *[2 marks]*
   Pathogenic variants in *KRAS*, *NRAS* or *BRAF* predict resistance to anti-EGFR monoclonal antibody therapy. [1 mark]
   This patient would receive chemotherapy without anti-EGFR monoclonal antibodies. [1 mark]
10. Real-time PCR has been used here rather than next-generation sequencing (NGS). State two advantages and two disadvantages of real-time PCR compared to next-generation sequencing (NGS). *[4 marks]*
    Advantages of real-time PCR: generally faster, generally works with lower-quality tissue, generally has lower neoplastic cell percentage requirement, will only detect variants of known clinical relevance so less complex reports, cheaper to implement, easier to analyse results

Disadvantages of real-time PCR: will only detect pre-specified variants and so will miss some, can generally only look at one (or a couple of genes) at a time, likely to be less tissue-efficient when looking at multiple targets, likely to be less cost-effective when looking at multiple targets or large numbers of cases

11. The real-time PCR assays detect only pathogenic variants. Explain what is meant by 'pathogenic' variant. *[1 mark]*
    A pathogenic variant is one for which there is good evidence of functional impact, and therefore good evidence for a causal relationship with disease. *[1 mark]*
12. Pathogenic variants in *KRAS*, *NRAS* and *BRAF* tend to be mutually exclusive. Explain what this means and why this is the case. *[3 marks]*
    Mutual exclusivity means that a pathogenic variant is seen only in one of the genes (at most). [1 mark]
    KRAS, NRAS and BRAF are all in the same signalling pathway. [1 mark]
    Therefore, if there is an activating variant in one of these genes the signalling pathway will be overactive; acquiring a second activating variant in the same pathway is unlikely to confer a cell a selective advantage over those with only one variant. [1 mark]

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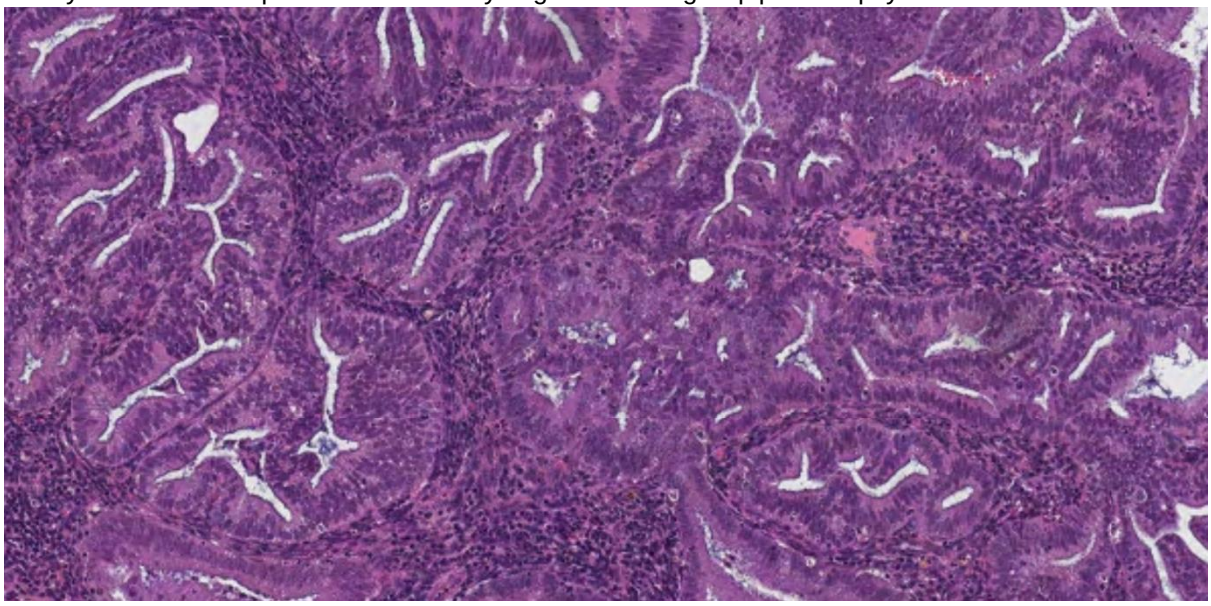
OSPE 3: Endometrial pipelle

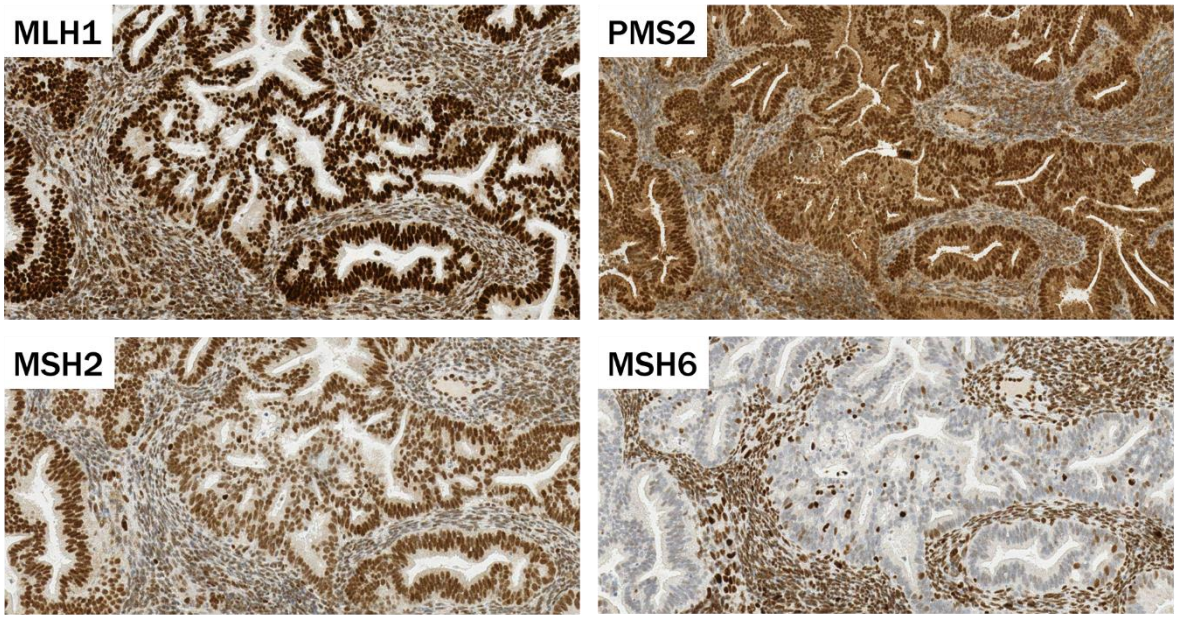
A 47 year old woman presents with heavy vaginal bleeding. A pipelle biopsy is taken:



Immunohistochemistry is undertaken.

p53:	Wild-type expression
ER:	Positive (Quick score 8/8)

Review the MMR immunohistochemistry results:



Answer the following questions.

1. State the diagnosis in this case. *[1 mark]*
2. Explain why p53 shows wild-type expression. *[3 marks]*
3. Describe the findings of MMR immunohistochemistry, and state the most likely underlying molecular alteration. *[5 marks]*
4. Explain the implications of this result both in terms of the likelihood of Lynch syndrome, and in terms of treatment options. *[2 marks]*

5. Explain how a diagnosis of Lynch syndrome can be confirmed in a living patient. *[3 marks]*
6. Describe the pattern of MMR staining which would typically be seen in non-neoplastic tissue from a patient with Lynch syndrome, and explain the reason for this. *[3 marks]*
7. *POLE* variant testing is requested. Explain how detection of a pathogenic *POLE* inactivating variant could account for the MMR staining seen above. *[3 marks]*

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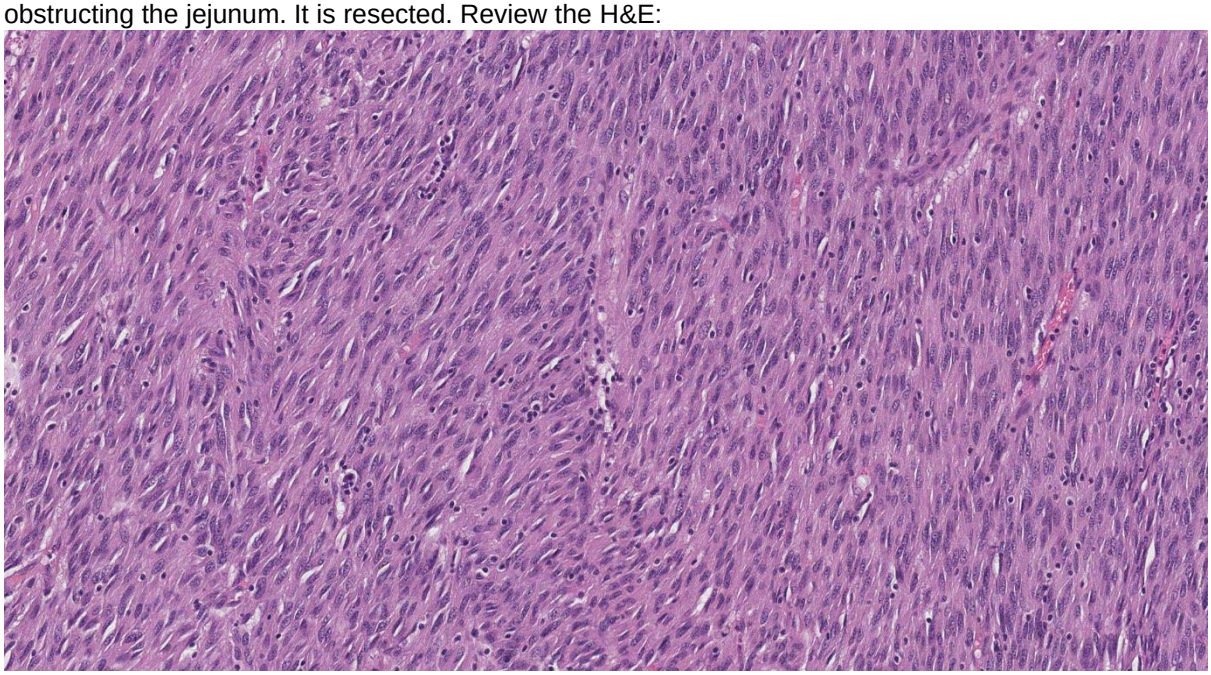
OSPE 3: Endometrial pipelle (answers)

Answer the following questions.

1. State the diagnosis in this case. *[1 mark]*
   Endometrioid adenocarcinoma [1 mark]
2. Explain why p53 shows wild-type expression. *[3 marks]*
   p53 is encoded by *TP53*. [1 mark]
   In the absence of inactivating *TP53* variants, p53 shows a wild-type pattern of expression. [1 mark]
   Low-grade endometrioid adenocarcinomas typically lack inactivating *TP53* variants. [1 mark]
3. Describe the findings of MMR immunohistochemistry, and state the most likely underlying molecular alteration. *[5 marks]*
   The tumour cells show isolated loss of expression of MSH6, with preserved expression of MSH2, MLH1 and PMS2. [4 marks]
   The most likely cause is a germline *MSH6* variant [1 mark], although somatic *MSH6* can rarely be the cause.
4. Explain the implications of this result both in terms of the likelihood of Lynch syndrome, and in terms of treatment options. *[2 marks]*
   This patient is likely to have Lynch syndrome, but confirmatory germline testing is required. [1 mark]
   This patient would be eligible for immune checkpoint inhibitors if systemic treatment is required. [1 mark]
5. Explain how a diagnosis of Lynch syndrome can be confirmed in a living patient. *[3 marks]*
   A peripheral blood sample would usually be taken. [1 mark]
   This would undergo sequencing of the MMR genes. [1 mark]
   A pathogenic variant detected in one of the MMR genes would support the diagnosis of Lynch syndrome. [1 mark]
6. Describe the pattern of MMR staining which would typically be seen in non-neoplastic tissue from a patient with Lynch syndrome, and explain the reason for this. *[3 marks]*
   There would usually be preserved nuclear expression for MLH1, PMS2, MSH2 and MSH6. [1 mark]
   Individuals with Lynch syndrome inherit a non-functional allele of one of the MMR genes but the other allele is intact, meaning that the protein can still be expressed. [1 mark]
   Inactivation of the second allele results in a cancer which, because it lacks any functional alleles, shows MMR immunohistochemical loss. [1 mark]
7. *POLE* variant testing is requested. Explain how detection of a pathogenic *POLE* inactivating variant could account for the MMR staining seen above. *[3 marks]*
   POLE encodes a protein which is involved in proof reading newly replicated DNA and correcting errors. [1 mark]
   Pathogenic inactivating variants in *POLE* results in the accumulation of huge numbers of sequence variants throughout the genome. [1 mark]
   Therefore, detection of a *POLE* inactivating variant increases the odds that an MMR defect detected by immunohistochemistry could be a result of a somatic sequence variant in an MMR gene. [1 mark]

OSPE 4: Small bowel tumour

A 47 year old man presents with small bowel obstruction. At laparotomy, there is a 5 cm mass obstructing the jejunum. It is resected. Review the H&E:



Immunohistochemistry is undertaken.

Cytokeratin AE1/3:	Negative
S100:	Negative
SMA:	Negative
Desmin:	Negative
CD34:	Negative
CD117:	Positive
DOG1:	Positive

Read the molecular report below.

MOLECULAR PRE-ANALYTIC ASSESSMENT:

Representative tumour block:	1E
Neoplastic cell percentage:	60%
Percentage area necrosis:	10%

GENOMIC TESTING:

REPORT SUMMARY:

No evidence of variants associated with therapeutic or prognostic implications in KIT or PDGFRA genes

RNA extract did not meet the minimum quantity threshold required for testing. Repeat sample requested.

Molecular testing of the KIT, PDGFRA, NTRK1, NTRK2 and NTRK3 genes has been requested to facilitate clinical management. Percentage of neoplastic cells, reported by referring centre, is estimated to be 60% in the FFPE sections provided (Block Ref: 21H00041512 1E).

Molecular analysis has not detected any clinically actionable variants in the KIT or PDGFRA genes. The RNA extract did not meet the minimum quantity threshold required for testing. If you still require testing, please send a repeat sample quoting our laboratory reference, D21.70102.

These results are dependent upon the information supplied being correct and complete.

Basis of test:
The Ion Torrent Genexus Next Generation Sequencing (NGS) platform has been used to sequence hotspot regions of genes covered by the pan cancer Oncomine Precision Assay (OPA) GX5 w2.6.0 DNA Panel. Data analysis was performed using the Ion Torrent Genexus Software 6.2.1. This test will detect sequence variants as detailed in attached pdf. Sensitivity of assay is 5% sequence variation in background wildtype DNA for a tumour cell content of 20%.

The NextSeq next generation sequencing (NGS) platform has been used to sequence the genes covered by the Archer FusionPlex® NHS vs.1 solid tumour NGS panel (see attached pdf). Data analysis was performed using the bioinformatics pipeline Archer Analysis 6.2.7. This test will detect fusion partners involving the exons listed within the targeted genes. Analysis requires a minimum RNA quality as assessed on the expression of 4 control genes (CHMP2A, GPI, RAB7A, and VCP).

Please note that tumour only analysis cannot exclude that detected variants may be germline in origin. Variant nomenclature used according to HGVS guidelines vs 20.05. DNA and RNA has been stored.

NTRK FISH TESTING:

NTRK1
FISH analysis of this specimen was performed using the Zytovision ZytoLight SPEC NTRK1 dual colour break apart probe (Z-2167-200). This detects translocations involving the chromosomal region 1q22-q23.1 harbouring the NTRK1 gene. Out of 50 cells observed there were no cells that showed evidence of a NTRK1 gene rearrangement.

NTRK2
FISH analysis of this specimen was performed using the Zytovision ZytoLight SPEC NTRK2 dual colour break apart probe (Z-2205-200). This detects translocations involving the chromosomal region 9q21.32q21.33 harbouring the NTRK2 gene. Out of 50 cells observed there were no cells showing evidence of a NTKR2 gene rearrangement.

NTRK3
FISH analysis of this specimen was performed using the Zytovision ZytoLight SPEC NTRK3 dual colour break apart probe (Z-2206-200). This detects translocations involving the chromosomal region 15q25.3-q26.1 harbouring the NTRK3 gene. Out of 50 cells observed there were 37 cells that showed evidence of NTRK3 gene rearrangement.

Positive for NTRK3 translocation

Answer the following questions.

1. State the diagnosis. *[1 mark]*
2. Here, the pathologist initiated molecular testing on making the diagnosis. State one advantage and one disadvantage of 'reflex testing'. *[2 marks]*
3. DNA-based NGS was successful but RNA-based NGS failed. Explain the likely reason for this. *[1 mark]*
4. State two advantages and two disadvantages of using FISH rather than next-generation sequencing (NGS) for detecting gene rearrangements/translocations/fusions. *[4 marks]*
5. Only 37 out of 50 cells showed evidence of *NTRK3* gene rearrangement. State one explanation that all 50 cells did not show evidence of the rearrangement. *[1 mark]*
6. Explain two ways of identifying the fusion partner of *NTRK3*. *[2 marks]*

7. Explain how a gene rearrangement may result in cancer. *[3 marks]*
8. Explain the significance of the *NTRK* result for this patient. *[1 mark]*

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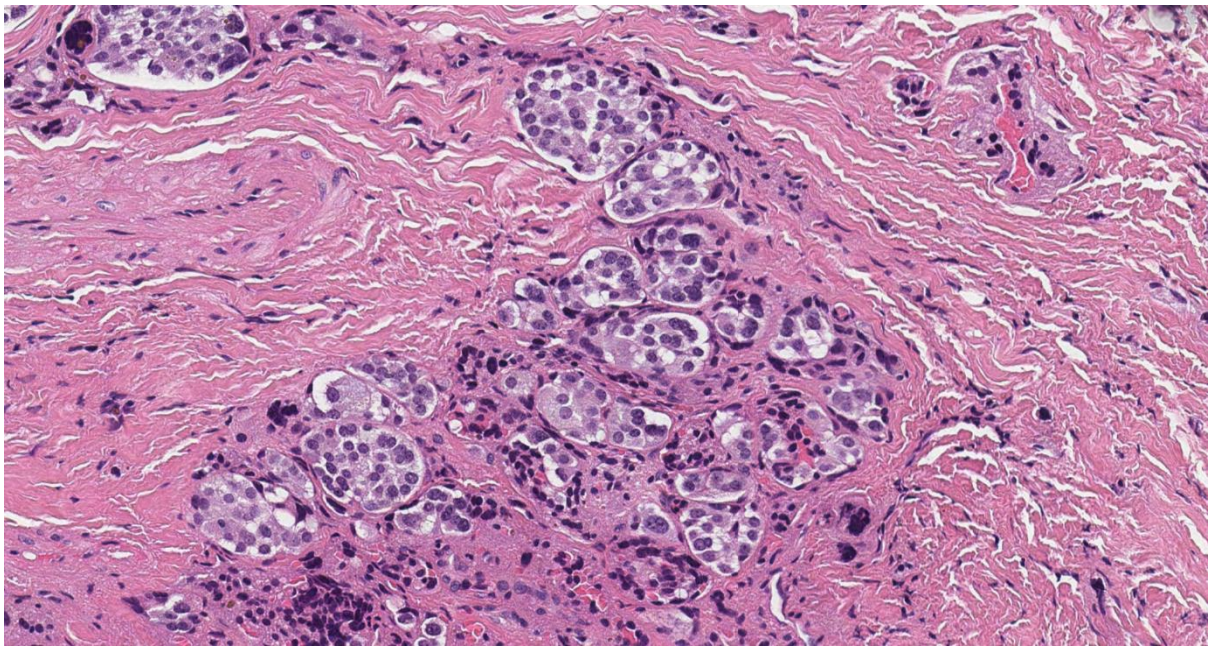
OSPE 4: Small bowel tumour (answers)

Answer the following questions.

1. State the diagnosis. *[1 mark]*
   Gastrointestinal stromal tumour.
2. Here, the pathologist initiated molecular testing on making the diagnosis. State one advantage and one disadvantage of 'reflex testing'. *[2 marks]*
   Advantages: shorter turnaround time to obtain results, lower likelihood of molecular testing being forgotten [1 mark]
   Disadvantages: some results will not be needed, so wastes pathologist/laboratory time and resources [1 mark]
3. DNA-based NGS was successful but RNA-based NGS failed. Explain the likely reason for this. *[1 mark]*
   RNA is inherently less stable than DNA and so RNA-based techniques are more prone to fail [1 mark]
4. State two advantages and two disadvantages of using FISH rather than next-generation sequencing (NGS) for detecting gene rearrangements/translocations/fusions. *[4 marks]*
   Advantages to FISH: often faster, often more reliable with low tumour cell content, less impacted by nucleic acid degradation, more cost-effective with lower throughput, does not require complex data analysis
   Disadvantages to FISH: subjective assessment, more hands-on time in set up and interpretation, can generally only examine one gene at once, less cost-effective and possibly more time consuming with large workloads, risk of false negative results with small-scale rearrangements
5. Only 37 out of 50 cells showed evidence of *NTRK3* gene rearrangement. State one explanation that all 50 cells did not show evidence of the rearrangement. *[1 mark]*
   Any of the following:
   Some of the negative cells were not actually neoplastic cells
   The non-rearranged cells may have represented an alternative clone of cells without an *NTRK3* rearrangement
6. Explain two ways of identifying the fusion partner of *NTRK3*. *[2 marks]*
   Any of the following:
   Use dual-colour dual-fusion FISH probes to assess for possible *NTRK3* fusions.
   Use reverse transcription PCR to identify possible fusion sequences.
   Use DNA-based NGS to attempt to identify the fusion (although this is not the optimal way of using NGS to identify fusions)
7. Explain how a gene rearrangement may result in cancer. *[3 marks]*
   When an oncogene fuses with a highly-expressed gene, the oncogene may also be highly expressed, resulting in intense signalling. [1 mark]
   The nature of the rearrangement may result in the encoded protein being constitutively active. [1 mark]
   The nature of the rearrangement may make the encoded protein resistant to degradation. [1 mark]
8. Explain the significance of the *NTRK* result for this patient. *[1 mark]*
   NTRK rearrangements predict sensitivity to TRK inhibitors. [1 mark]

OSPE 5: Mediastinal mass

A 64 year old man is incidentally found to have a mediastinal mass which is resected. Review the H&E:



Immunohistochemistry is undertaken.

Cytokeratin AE1/3:	Positive
TTF1:	Positive
Synaptophysin:	Positive
Chromogranin A:	Positive
CD56:	Positive
Calcitonin:	Positive

Read the molecular report below.

MOLECULAR PRE-ANALYTIC ASSESSMENT:

Representative tumour block: 1B
Neoplastic cell percentage: 30%
Percentage area necrosis: 0%

GENOMIC TESTING:

REPORT SUMMARY:

Detection of RET variant, c.1804A>G p.(Ile602Val)

Molecular testing of the RET gene has been requested to facilitate clinical management. Percentage of neoplastic cells, reported by referring centre, is estimated to be 30% in the FFPE sections provided (Block Ref: 21H034521 1B).

Molecular analysis detected RET gene sequence variant, c.1804A>G p.(Ile602Val).

These results are dependent upon the information supplied being correct and complete.

Basis of test:

The Ion Torrent Genexus Next Generation Sequencing (NGS) platform has been used to sequence hotspot regions of genes covered by the pan cancer Oncomine Precision Assay (OPA) GX5 w2.6.0 DNA Panel. Data analysis was performed using the Ion Torrent Genexus Software 6.2.1. This test will detect sequence variants as detailed in attached pdf. Sensitivity of assay is 5% sequence variation in background wildtype DNA for a tumour cell content of 20%.

Please note that tumour only analysis cannot exclude that detected variants may be germline in origin. Variant nomenclature used according to HGVS guidelines vs 20.05. DNA has been stored.

Answer the following questions.

1. State the diagnosis. *[1 mark]*
2. Explain the difference between a *RET* variant like the one identified here, and a *RET* structural variant or rearrangement. *[2 marks]*
3. Explain two consequences for the patient of the variant detected here. *[2 marks]*
4. How might it be possible to gain an idea of whether a variant is germline, based on next-generation sequencing (NGS) data from tumour testing. *[2 marks]*
5. Explain why formalin fixation might increase the risk of failure with next-generation sequencing (NGS). *[3 marks]*

OSPE 5: Mediastinal mass (answers)

Answer the following questions.

1. State the diagnosis. *[1 mark]*
   Metastatic medullary thyroid carcinoma.
2. Explain the difference between a *RET* variant like the one identified here, and a *RET* structural variant or rearrangement. *[2 marks]*
   The variant here is a small-scale substitution. [1 mark]
   A structural rearrangement involves the movement of a large amount of DNA to an abnormal location in the genome. [1 mark]
3. Explain two consequences for the patient of the variant detected here. *[2 marks]*
   The patient is eligible for tyrosine kinase inhibitor therapy. [1 mark]
   It raises the possibility that this may be a germline variant, and therefore that the patient may have MEN2. [1 mark]
4. How might it be possible to gain an idea of whether a variant is germline, based on next-generation sequencing (NGS) data from tumour testing. *[2 marks]*
   Whether a variant is germline or not can be confirmed only through testing of non-neoplastic tissue (e.g. blood). [1 mark]
   However, the presence of a very high variant allelic frequency (VAF) on testing of neoplastic tissue raises the possible of a variant being germline. [2 marks]
5. Explain why formalin fixation might increase the risk of failure with next-generation sequencing (NGS). *[3 marks]*
   Formalin induces protein-nucleic acid crosslinks which must be broken in the process of molecular testing. [1 mark]
   This results in fragmentation of nucleic acids which makes testing less likely to succeed. [1 mark]
   Formalin can also directly change the sequence of nucleic acids. [1 mark]

OSPE 6: Breast cancer

Read the histology report below.

SPECIMEN TYPE:
Right axillary lymph node biopsy

CLINICAL DETAILS:
Previous breast cancer. Now enlarged axillary lymph nodes. ? lymphoma ? recurrence.

MACROSCOPY:
Three cores of cream-coloured tissue measuring up to 6, 7 and 7 mm. One core each processed in blocks 1A, 1B and 1C.

MICROSCOPY:
Sections show cores of entirely lesional tissue. This comprises solid sheets of atypical cohesive cells. There is a focal suggestion of gland formation. On immunohistochemistry, the tumour cells are positive for cytokeratin A1/E, cytokeratin 7 and GATA3.

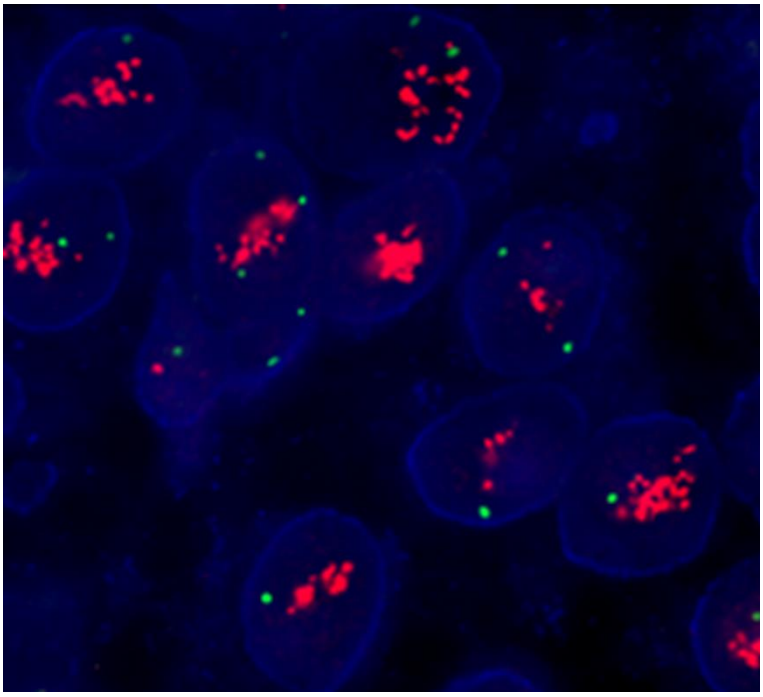
ER: Positive (Quick score 7/8)
PR: Positive (Quick score 5/8)

DIAGNOSIS:
Right axillary lymph node biopsy – Metastatic adenocarcinoma, consistent with breast origin

MOLECULAR PRE-ANALYTIC ASSESSMENT:
Representative tumour block: 1C
Neoplastic cell percentage: 40%
Percentage area necrosis: 0%

HER2 IMMUNOHISTOCHEMISTRY:
The tumour cells show HER2 protein expression which is scored as 2+ (borderline).

Study the *HER2* FISH image below. Green signals = CEP17; orange signals = *HER2*. This image is representative of all tumour cells in the sample.



https://journals.plos.org/plosone/article/figure?id=10.1371/journal.pone.0132824.g003

Answer the following questions.

1. Explain how *HER2* gene amplification can result in cancer. *[2 marks]*
2. Explain how HER2 immunohistochemistry can be used to determine *HER2* amplification status in breast cancer. *[4 marks]*
3. Explain what is meant by in situ hybridisation (ISH), and the different visualisation methods which can be used with ISH. *[4 marks]*
4. State one advantage and one disadvantage of using FISH, compared to CISH/ddISH. *[2 marks]*
5. Explain why a *HER2* and a CEP17 probe is used for *HER2* FISH. *[2 marks]*
6. State what systemic treatment this patient is likely to receive. *[1 mark]*
7. This patient eventually progresses on treatment. She exhausts all standard systemic treatment options. State two molecular testing options which could provide the patient with further systemic treatment, and explain how those options would open up more options. *[4 marks]*

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06/09/2022

OSPE 6: Breast cancer (answers)

Answer the following questions.

1. Explain how *HER2* gene amplification can result in cancer. *[2 marks]*
   Amplification results in extra copies of the *HER2* gene which result in overexpression of the HER2 protein. [1 mark]
   The presence of more HER2 protein increases the level of HER2 signalling, which results in uncontrolled cell growth and proliferation. [1 mark]
2. Explain how HER2 immunohistochemistry can be used to determine *HER2* amplification status in breast cancer. *[4 marks]*
   HER2 IHC is scored on the basis of the proportion of invasive carcinoma cells showing membranous staining, and the intensity of that staining. [1 mark]
   When HER2 IHC is negative (0) or only very slightly positive (1+), *HER2* amplification can confidently be excluded. [1 mark]
   When HER2 IHC is positive (3+), *HER2* amplification can be considered present. [1 mark]
   For intermediated levels of expression (2+), it cannot be determined whether *HER2* amplification is or is not present, and confirmatory molecular methods are required. [1 mark]
3. Explain what is meant by in situ hybridisation (ISH), and the different visualisation methods which can be used with ISH. *[4 marks]*
   ISH involves hybridising nucleic acid probes against nucleic acid sequences of interest. [1 mark]
   This allows the presence, number and/or location of particular nucleic acid sequences to be determined within the tissue. [1 mark]
   The probes can be labelled with silver (SISH), chromogenic markers (CISH) or a combination of both (DDISH). [1 mark]
   The probes can also be labelled with fluorescent markers (FISH). [1 mark]
4. State one advantage and one disadvantage of using FISH, compared to CISH/ddISH. *[2 marks]*
   Advantages: more probes available, may be more sensitive to low-level amplification, can be used to identify rearrangements as well as copy number variants
   Disadvantages: requires fluorescence microscopy, slides fade rapidly, requires more hands-on set up
5. Explain why a *HER2* and a CEP17 probe is used for *HER2* FISH. *[2 marks]*
   Genuine amplification involves an increase in *HER2* copy number without a significant increase in *HER2*:CEP17 ratio. [1 mark]
   An increase in *HER2* copy number with no significant increase in HER2:CEP17 ratio suggests that the increase in *HER2* copies is likely due to chromosome 17 polysomy rather than amplification. [1 mark]
6. State what systemic treatment this patient is likely to receive. *[1 mark]*
   She will likely receive anti-HER2 monoclonal antibodies (e.g. trastuzumab) as part of her treatment. [1 mark]
7. This patient eventually progresses on treatment. She exhausts all standard systemic treatment options. State two molecular testing options which could provide the patient with further systemic treatment, and explain how those options would open up more options. *[4 marks]*
   Any of:
   NTRK rearrangement testing. [1 mark]. A patients whose tumour harbours *NTRK* rearrangements, regardless of tumour type, and who has exhausted standard treatment options is able to access TRK inhibitors. [1 mark]
   Wide next-generation sequencing (NGS) panel. [1 mark]. This may identify non-standard targets which may permit the patient to enrol in a clinical trial, or to be prescribed treatment on a compassionate-use basis. [1 mark]
   Whole-exome sequencing (WES) or whole-genome sequencing (WGS). [1 mark]. This may identify non-standard targets which may permit the patient to enrol in a clinical trial, or to be prescribed treatment on a compassionate-use basis. [1 mark]

OSPE 7: Melanoma

Read the histology report below.

SPECIMEN TYPE:
Left groin biopsy

CLINICAL DETAILS:
Inguinal lymphadenopathy. ? lymphoma.

MACROSCOPY:
Two cores of cream-coloured tissue measuring up to 6 and 7 mm. One core each processed in blocks 1A and 1B.

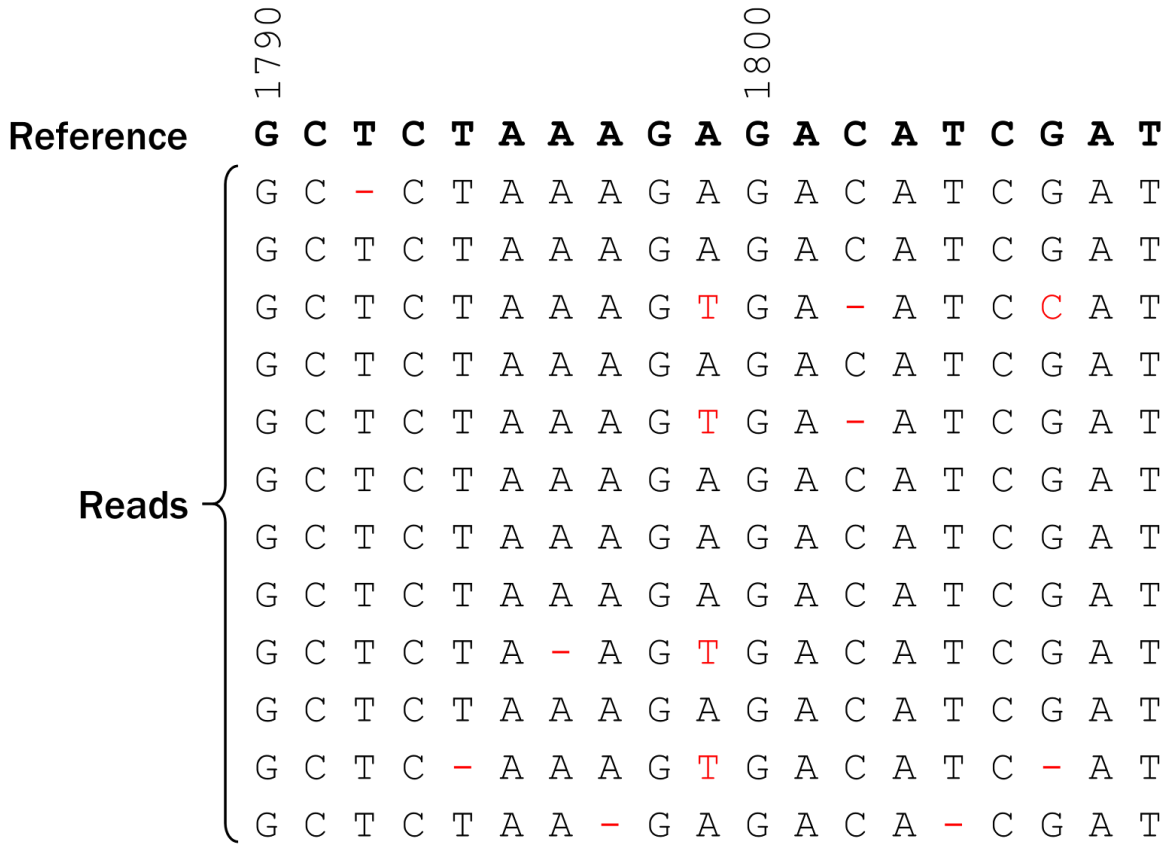
MICROSCOPY:
Sections show cores of lymph node parenchyma which are extensively replaced by tumour. The latter comprises sheets of loosely cohesive cells with fair amounts of cytoplasm and enlarge, irregular nuclei with very prominent nucleoli. Many of the cells contain cytoplasmic pigment. On immunohistochemistry, the tumour cells are positive for S100 and MelanA; they are negative for CD45 and cytokeratin AE1/3.

DIAGNOSIS:
Left groin biopsy – Metastatic melanoma

MOLECULAR PRE-ANALYTIC ASSESSMENT:

Representative tumour block:	1A
Neoplastic cell percentage:	60%
Percentage area necrosis:	0%

The *BRAF* gene is sequenced by next-generation sequencing (NGS). Raw data are provided below.



Answer the following questions.

1. You may assume that the thymine (T) detected at position 1799 is a genuine variant. Express the variant using standard nomenclature. *[4 marks]*
2. What is the depth of sequencing at position 1799? *[1 mark]*
3. State the variant allelic frequency (VAF) for the variant at position 1799. *[1 mark]*
4. Explain the difference between a synonymous and non-synonymous variant. *[2 marks]*
5. The variant at position 1799 represents a *BRAF* V600E variant. Explain the significance of this variant in this clinical setting. *[1 mark]*
6. Explain the difference between an oncogene and a tumour suppressor gene, and explain whether *BRAF* is an oncogene or tumour suppressor gene. *[3 marks]*
7. Immunohistochemistry is available to detect the protein product of the *BRAF* V600E variant. Explain one advantage and one disadvantage of using this stain rather than a molecular technique. *[2 marks]*

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06/09/2022

OSPE 7: Melanoma (answers)

Answer the following questions.

1. You may assume that the thymine (T) detected at position 1799 is a genuine variant. Express the variant using standard nomenclature. *[4 marks]*
   c.1799A>T [4 marks]
2. What is the depth of sequencing at position 1799? *[1 mark]*
   12× [1 mark] (the number of reads in which this nucleotide was called)
3. State the variant allelic frequency (VAF) for the variant at position 1799. *[1 mark]*
   $\frac{4}{12} = 33\%$ [1 mark]
4. Explain the difference between a synonymous and non-synonymous variant. *[2 marks]*
   A synonymous variant is one whether the DNA sequence is changed, but the change does not affect the amino acid expressed. [1 mark]
   A non-synonymous variant results in a change of the amino acid encoded. [1 mark]
5. The variant at position 1799 represents a *BRAF* V600E variant. Explain the significance of this variant in this clinical setting. *[1 mark]*
   The presence of a *BRAF* V600E variant in advanced melanoma makes the patient eligible for *BRAF* and *MEK* inhibitors. [1 mark]
6. Explain the difference between an oncogene and a tumour suppressor gene, and explain whether *BRAF* is an oncogene or tumour suppressor gene. *[3 marks]*
   The function of an oncogene tends to encourage carcinogenesis (e.g. driving cell growth, survival or proliferation). [1 mark]
   The function of a tumour suppressor gene tends to oppose carcinogenesis (e.g. halting the cell cycle, repairing DNA, triggering apoptosis). [1 mark]
   The BRAF protein is downstream of EGFR and transduces its pro-growth and pro-proliferation signalling, and is therefore an oncogene. [1 mark]
7. Immunohistochemistry is available to detect the protein product of the *BRAF* V600E variant. Explain one advantage and one disadvantage of using this stain rather than a molecular technique. *[2 marks]*
   Advantages:
   It is often faster than nucleic acid-based techniques, especially compared to NGS.
   It is more reliable in samples with low neoplastic cell percentage. Because the cells can be directly visualised, whether they are positive or negative can easily be determined irrespective of the neoplastic cell percentage.
   It is more reliable in samples where nucleic acids have been degraded, for example, by excessive fixation.
   It is easily implemented in histopathology laboratories, since they will already have automated immunostainers which are in daily use.
   It may require less tissue than nucleic acid-based techniques if the question being asked is simply whether there is a *BRAF* V600E variant or not. If more variants or genes are relevant, it is likely that nucleic acid-based techniques will require less tissue.
   Disadvantages:
   It only detects V600E variants, and therefore a negative result could indicate either that there is no *BRAF* variant or that there is a *BRAF* non-V600E variant. If non-V600E variants are clinically relevant, follow-up nucleic acid-based testing would be needed on negative cases.
   It only examines one gene, unlike many nucleic acid-based tests which can examine multiple genes in parallel. It is likely to become more expensive and tissue-inefficient if several genes need to be assessed using separate techniques.

OSPE 8: High-grade ovarian serous carcinoma

Read the cytology report below.

SPECIMEN TYPE:
Ascitic fluid

CLINICAL DETAILS:
Omental cake. ? ovarian cancer.

MACROSCOPY:
43 ml of clear, straw-coloured fluid received.

MICROSCOPY:
1. The cytological preparations show a cellular, bloodstained sample comprising reactive mesothelial cells and mixed inflammatory cells. There is a further population of cells arranged in large, disorderly clusters, many with papillaroid morphology. The cells have enlarged, hyperchromatic nuclei with prominent nucleoli.

2. Section from the cell block preparation shows representation by the atypical cells described above. On immunohistochemistry, they are positive for PAX8, WT1 and ER (Quick score 6/8); they show aberrant overexpression of p53.

DIAGNOSIS:
Ascitic fluid – High-grade serous carcinoma, consistent with extrauterine (tubo-ovarian or primary peritoneal) origin

MOLECULAR PRE-ANALYTIC ASSESSMENT:

Representative tumour block:	2A
Neoplastic cell percentage:	50%
Percentage area necrosis:	0%

GENOMIC TESTING:
REPORT SUMMARY:
Myriad HRD Test Result - Supporting report
HRD Status: HOMOLOGOUS RECOMBINATION DEFICIENCY INDICATED
Genomic Instability Score (GIS): 68
Tumour BRCA1/2 variants: BRCA1: c.2315del p.(Val772Aspfs*20)

Formalin-fixed paraffin-embedded (FFPE) tissue from an ascitic fluid with high-grade serous ovarian cancer origin (slide ref: 21H00029749/A1, neoplastic cell content provided with referral ~50%) from Janet Smith has been sent to Myriad for the myChoice CDx test (GIS+BRCA1/2) due to a personal diagnosis of ovarian cancer.

These results are dependent upon the information supplied being correct and complete.

Basis of test:
Testing performed by Myriad Genetics Laboratories, Inc. For further information regarding this assay and results please refer to the Myriad myChoice CDx Test Results report attached; please note the content of the Myriad report is for the US market and might not reflect UK practice.

Answer the following questions.

1. Explain what is meant by 'homologous recombination deficiency' (HRD) and how it predisposes to cancer. *[2 marks]*
2. Explain the relationship between pathogenic *BRCA1/2* variants and HRD. *[2 marks]*

3. Explain the most appropriate next step in determining whether this patient's *BRCA1* variant is germline. *[2 marks]*
4. Explain the clinical implication of this HRD result for the patient. *[1 marks]*
5. Explain how patterns of p53 immunohistochemical expression can be used to infer the presence of underlying *TP53* variants. *[4 marks]*
6. State one advantage and one disadvantage of using cytological samples for nucleic acid-based molecular testing. *[2 marks]*

OSPE 8: High-grade ovarian serous carcinoma (answers)

Answer the following questions.

1. Explain what is meant by 'homologous recombination deficiency' (HRD) and how it predisposes to cancer. *[2 marks]*
   Homologous recombination is the technique which cells use to repair double-strand DNA breaks. HRD is the state in which cells have a dysfunctional homologous recombination system. [1 mark]
   Cells with HRD cannot repair double-strand DNA breaks. They therefore accumulate severe DNA damage which cannot be repaired and can be passed on to their daughter cells. This may eventually result in cancer. [1 mark]
2. Explain the relationship between pathogenic *BRCA1/2* variants and HRD. *[2 marks]*
   Pathogenic *BRCA1/2* variants result in HRD. [1 mark]
   However, HRD may result from phenomena other than pathogenic *BRCA1/2* variants. [1 mark]
3. Explain the most appropriate next step in determining whether this patient's *BRCA1* variant is germline. *[2 marks]*
   The patient should undergo *BRCA1* sequencing on non-neoplastic tissue (usually blood). [1 mark]
   If the variant is detected in non-neoplastic tissue, the variant is considered germline and the patient requires referral to clinical genetics; if no variant is detected in non-neoplastic tissue, the variant detected in the tumour is likely somatic. [1 mark]
4. Explain the clinical implication of this HRD result for the patient. *[1 marks]*
   It suggests that the patient may be eligible for PARP inhibitors. [1 mark]
5. Explain how patterns of p53 immunohistochemical expression can be used to infer the presence of underlying *TP53* variants. *[4 marks]*
   Inactivating *TP53* variants may result in one of four patterns of p53 expression:
   The null pattern is when the tumour cell nuclei are completely negative. [1 mark]
   The overexpression pattern is when at least 80% of tumour cell nuclei show strong expression. [1 mark]
   The cytoplasmic pattern is when the tumour cells show aberrant cytoplasmic expression. [1 mark]
   The wild-type pattern is what is normally seen in the absence of inactivating *TP53* variants, but is rarely seen even when a variant is present; it manifests as variable intensity of nuclear staining. [1 mark]
6. State one advantage and one disadvantage of using cytological samples for nucleic acid-based molecular testing. *[2 marks]*
   Advantages:
   Less invasive sample acquisition
   Usually more rapid histological diagnosis
   Disadvantages:
   Molecular techniques may not be validated for cytological samples
   Macrodissection is not usually helpful in increasing neoplastic cell percentage
   It may not be possible to distinguish between invasive and in situ malignancy

OSPE 9: Small round blue cell tumour

Read the histology report below.

SPECIMEN TYPE:
Pleural biopsy

CLINICAL DETAILS:
17 year old. Chest wall mass with multiple pleural metastases.

MACROSCOPY:
Three single core of cream-coloured tissue measuring up to 6, 7 and 12 mm. Each processed in a separate cassette.

MICROSCOPY:
Sections show cores of lesional tissue. It comprises sheets of loosely cohesive cells with extremely high nuclear-cytoplasmic ratios. The cells have slightly irregular nuclear outlines, coarse chromatin patterns and occasional prominent nucleoli. There is a focal suggestion of rosette formation.

Immunohistochemistry shows the following:

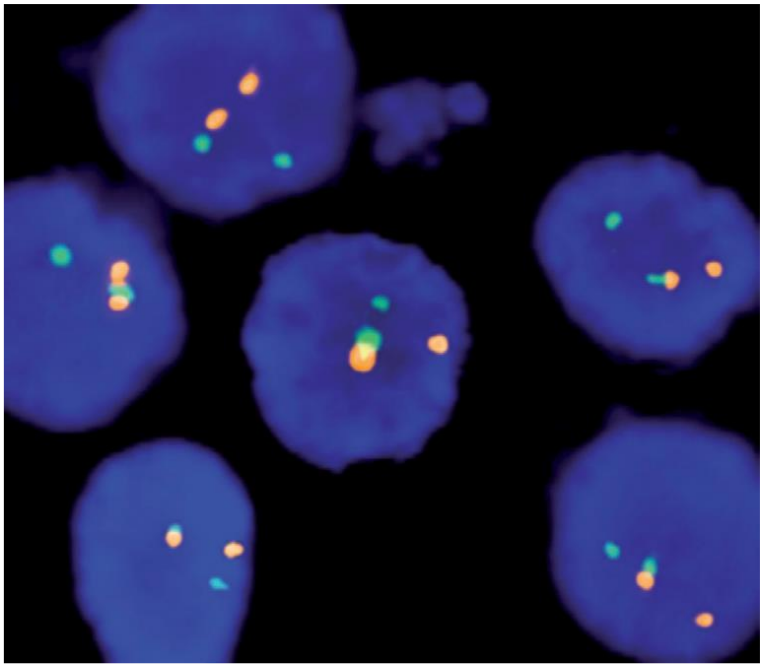
Cytokeratin AE1/3:	Negative
CAM5.2:	Negative
LCA:	Negative
S100:	Negative
MelanA:	Negative
CD99:	Positive
Myogenin:	Negative
Desmin:	Negative
CD34:	Negative

DIAGNOSIS:
Pleural biopsy – Small round blue cell tumour

MOLECULAR PRE-ANALYTIC ASSESSMENT:

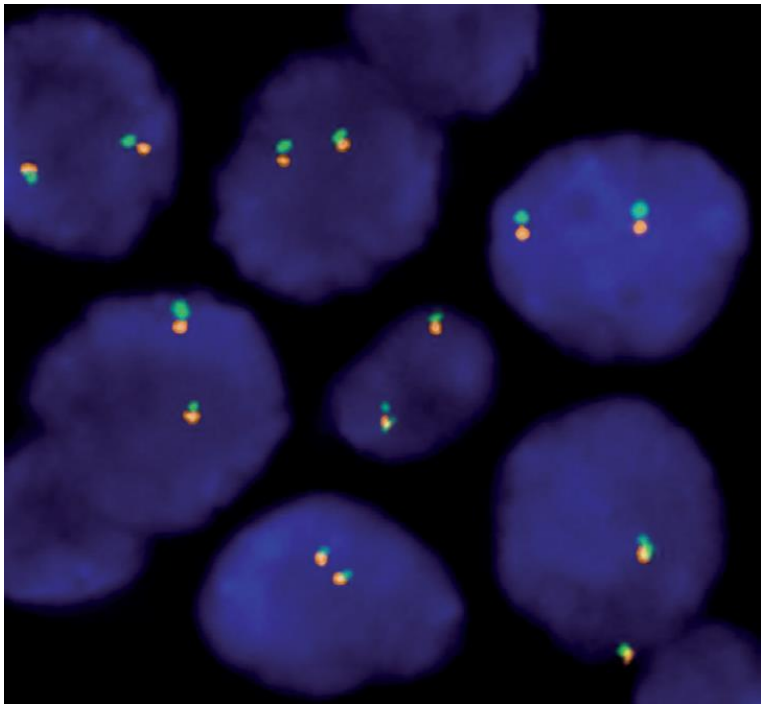
Representative tumour block:	1A
Neoplastic cell percentage:	40%
Percentage area necrosis:	0%

Study the *EWSR1* FISH image below (dual-colour breakapart probes).



https://www.zytovision.com/downloads_products/datasheets/z-2096-ce-ivd.pdf

Study the *SS18* FISH image below (dual-colour breakapart probes).



https://www.zytovision.com/downloads_products/datasheets/z-2097-ce-ivd.pdf

Study the molecular testing results below.

REVERSE TRANSCRIPTION PCR:	
REPORT SUMMARY:	
EWSR1-FLI1 (type 1):	**Negative**
EWSR1-FLI1 (type 2):	**Negative**
EWSR1-FLI1 (type 3):	**Negative**
EWSR1-ERG:	**Negative**
EWSR1-WT1:	**Negative**

Answer the following questions.

1. State the most likely diagnosis in this case. *[1 mark]*
2. Explain the rationale for processing each biopsy in a separate cassette. *[2 marks]*
3. Describe the findings of *EWSR1* FISH. *[2 marks]*
4. Describe the findings of *SS18* FISH. *[2 marks]*
5. Provide three possible explanations for reverse transcription PCR being negative, in view of the results of *EWSR1* FISH. *[3 marks]*
6. Explain how dual-colour dual-fusion FISH probes could be used to identify the fusion partner of a gene. *[2 marks]*

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06/09/2022

OSPE 9: Small round blue cell tumour (answers)

Answer the following questions.

1. State the most likely diagnosis in this case. *[1 mark]*
   Ewing sarcoma [1 mark]
2. Explain the rationale for processing each biopsy in a separate cassette. *[2 marks]*
   This maximises efficiency of tissue use. [1 mark]
   One block can be used for diagnostic IHC, one for molecular testing, and one can be a backup. [1 mark]
3. Describe the findings of *EWSR1* FISH. *[2 marks]*
   There are two split signals and one fused signal. [1 mark]
   This indicates that a rearrangement involving *EWSR1* is present. [1 mark]
4. Describe the findings of *SS18* FISH. *[2 marks]*
   There are two fused signals. [1 mark]
   There is no evidence of an *SS18* rearrangement. [1 mark]
5. Provide three possible explanations for reverse transcription PCR being negative, in view of the results of *EWSR1* FISH. *[3 marks]*
   Any of:
   It could be that there is an *EWSR1* fusion, but that it does not involve any of *FLI1*, *ERG* or *WT1*.
   It could be that there is an *EWSR1* fusion involving one of *FLI1*, *ERG* or *WT1*, but that the specific fusion is not covered by the probes included in the assay.
   It could be that the neoplastic cell content of the sample is below the minimum required for the assay, giving a false negative result (although this seems unlikely given the neoplastic cell content of 40%)
   It could be that there is an *EWSR1* rearrangement, but that it is non-productive and does not give rise to an *EWSR1* fusion transcript
6. Explain how dual-colour dual-fusion FISH probes could be used to identify the fusion partner of a gene. *[2 marks]*
   Dual-colour dual-fusion probes look at the locations of two genes to identify whether they have been fused together. [1 mark]
   A series of probes to common gene fusions could be applied to identify the partner gene. [1 mark]

OSPE 10: Whole genome sequencing

A seven year old boy is diagnosed with neuroblastoma. He is later diagnosed with metastatic disease. Repeat biopsy is undertaken to obtain tissue for whole genome sequencing. Read the histology and whole genome sequencing report which follows, and answer the questions.

SPECIMEN TYPE:
Abdominal biopsy

CLINICAL DETAILS:
7 year old, metastatic neuroblastoma. Biopsy for WGS.

MACROSCOPY:
Three cores of cream-coloured tissue, the longest measuring up to 8 mm. One processed in each of blocks 1A, 1B and 1C.

MICROSCOPY:
Sections show cores of lesional tissue whose appearances are consistent with the known diagnosis of neuroblastoma. Tissue has been submitted for whole genome sequencing.

DIAGNOSIS:
Abdominal biopsy – Consistent with neuroblastoma (WGS in progress)

MOLECULAR PRE-ANALYTIC ASSESSMENT:
Representative tumour block: 1A
Neoplastic cell percentage: 90%
Percentage area necrosis: 0%

Questions

1. Explain why a new biopsy was taken rather than using an archival tissue block for whole genome sequencing. *[2 mark]*
2. Explain the advantage of processing each biopsy in a separate cassette, from the perspective of molecular testing. *[2 marks]*

Whole Genome Analysis

Patient

Referral ID	Interpreting Organisation	Requesting Organisation	Patient ID	Gender	Clinical Indication

r643267329	699P0 Central and South Genomic Laboratory Hub	University Hospital Southampton NHS Foundation Trust	p363825423	Male	Neuroblastoma – Paediatric

Tumour details

Histopathology Lab ID	Tumour Type	Primary or Metastatic	Presentation	Tumour Diagnosis Date
22HS15326S	Solid Tumour Metastatic	Metastatic	First Presentation	01/05/2022

Tumour sample

Sample ID	Dispatched Sample LSID	Storage Medium	Source	Tumour Content	Tumour Sample Cross-contamination	Calculated Overall Ploidy	Calculated Chromosome Count	Calculated Tumour Content
LP5000542-DNA_A01	547324623	FF	Tumour	90%	Pass	1.88	41.25	86.5%

Germline sample

Sample ID	Dispatched Sample LSID	Storage Medium	Source
LP5000642-DNA_B01	4328473298	EDTA	Blood

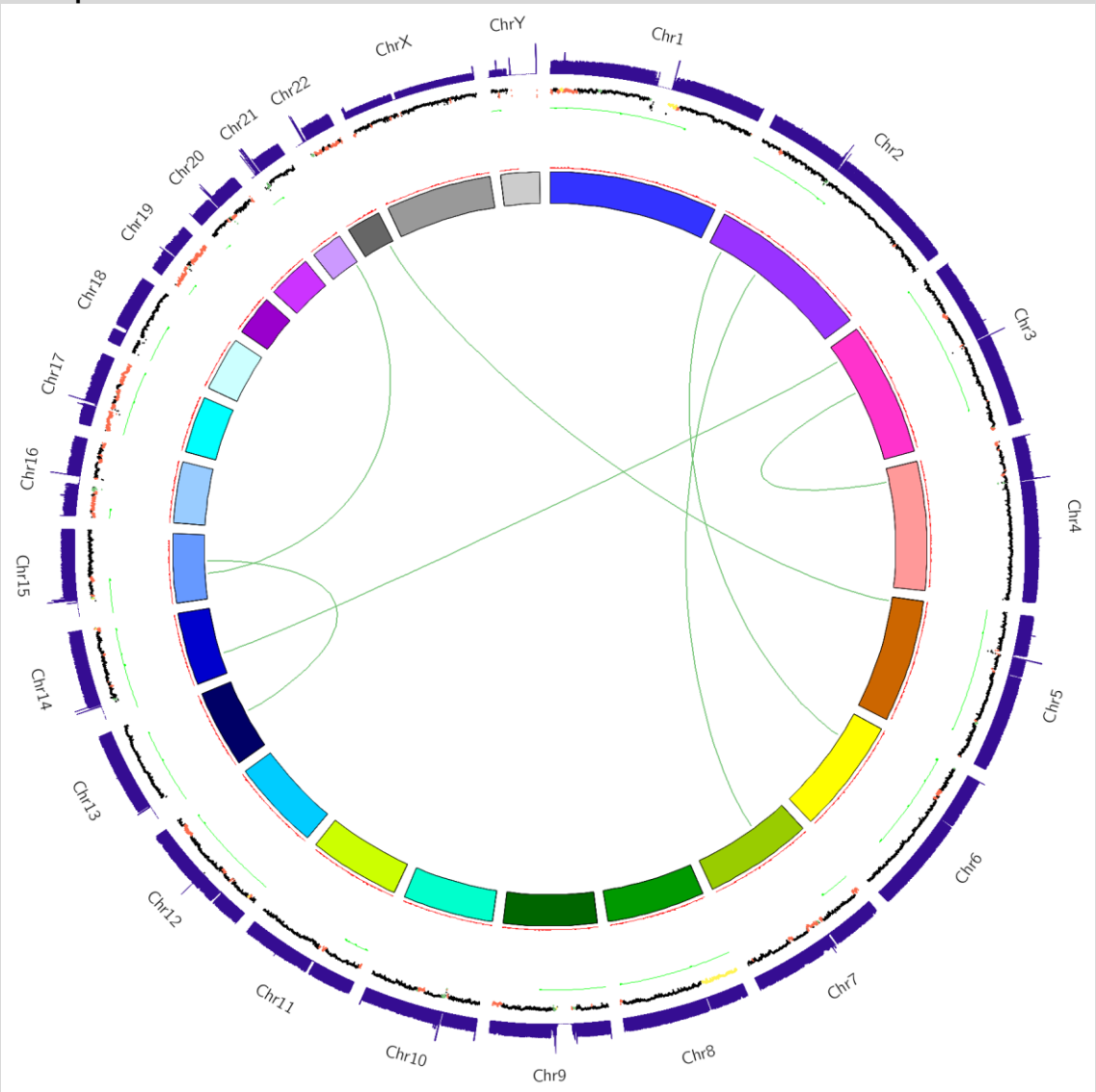
Sequencing quality information

Sample type	Mapped reads, %	Chimeric DNA fragments, %	Insert size median, bp	Genome-wide coverage mean, x	Unevenness of local genome coverage, x	COSMIC content with low coverage (<30×), %	Total somatic SNVs	Total somatic indels	Total somatic SVs
Germline	97.99	0.82	485.6	33.57	6.71	N/A	N/A	N/A	N/A
Tumour	98.41	1.04	497.3	84.63	12.24	0.76	919	44	21

Questions

3. Explain what is meant by ploidy, and what the calculated overall ploidy presented here means. *[2 marks]*
4. Explain what is meant by percentage mapped reads. *[1 mark]*
5. State one factor which may prevent reads from being mapped. *[2 marks]*
6. Explain what is meant by median insert size. *[1 mark]*
7. Explain what is meant by genome-wide coverage mean, and explain the meaning of the difference in values between the germline and tumour samples. *[2 marks]*

Circos plot



Track 1 (innermost): chromosomes
Track 2 (red): number of somatic SNVs
Track 3 (green): number of somatic indels
Track 4: ratio of normalised depth of coverage of tumour vs. normal; losses in red, gains in green, copy-neutral LOH in yellow
Track 5 (outermost): absolute depth of coverage

Structural variants are in the centre: translocations in green, inversions in purple, duplications in blue, deletions in red

Questions

8. Based on this Circos plot, state whether this tumour's genome is rich in aberrations or not. *[1 mark]*

Small somatic variants

Domain 1 variants

Gene	GRCh38 coordinates ref/alt allele	Transcript	CDS change and protein change	Predicted consequences	Population germline allele frequency (GE | gnomAD)	VAF	Alt allele/total read depth	COSMIC ID	Gene mode of action
NF1	17:31356999 T>A	ENST00000358273 ENSP00000351015	c.7778T>A p.Leu2593Ter	stop_gained	- | -	0.1	8/81	N/A	Tumour suppressor gene

Domain 2 variants
No variants in domain 2

Domain 3 variants
[not listed]

Questions

9. Based on the information provided here, state the likely biological effect of this variant with respect to carcinogenesis *[1 mark]*
10. Taking into account the neoplastic cell percentage of this sample, state the implication of the variant allelic frequency (VAF) provided here. *[1 mark]*

Structural variants (including Copy Number Variants)

Domain 1 variants

Gene	Transcript	Impacted transcript region	Variant GRCh38 coordinates	Variant type	Variant cytological bands	Confidence/support	Gene mode of action
AKT1	ENST00000555528	Full transcript	14:101917656-106114205	LOSS(1)	del(14)(q32.31;q32.33)	HC-11	Oncogene

[others not listed]

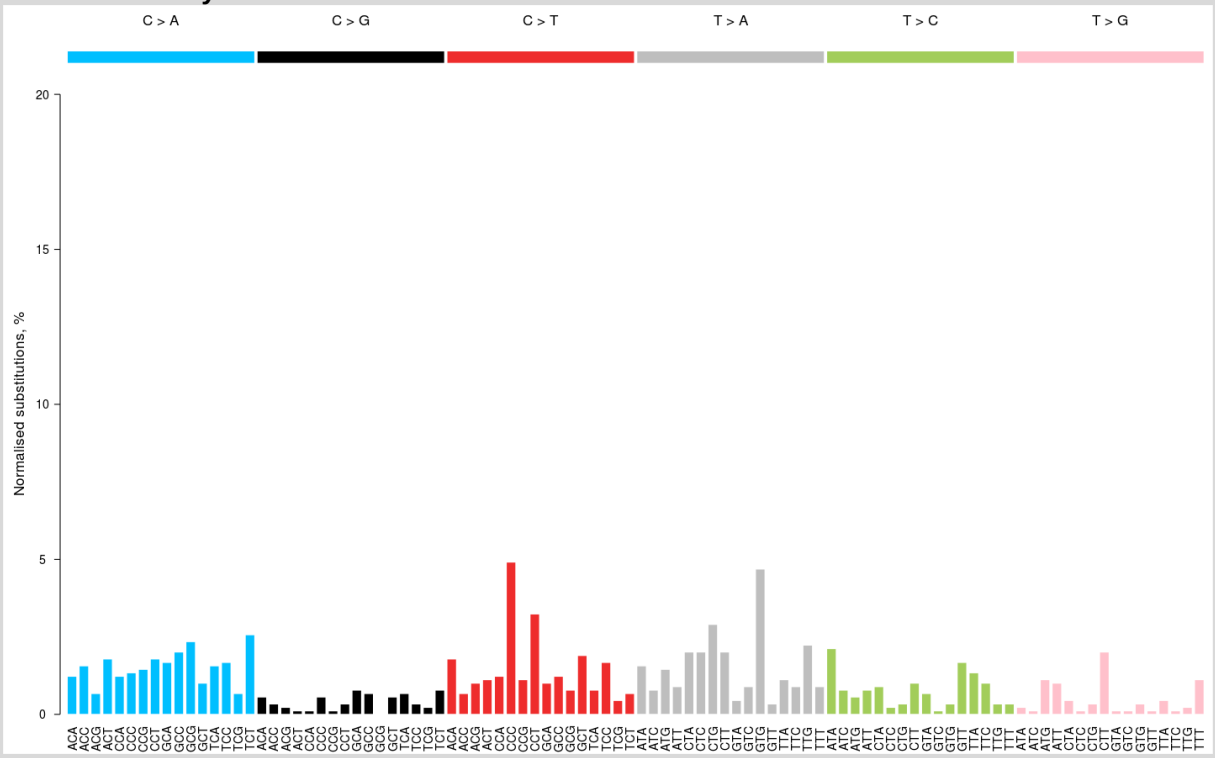
Domain 2 variants
[not listed]

Domain 3 variants
[not listed]

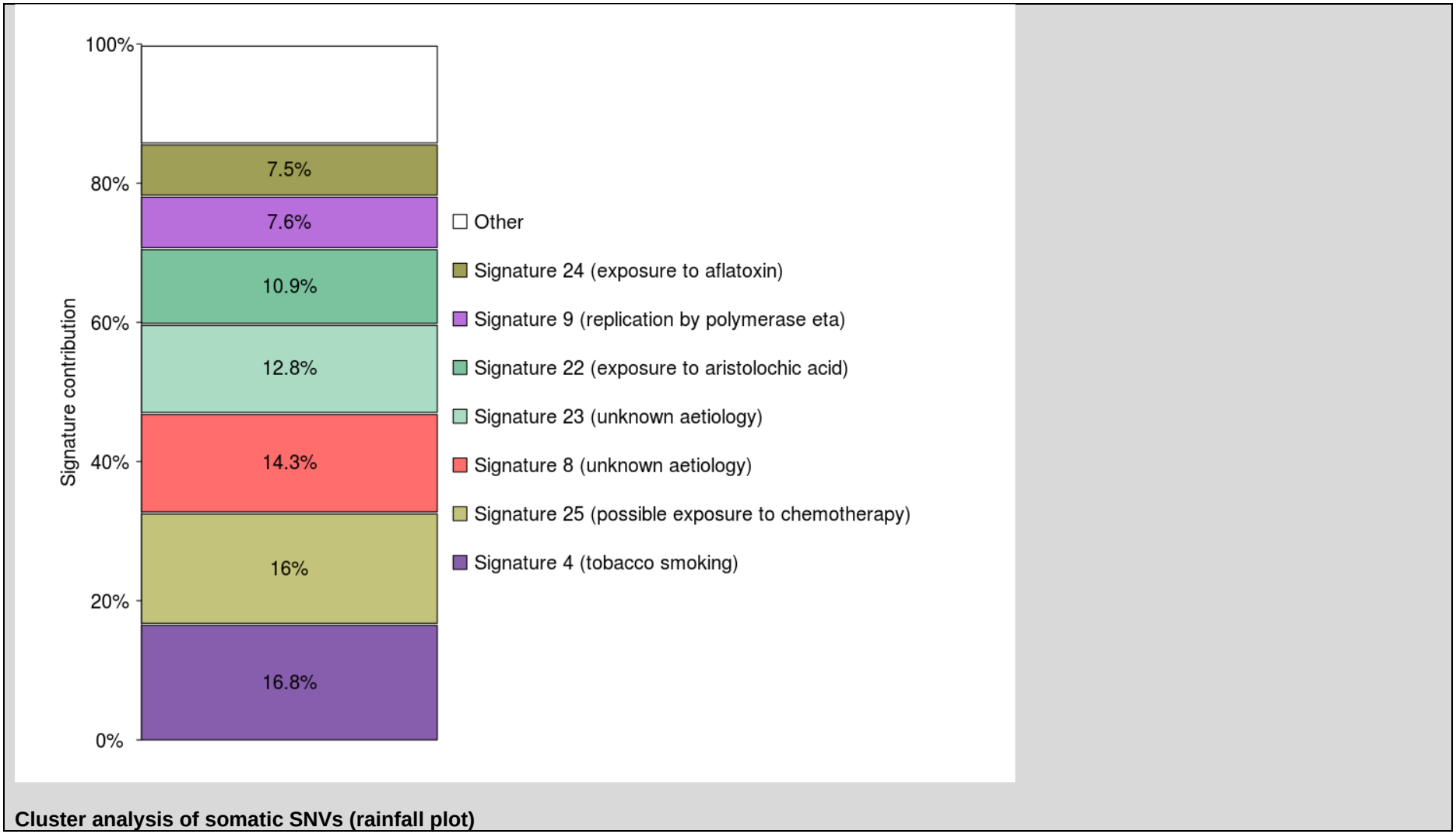
Global analyses of somatic variants

Somatic mutation prevalence (tumour mutational burden)
Total number of somatic non-synonymous small variants per megabase (coding region): 0.18

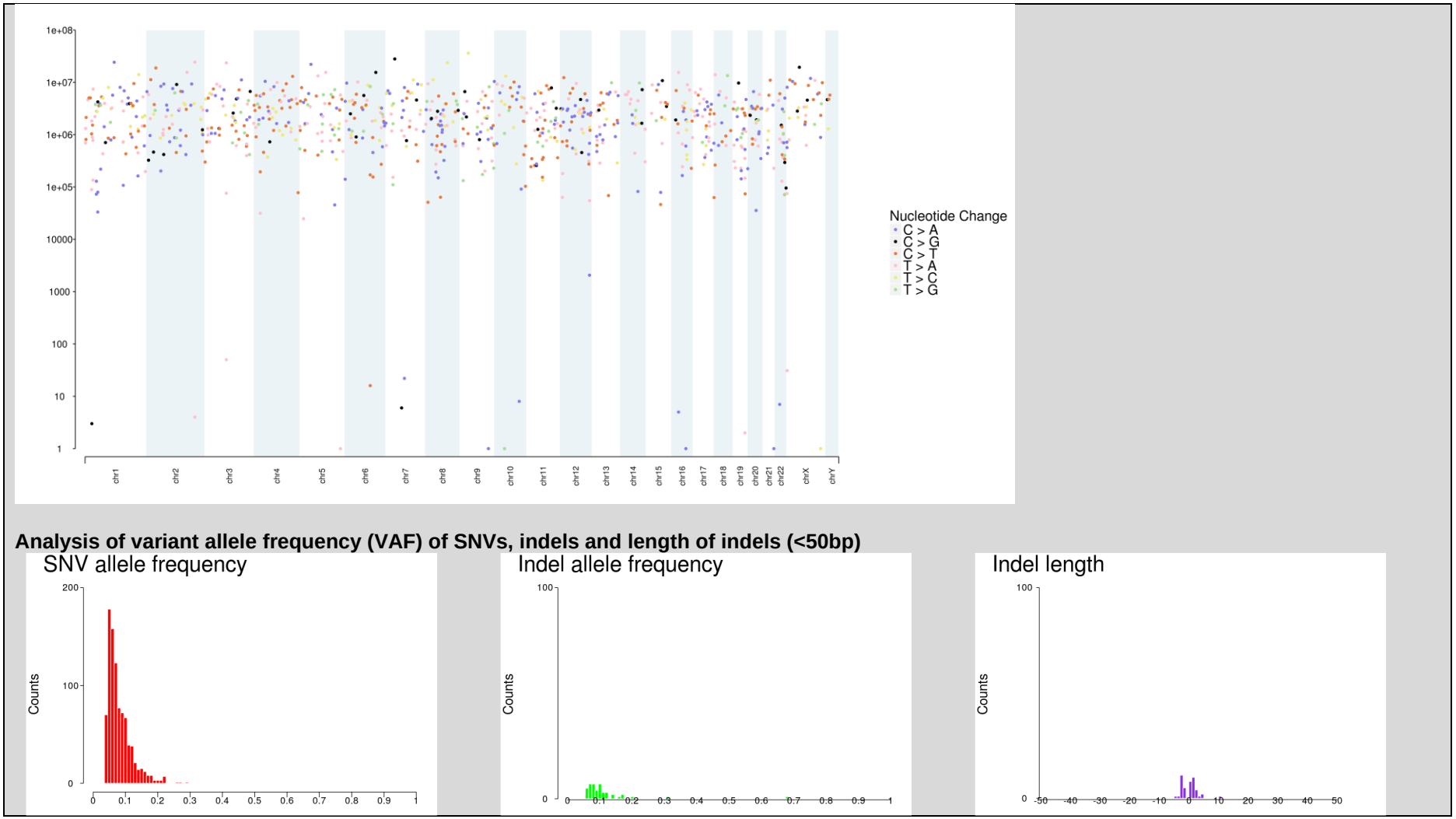
Contextual analysis of somatic SNVs



Mutational signature analysis



Cluster analysis of somatic SNVs (rainfall plot)



Questions

11. Explain the potential clinical utility of tumour mutational burden (TMB), and state whether this tumour would generally be regarded as having high or low TMB. *[3 marks]*

12. Explain how mutational signatures may be helpful in identifying tumours which harbour inactivation of tumour suppressor genes. *[1 mark]*
13. Explain the likely significance of signatures 4 and 25 detected in this tumour. *[2 marks]*
14. Explain what is demonstrated by the SNV allele frequency and indel allele frequency plots. *[2 marks]*

Analysis of germline variants

Variants in cancer susceptibility genes

Tier 1 variants
No pertinent germline findings detected in tier 1

Tier 3 variants

Gene	GRCh38 coordinates ref/alt allele	Transcript	CDS change and protein change	Predicted consequences	Population germline allele frequency (GE | gnomAD)	Alt allele/total read depth	Genotype	ClinVar ID	ClinVar data	Gene mode of action
SLX4	16:3591279 C>T	ENST00000294008 ENSP00000294008	c.2359G>A p.Glu787Lys	Missense variant	0.0014 | 0.0012	20/38	0/1	407938	Conflicting interpretations of pathogenicity	N/A
POLE	12:132687300 C>G	ENST00000320574 ENSP00000322570	c.16G>C p.Gly6Arg	Missense variant	0.0046 | 0.0025	15/31	0/1	218680	Conflicting interpretations of pathogenicity	Tumour suppressor gene

Questions

15. Explain the relevance of population germline allele frequency in determining whether a germline variant is pathogenic, and explain its relevance in this case. *[2 marks]*
16. Explain what is meant by alt allele/total read depth, and state whether this individual is likely heterozygous or homozygous for the variants detected. *[2 marks]*

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06/09/2022

OSPE 10: Whole genome sequencing (answers)

1. Explain why a new biopsy was taken rather than using an archival tissue block for whole genome sequencing. *[2 mark]*
   Whole genome sequencing requires high-quality DNA. [1 mark]
   Formalin-fixed tissue contains DNA of insufficient quality for whole-genome sequencing, and so a new biopsy is required to undergo sequencing as fresh frozen tissue. [1 mark]
2. Explain the advantage of processing each biopsy in a separate cassette, from the perspective of molecular testing. *[2 marks]*
   It improves efficiency of tissue use. [1 mark]
   One block can be used for diagnostic immunohistochemistry, preserving all the tissue in the remaining blocks for molecular testing. [1 mark]
3. Explain what is meant by ploidy, and what the calculated overall ploidy presented here means. *[2 marks]*
   Ploidy is the number of sets of chromosomes in a cell. [1 mark]
   Here, calculated overall ploidy is 1.88 which means that each cell has around two sets of chromosomes which is normal. [1 mark]
4. Explain what is meant by percentage mapped reads. *[1 mark]*
   It is the percentage of all reads which can be successfully aligned against the reference genome. [1 mark]
5. State one factor which may prevent reads from being mapped. *[2 marks]*
   Any of:
   Short read length
   Repetitive DNA sequences
6. Explain what is meant by median insert size. *[1 mark]*
   Before NGS, the sample DNA is broken up into small fragments to which are attached adapters; the DNA fragment is called the insert, and so the median insert size is the mean length of DNA fragments used for sequencing. [1 mark]
7. Explain what is meant by genome-wide coverage mean, and explain the meaning of the difference in values between the germline and tumour samples. *[2 marks]*
   Coverage is the number of times that each nucleotide is sequenced in the various reads, and so the mean coverage is the mean number of times each nucleotide in the genome has been sequenced. [1 mark]
   The value for the tumour sample is higher than that for the germline sample, suggesting that the confidence of the derived sequence for the tumour sample will be higher than that for the germline sample. [1 mark]
8. Based on this Circos plot, state whether this tumour's genome is rich in aberrations or not. *[1 mark]*
   Overall, this is a fairly 'quiet' Circos plot, indicating that there are relatively few aberrations. [1 mark]
9. Based on the information provided here, state the likely biological effect of this variant with respect to carcinogenesis *[1 mark]*
   This is a nonsense (therefore inactivating) variant in a tumour-suppressor gene, and is therefore likely to promote carcinogenesis. [1 mark]
10. Taking into account the neoplastic cell percentage of this sample, state the implication of the variant allelic frequency (VAF) provided here. *[1 mark]*
    The VAF is 10%, meaning that 10% of alleles of NF1 in the tumour sample represent this variant. The reported neoplastic cell content was 90%, which means that only a minority of tumour cells must harbour this variant. [1 mark]
11. Explain the potential clinical utility of tumour mutational burden (TMB), and state whether this tumour would generally be regarded as having high or low TMB. *[3 marks]*

TMB is a measure of how mutated a tumour's genome is. [1 mark]
The more variants in a tumour's genome, the more abnormal proteins the tumour will express and the more likely the tumour will be to incite an anti-tumour immune response. Therefore, high TMB may predict response to immune checkpoint inhibitors. [1 mark]
This tumour would generally be regarded as having low TMB. [1 mark] (10 mutations per megabase is often taken as the cut-off between low and high)

12. Explain how mutational signatures may be helpful in identifying tumours which harbour inactivation of tumour suppressor genes. *[1 mark]*
    Tumour's with inactivation of DNA repair mechanisms sustain DNA damage which may occur in such a way as to be specific to the DNA repair mechanism which has failed. It may therefore be possible to work out which DNA repair pathway has failed from the type of damage sustained. [1 mark]
13. Explain the likely significance of signatures 4 and 25 detected in this tumour. *[2 marks]*
    Signature 4 is associated with tobacco smoking; in a paediatric cancer, this is highly likely to be a spurious finding. [1 mark]
    Signature 25 is associated with chemotherapy exposure; this is a metastatic tumour deposit, and so it may well be that the patients has already received chemotherapy which could account for this signature. [1 mark]
14. Explain what is demonstrated by the SNV allele frequency and indel allele frequency plots. *[2 marks]*
    SNV allele frequency is the allele frequency of single nucleotide variants, and indel allele frequency is the allele frequency of insertions and deletions. [1 mark]
    Given the neoplastic cell content of 90%, the fact that almost all variants are present with a frequency of no more than 20% suggests that all detected variants are present in only a subset of neoplastic cells. [1 mark]
15. Explain the relevance of population germline allele frequency in determining whether a germline variant is pathogenic, and explain its relevance in this case. *[2 marks]*
    Germline variants which are found at high frequencies in a population are unlikely to be pathogenic. [1 mark]
    Here, both variants are reported at very low frequencies in the population which is not particularly helpful in establishing their pathogenicity. [1 mark]
16. Explain what is meant by alt allele/total read depth, and state whether this individual is likely heterozygous or homozygous for the variants detected. *[2 marks]*
    This is the number of reads which contain the variant, out of the total number of reads in which that position was sequenced. [1 mark]
    For both variants, the variant is detected in around half the reads, indicating that the individual is heterozygous for this variant. [1 mark]