

Germline Variant Calling in Formalin-fixed Paraffin-embedded Tumours

Eva Yap, M.Sc. Student

Thesis defence – Dec 12, 2017

Overview

- ① Background
- ② Research Question
- ③ Thesis Aims
- ④ Aim 1: Formalin-induced DNA damage could be mitigated by using shorter amplicons and avoiding older FFPE blocks.
- ⑤ Aim 2: FFPE tumour DNA is a reliable source for germline variant calling.
- ⑥ Aim 3: The use of a VAF cut-off could result in high sensitivity in identifying germline variants in FFPE tumours with an acceptable positive predictive value.
- ⑦ Conclusions

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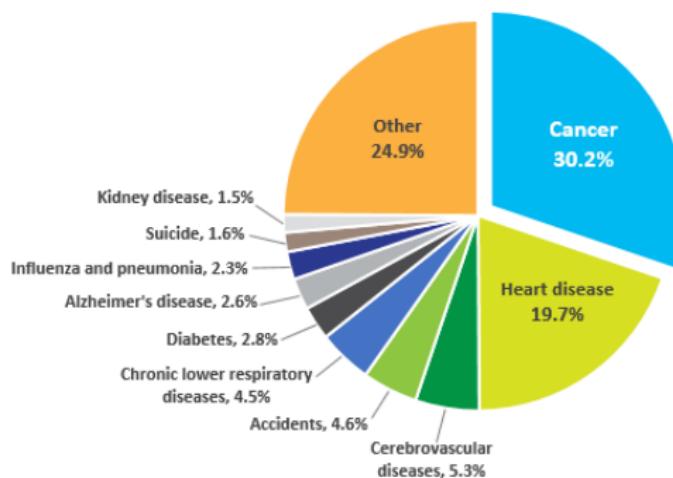
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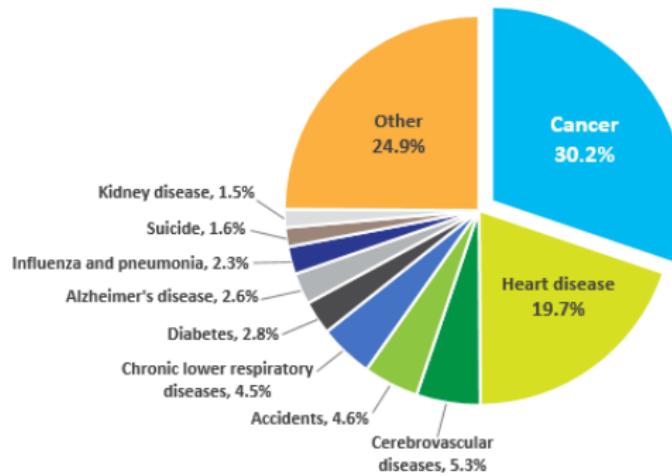
Cancer is a leading cause of death in Canada

Proportion of deaths due to cancer and other causes, Canada, 2012



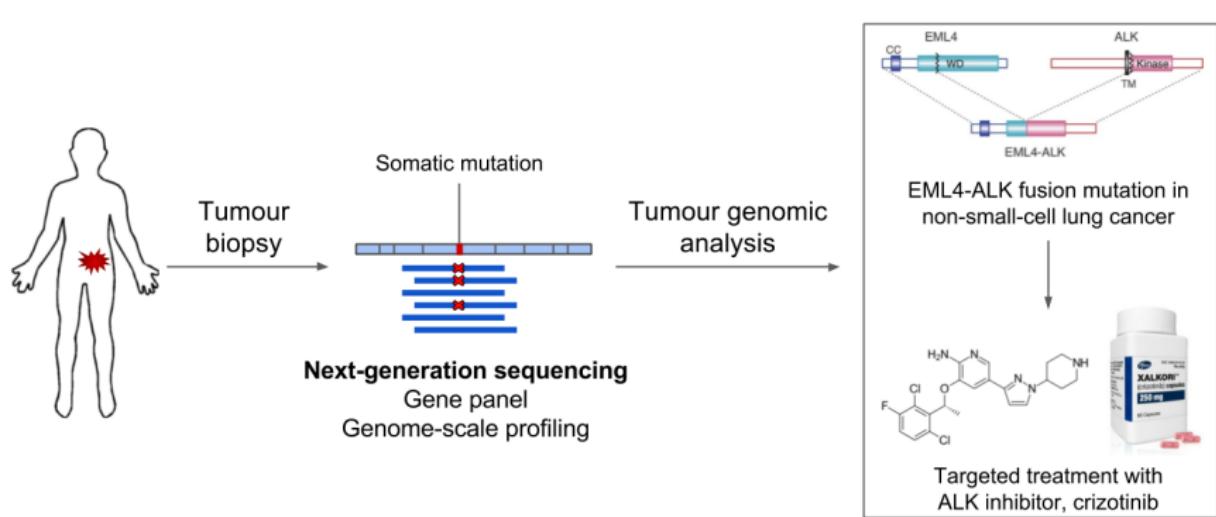
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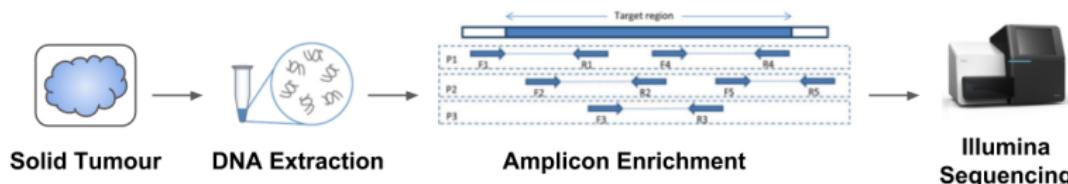
In 2017, the estimate number of new cancer cases is 206,200 and deaths from cancer is 80,800.

Genomic-driven cancer medicine has great potential in guiding oncologic care



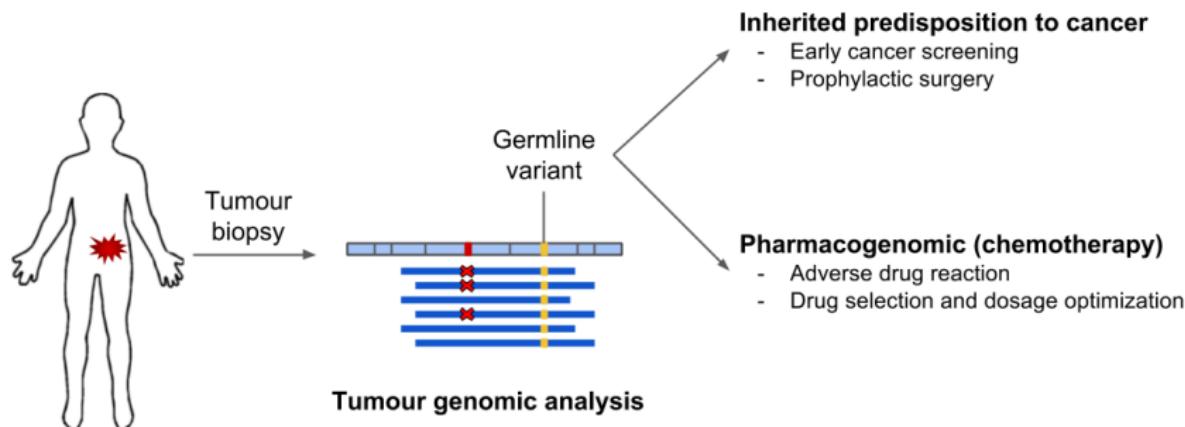
S. Roychowdhury & A.M. Chinnaian, 2014. Annu. Rev. Genomics Hum. Genet, 15:395-415.
H. Mano, 2015. Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci, 91(5):193-201.

British Columbia Cancer Agency's OncoPanel

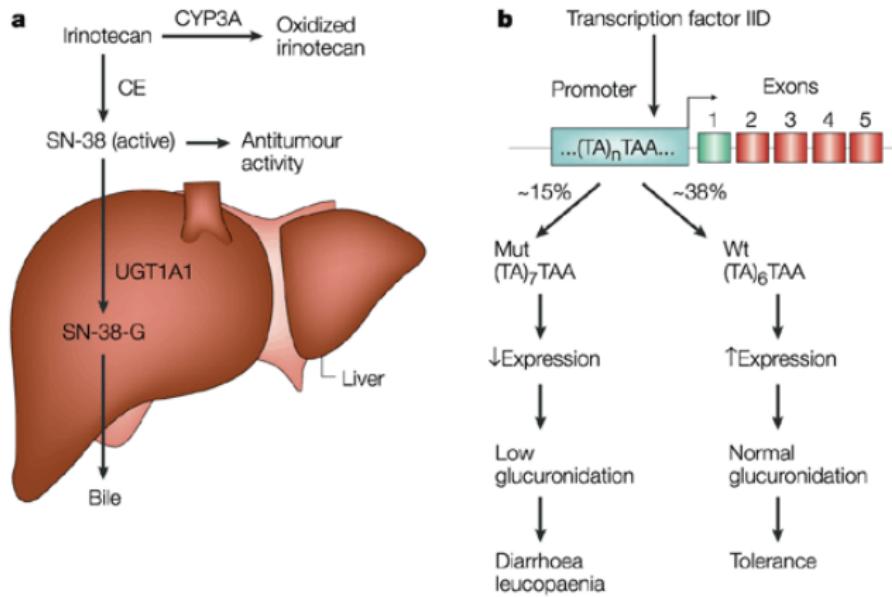


- ① Targeted amplicon-based next-generation sequencing panel for solid tumours
- ② Detects single nucleotide variants (SNVs) and small indels in cancer- and pharmacogenomic-related genes

Germline variants can have clinical implications for both cancer patients and their families

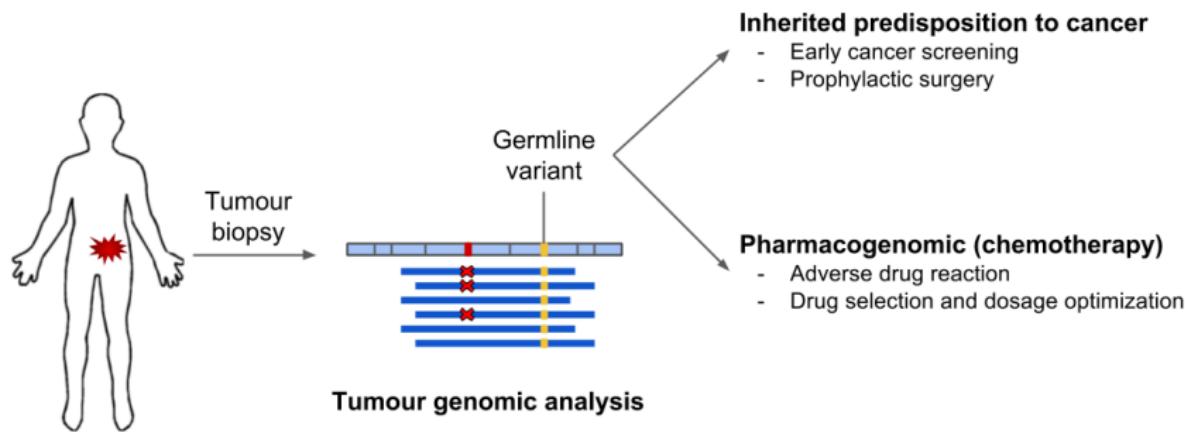


UGT1A1 promoter polymorphism leads to irinotecan toxicity



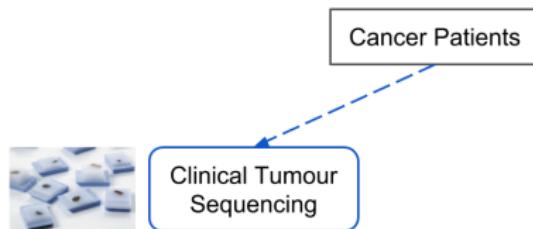
Nature Reviews | Cancer

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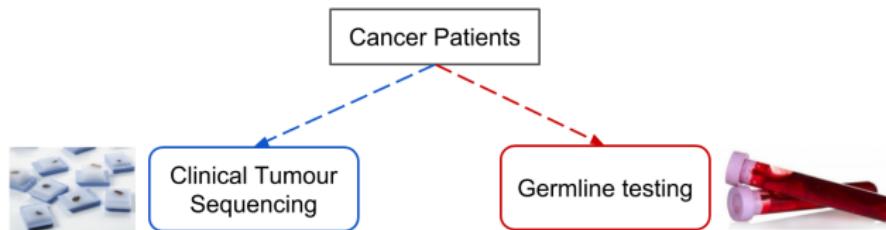


Reporting clinically important germline variants can improve clinical outcomes for both cancer patients and their families.

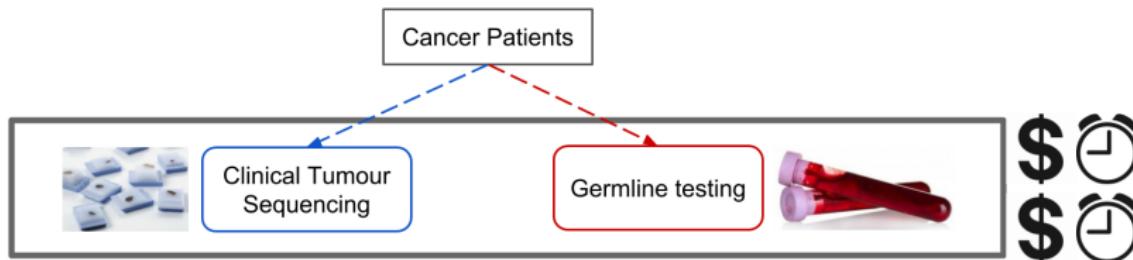
Clinical tumour sequencing presents an opportunity to pre-screen for germline variants



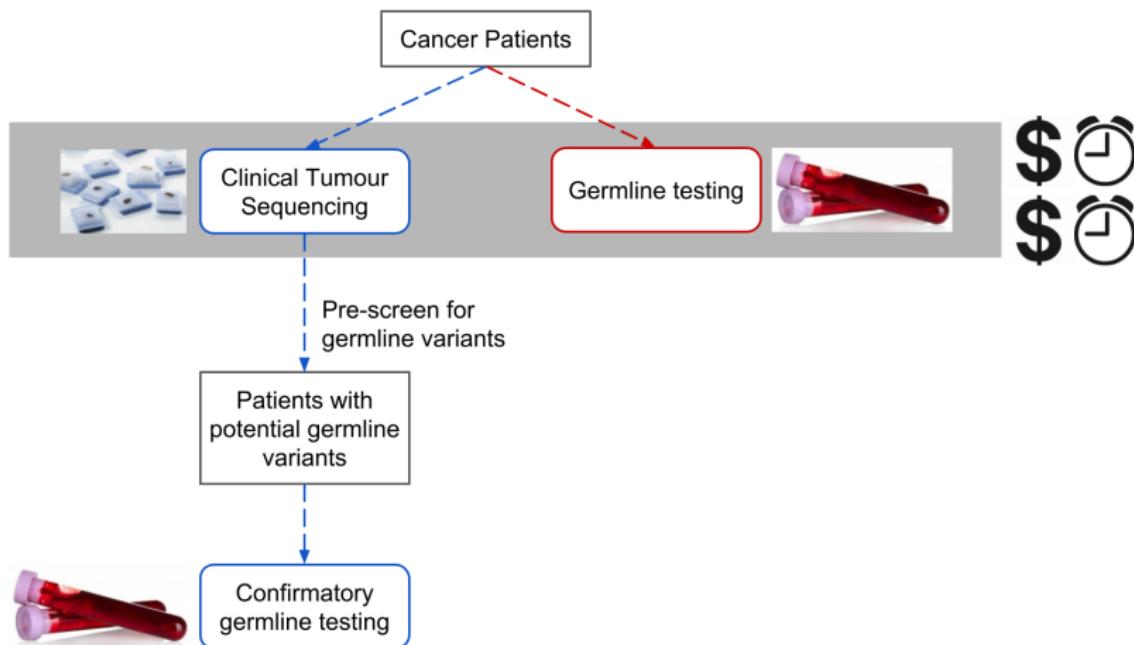
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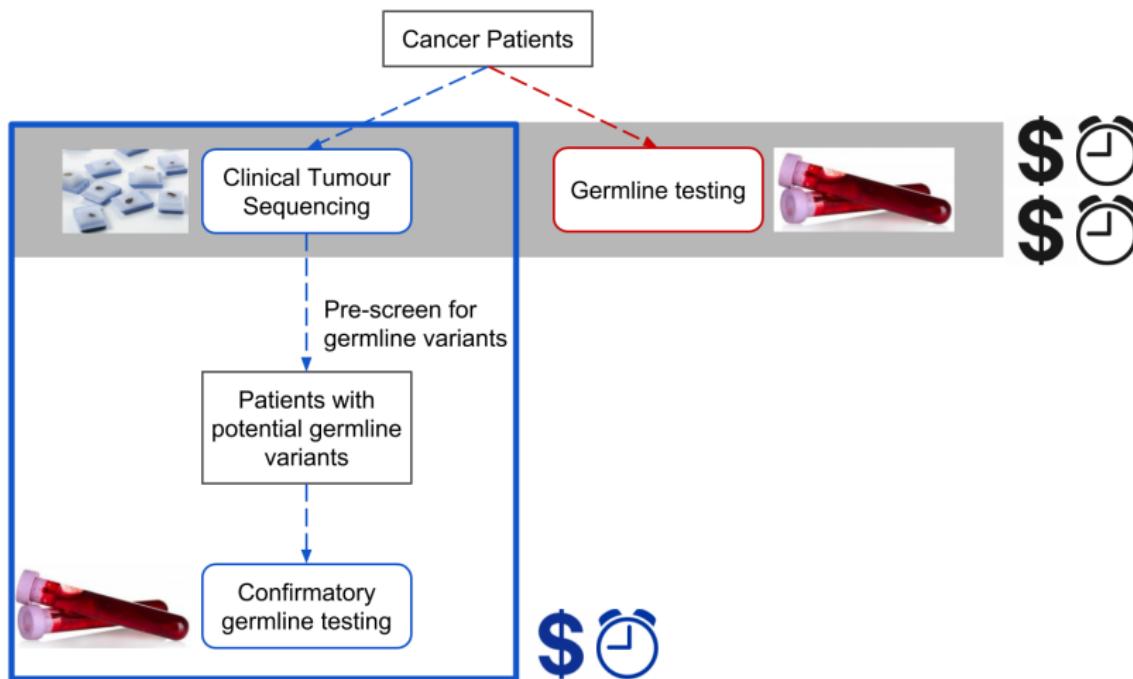
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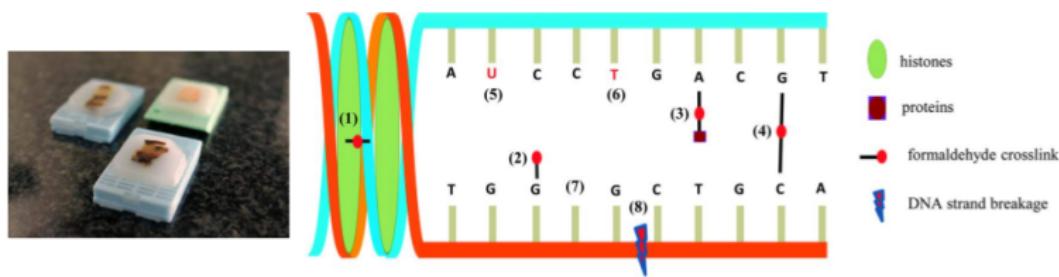


Clinical tumour sequencing presents an opportunity to pre-screen for germline variants



Challenges in identifying germline variants using clinical tumour-only sequencing

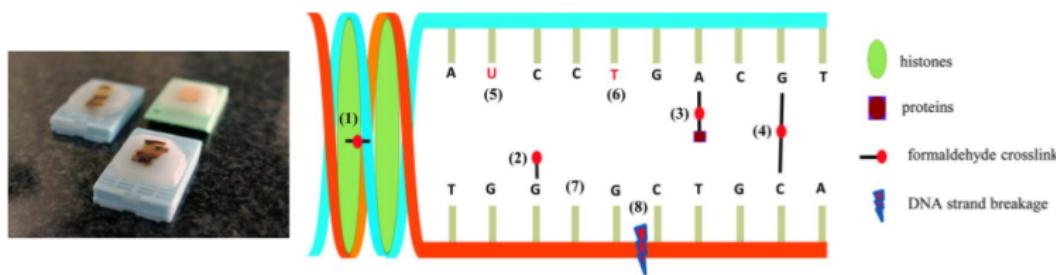
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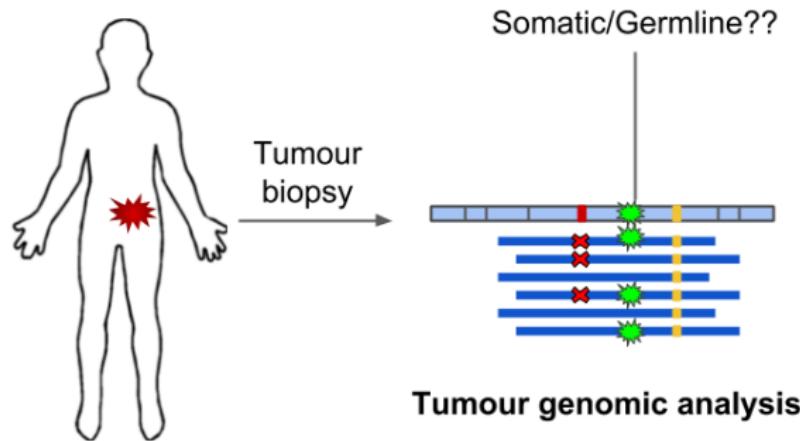
1: Formalin-induced DNA damage



- Tumours are usually formalin-fixed and paraffin-embedded (FFPE) for pathologic assessment.
- Formaldehyde exposure causes fragmentation and sequence artifacts (e.g. C>T/G>A).

Challenges in identifying germline variants using clinical tumour-only sequencing

2: Distinguishing between germline and somatic variants



Low accuracy in distinguishing between germline and somatic variants using unmatched normal sample and public databases

C

Matched tumor/normal sample analyses			Tumor-only analyses			
	Total sequence coverage	Number of samples analyzed	Somatic mutations per tumor	Candidate mutations per tumor	Candidate mutations per tumor after removal of common germline variants (dbSNP)	Alterations in actionable genes after dbSNP filter
Whole exome	199x	100	135	Total alterations	1401	382
				True positives (somatic)	135 (10%)	133 (35%)
				False positives (germline)	1266 (90%)	249 (65%)
Targeted	1052x	58	4.34	Total alterations	11.53	6.28
				True positives (somatic)	4.34 (38%)	4.33 (69%)
				False positives (germline)	7.19 (62%)	1.95 (31%)

Whole exome = 20,766 coding genes; Targeted = 111 genes

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Whole exome	199x	100	135	Total alterations	1401	382	2.41
				True positives (somatic)	135 (10%)	133 (35%)	1.61 (67%)
				False positives (germline)	1266 (90%)	249 (65%)	0.80 (33%)
Targeted	1052x	58	4.34	Total alterations	11.53	6.28	2.31
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An approach to separate germline from somatic variants in FFPE tumour-only analyses must be established.

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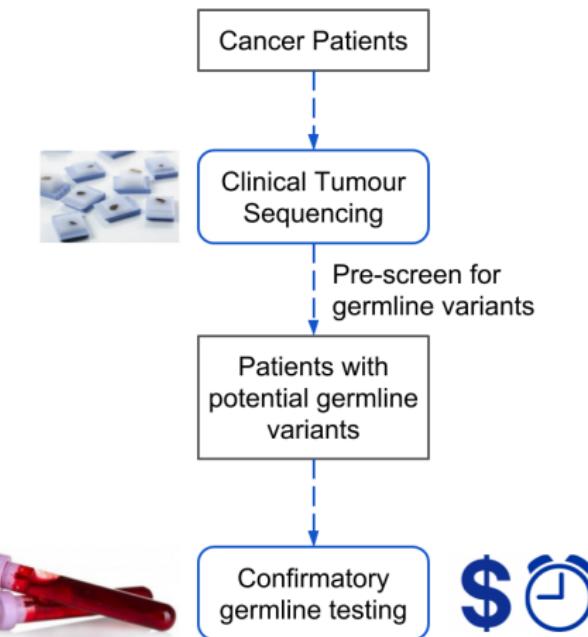
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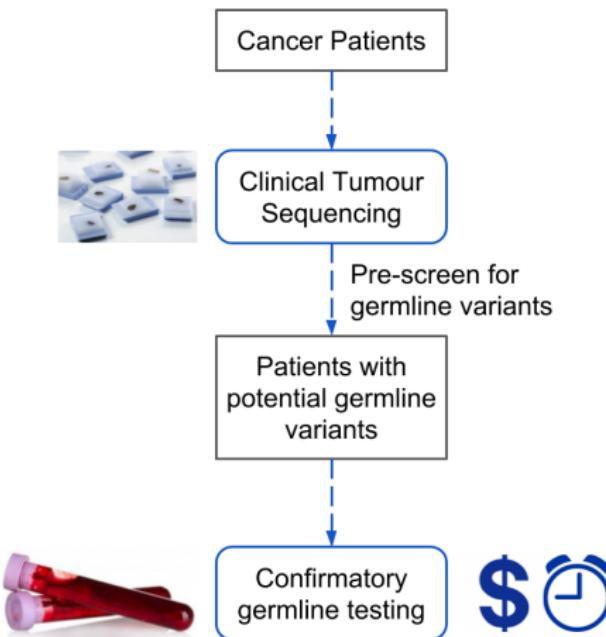
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Research Question

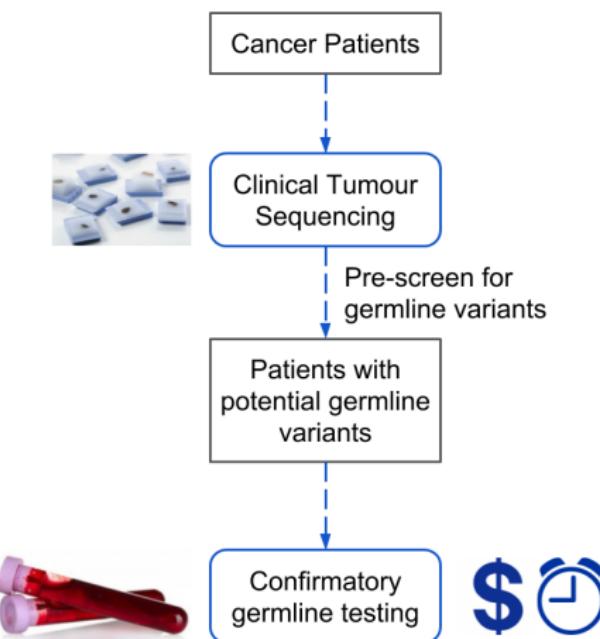


Research Question

Can we use clinical tumour sequencing to identify germline variants?



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Challenges:

- Formalin-induced DNA damage
- Distinguishing between germline and somatic variants

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Challenge 1: Formalin-induced DNA damage

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Aim 1: Characterize formalin-induced DNA damage in amplicon sequencing data

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Aim 1: Characterize formalin-induced DNA damage in amplicon sequencing data

Challenge 2: Distinguishing between germline and somatic variants

Aim 2: Determine the retention rate of germline variants in tumours

Thesis Aims

Can we use clinical tumour sequencing to identify germline variants?

Challenge 1: Formalin-induced DNA damage

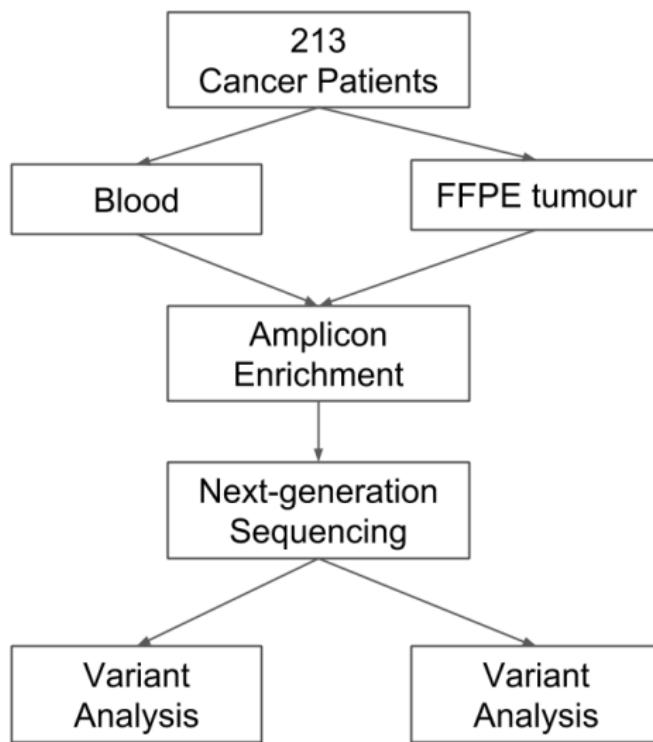
Aim 1: Characterize formalin-induced DNA damage in amplicon sequencing data

Challenge 2: Distinguishing between germline and somatic variants

Aim 2: Determine the retention rate of germline variants in tumours

Aim 3: Evaluate the use of variant allele frequency (VAF) thresholds to separate germline from somatic variants in FFPE tumours

Study Design: The OncoPanel Pilot (TOP) Study



Tumour types in the TOP cohort

Cancer Type	Number of Cases	Percentage (%)
Colorectal	97	46
Lung	59	28
Melanoma	18	8
Other	17	8
GIST	7	3
Sarcoma	4	2
Neuroendocrine	4	2
Cervical	2	0.9
Ovarian	2	0.9
Breast	2	0.9
Unknown	1	0.5

Cancer-related genes in the OncoPanel

Gene	Protein
AKT1	Protein kinase B
ALK	Anaplastic lymphoma receptor tyrosine kinase
BRAF	Serine/threonine-protein kinase B-Raf
EGFR	Epidermal growth factor receptor
HRAS	GTPase HRas
MAPK1	Mitogen-activated protein kinase 1
MAP2K1	Mitogen-activated protein kinase kinase 1
MTOR	Serine/threonine-protein kinase mTOR
NRAS	Neuroblastoma RAS viral oncogene homolog
PDGFRA	Platelet-derived growth factor receptor alpha
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PTEN	Phosphatase and tensin homolog
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
TP53	Tumor protein P53

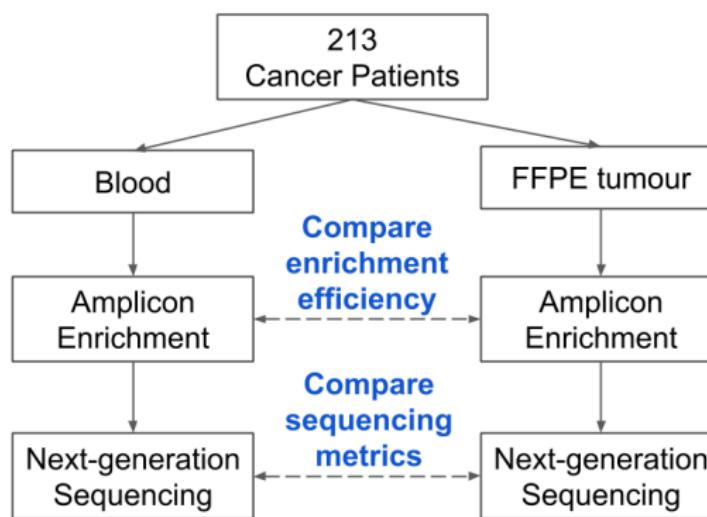
Pharmacogenomic-related genes in the OncoPanel

Gene	Protein	Chemotherapy
DPYD	Dihydropyrimidine dehydrogenase	5-FU
GSTM1	Glutathione S-transferase pi 1	Oxaliplatin
MTHFR	Methylenetetrahydrofolate reductase	5-FU
TYMP	Thymidine phosphorylase	5-FU
TYMS	Thymidylate synthetase	5-FU
UGT1A1	Uridine diphosphate (UDP)-glucuronosyl transferase 1A1	Irinotecan

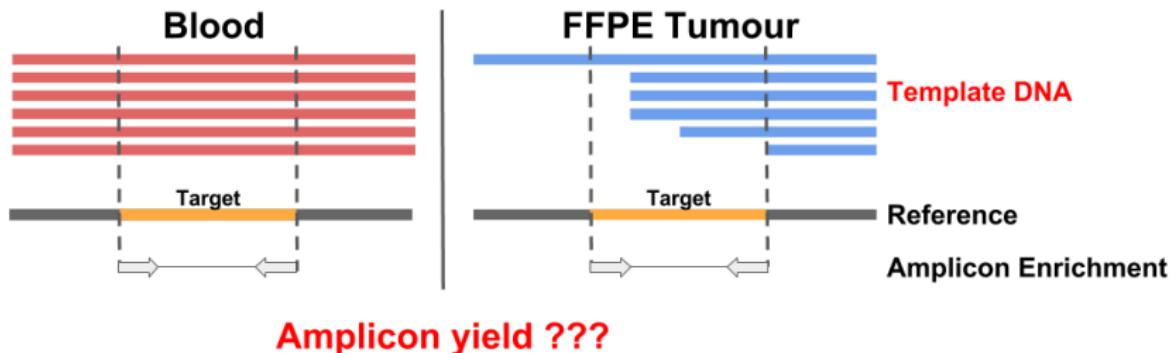
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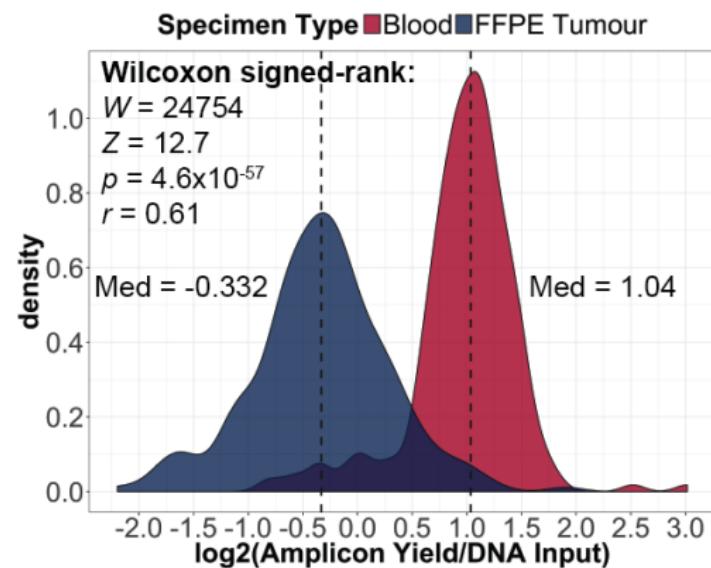
Formalin-induced DNA fragmentation could affect amplicon enrichment



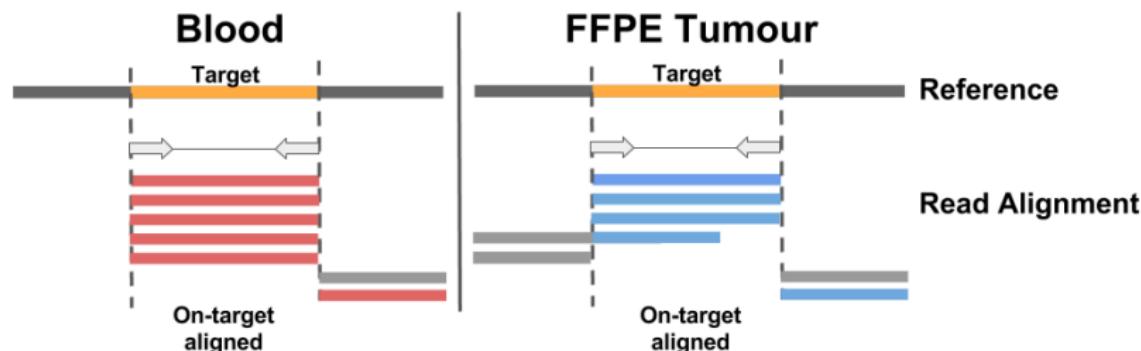
FFPE specimens demonstrate reduced amplicon enrichment

Enrichment efficiency:

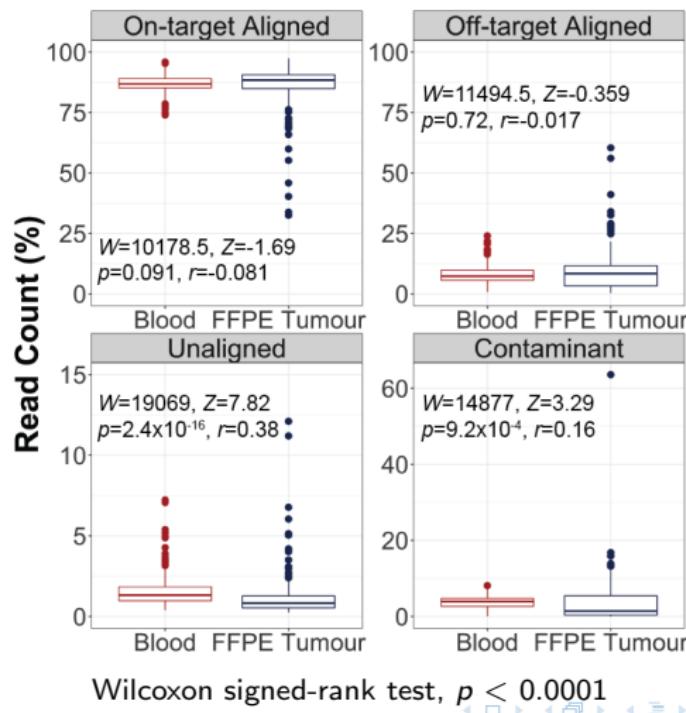
$$\log_2 \frac{\text{Amplicon Yield (ng)}}{\text{DNA Input (ng)}}$$



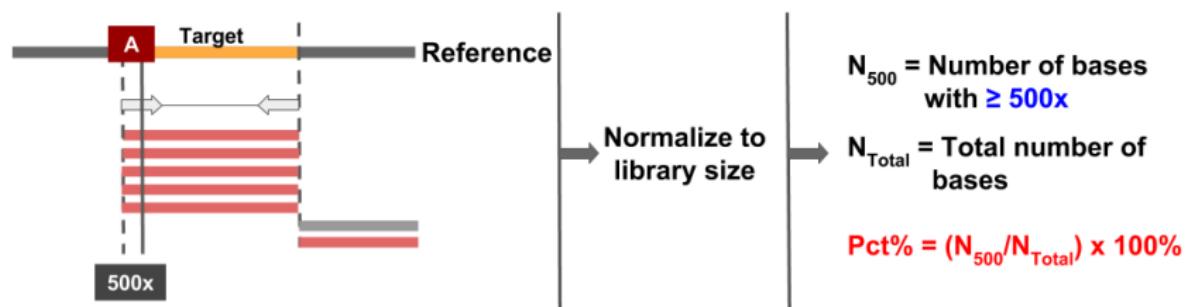
Formalin-induced DNA damage could affect read alignments



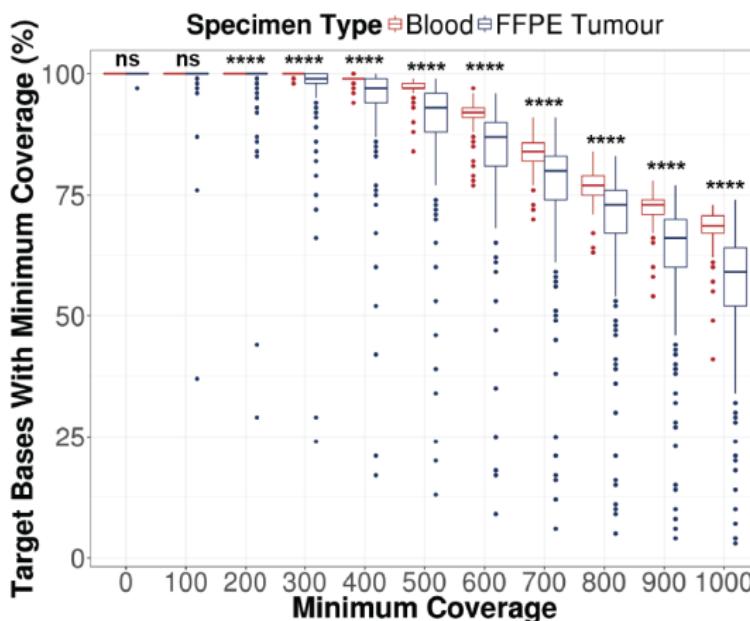
FFPE and blood specimens show comparable percentage of on-target aligned reads



Measuring coverage uniformity as the percentage of target bases that met increasing coverage thresholds

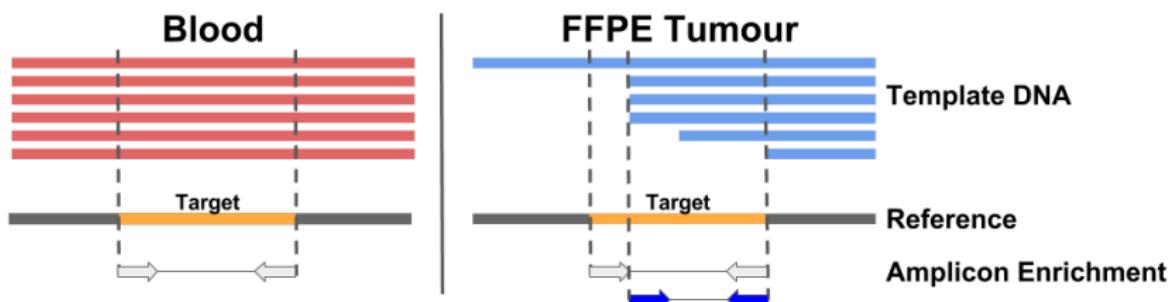


FFPE specimen show lower coverage uniformity at coverage thresholds >100x



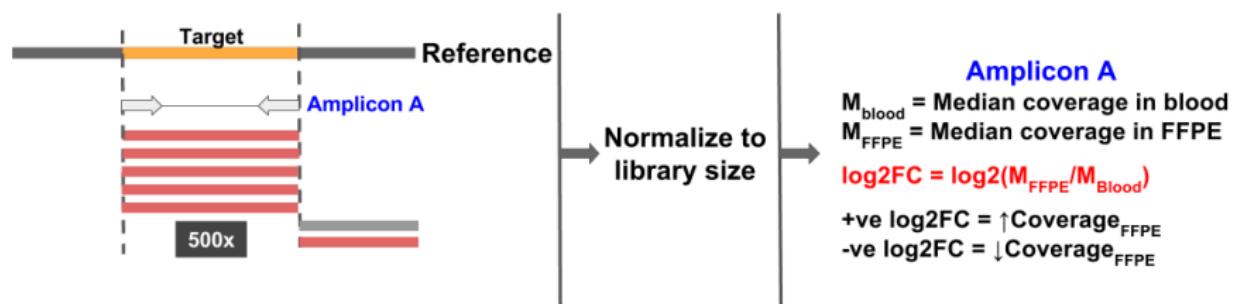
Wilcoxon signed-rank test, **** $p < 0.0001$, ns = not significant

OncoPanel consists of 416 amplicons



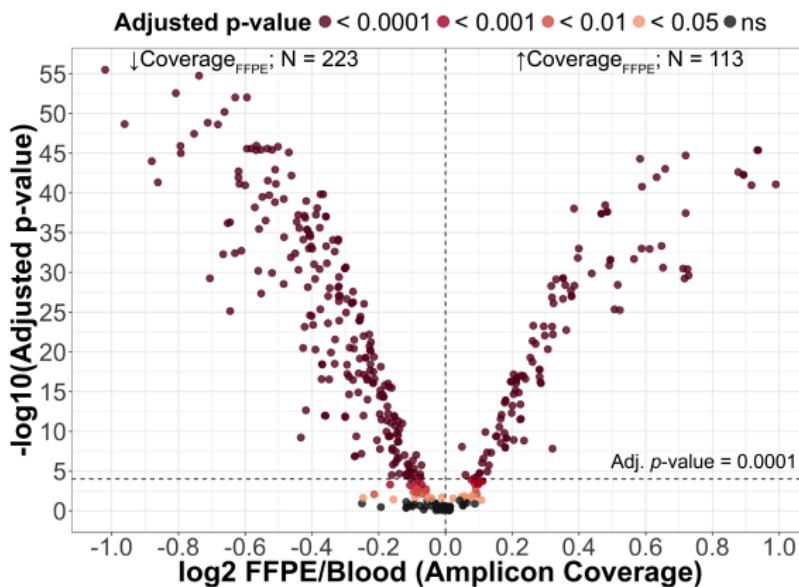
Shorter amplicons might yield greater coverage depth in FFPE specimens due to fragmentation damages in template DNA.

Analysis of amplicon-specific coverage depth



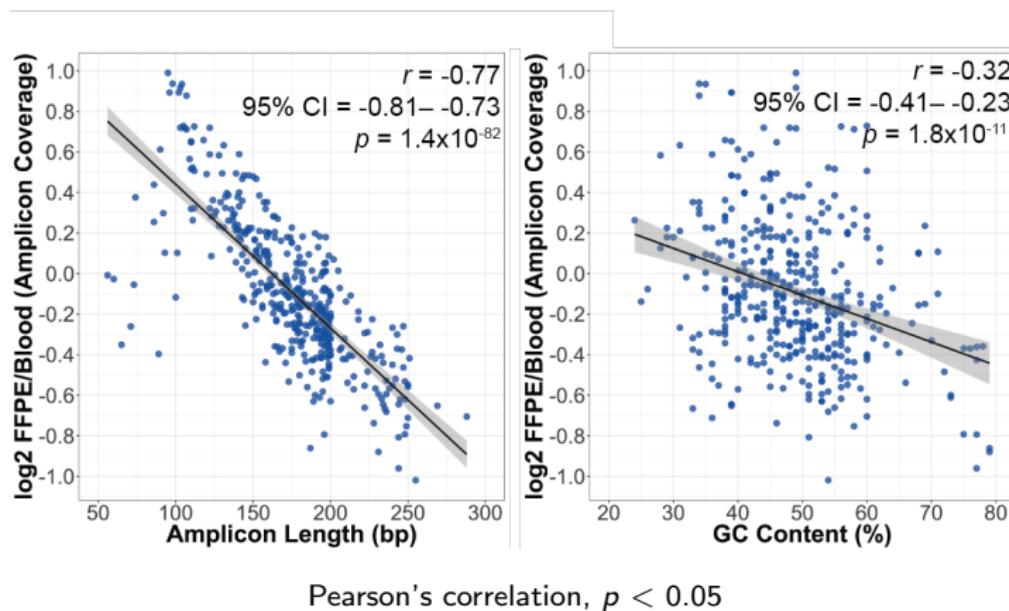
Comparison of amplicon coverage was performed with the Wilcoxon signed-rank test.

There are more amplicons with lower coverage depth in FFPE specimens relative to blood specimens



Wilcoxon signed-rank test with Benjamini Hochberg correction

Decreased amplicon coverage in FFPE specimens is correlated with increased amplicon length and GC content



Reduced amplicon coverage in FFPE specimens is more pronounced for longer amplicons

Multiple regression of amplicon length and GC content as predictors for log2FC between amplicon coverage in FFPE specimens and blood.

Variable	Unstandardized	S.E.	Standardized	p-value
Length (bp)	-6.97×10^{-3}	2.59×10^{-4}	-7.56×10^{-1}	7.45×10^{-93}
GC Content (%)	-1.03×10^{-2}	1.01×10^{-3}	-2.88×10^{-1}	4.71×10^{-22}
Intercept = 1.63, Adjusted R ² = 0.673 $F(2, 413) = 427.6$, p-value = 2.41×10^{-101}				

- ① 1 s.d. increase in amplicon length = 0.756 s.d. decrease in log2FC

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- ② 1 s.d. increase in amplicon GC content = **0.288** s.d. decrease in log2FC

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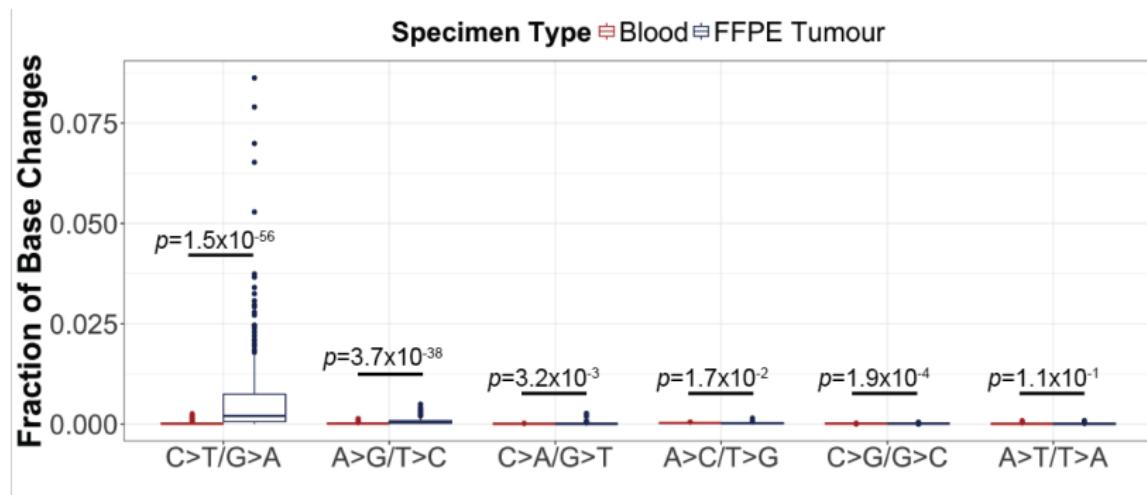
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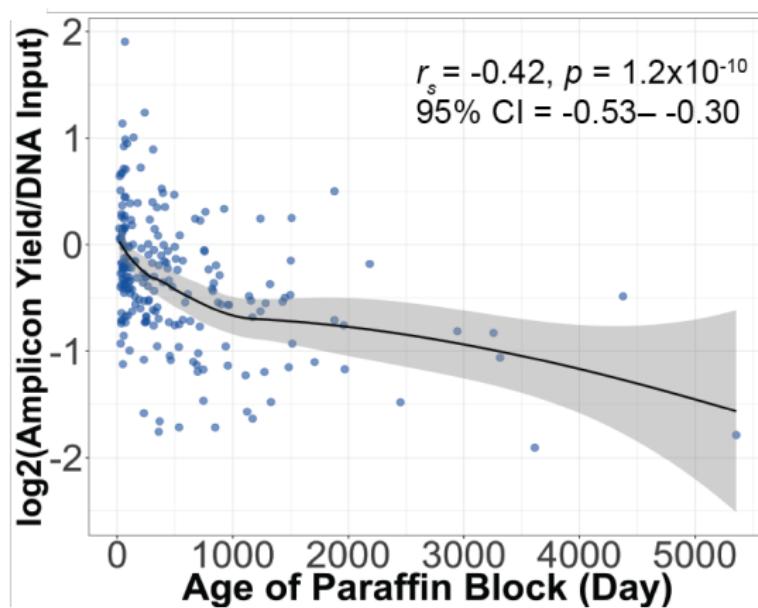
Discrepancy in coverage depth between FFPE and blood specimens could be mitigated by using shorter amplicons.

FFPE specimens demonstrate increased C>T/G>A sequence artifacts

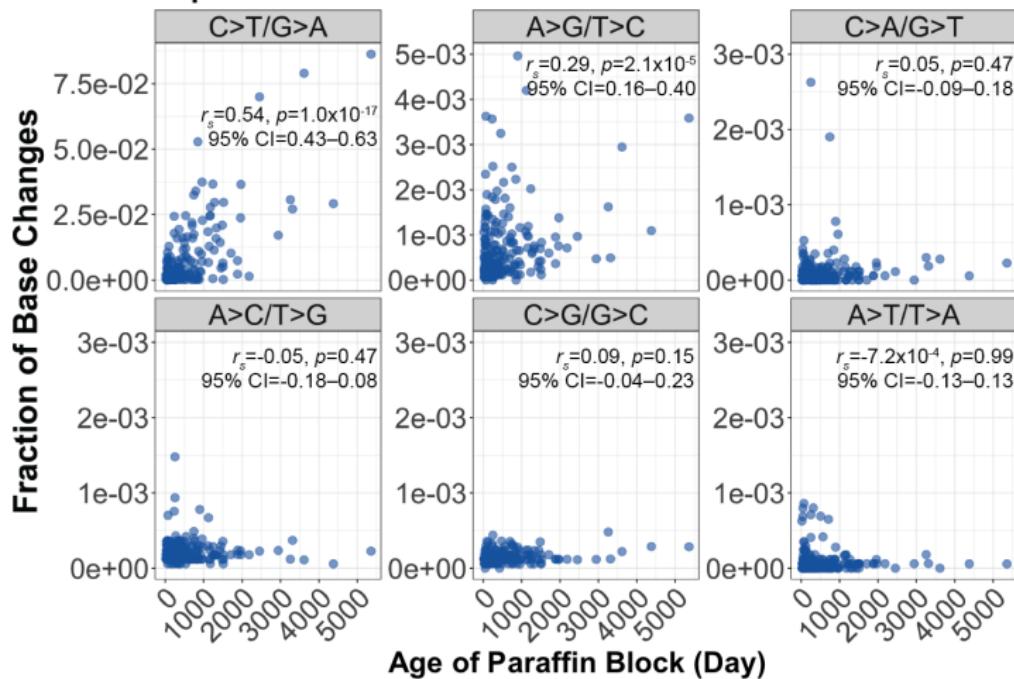


Wilcoxon signed-rank, $p < 0.0001$

Increased age of paraffin block results in lower amplicon enrichment



Increased age of paraffin block results in elevated events of C>T/G>A sequence artifacts



Spearman's rank correlation, $p < 0.05$

Summary for Aim 1

- ① FFPE specimens demonstrated reduced amplicon enrichment.

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- ③ Discrepancy in coverage depth between blood and FFPE specimens was more pronounced for longer amplicons.

Recommendation: Use shorter amplicons

Summary for Aim 1

- ④ Increased C>T/G>A and A>G/T>C artifacts were observed in FFPE specimens, but these differences were minor.

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- ⑤ Increased age of paraffin block resulted in lower amplicon enrichment and elevated events of artifactual transitions.

Summary for Aim 1

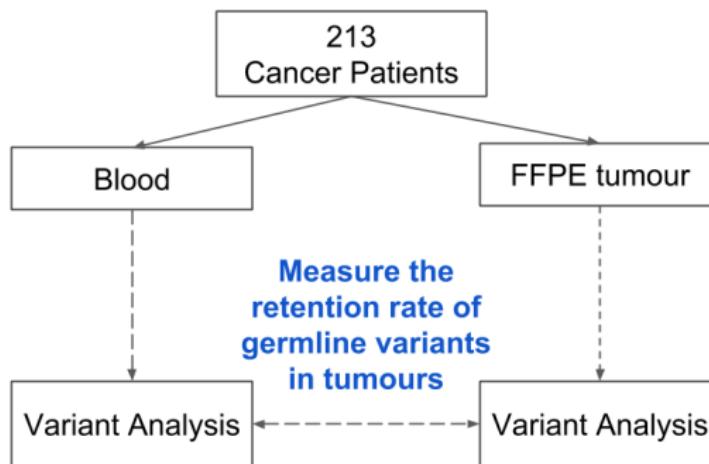
- ④ Increased C>T/G>A and A>G/T>C artifacts were observed in FFPE specimens, but these differences were minor.
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Recommendation: Avoid long-term storage of FFPE blocks

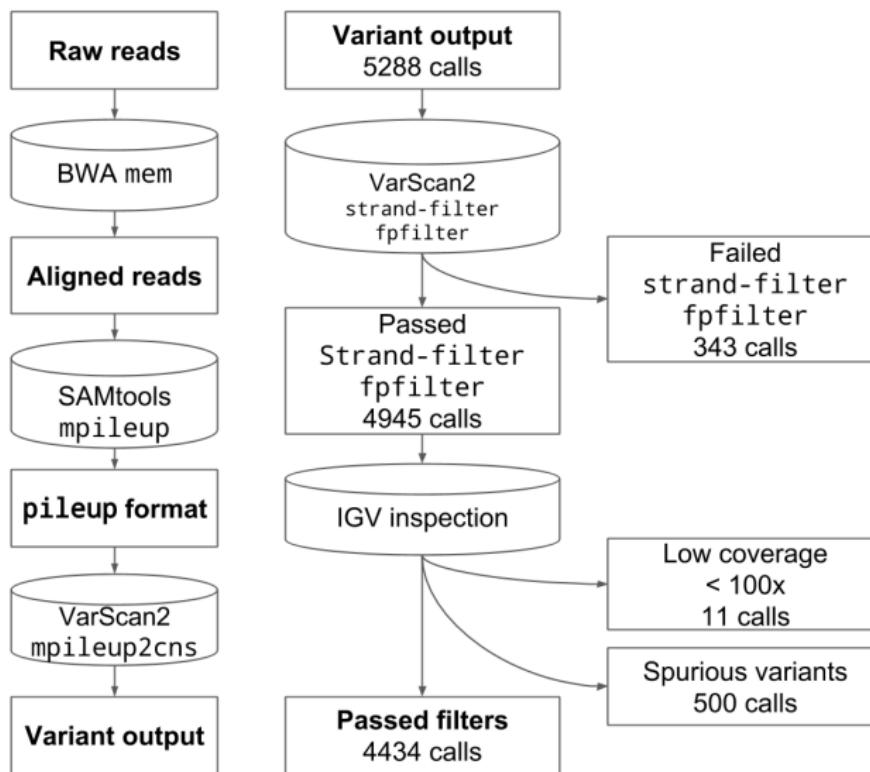
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Aim 2: Determine the retention rate of germline variants in tumours



Variant analysis pipeline



Eight drug-response-related germline variants are identified with the ClinVar database

Gene	Variant	dbSNP ID	Frequency [†]
TP53	p.Arg72Pro/c.215G>C	rs1042522	97
DPYD	p.Asp949Val/c.2846A>T	rs67376798	2
	c.1906G>A	rs3918290	1
	p.Met166Val/c.496A>G	rs2297595	34
GSTP1	p.Ile105Val/c.313A>G	rs1695	109
MTHFR	p.Glu429Ala/c.1286A>C	rs1801131	102
	p.Alanine222Val/c.665C>T	rs1801133	110
TYMS	c.447_452delTTAAAG	rs151264360	132

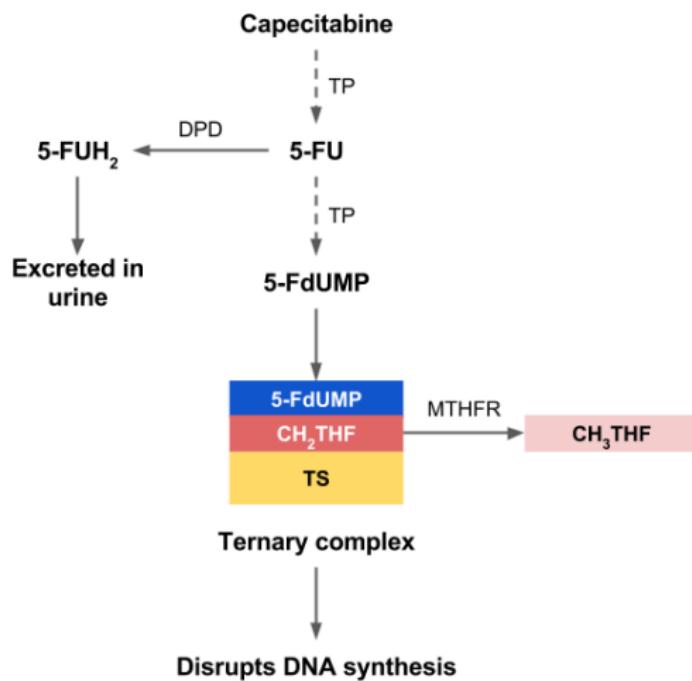
[†]Out of 213 patients.

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GSTP1	p.Met166Val/c.496A>G	rs2297595	34
MTHFR	p.Ile105Val/c.313A>G	rs1695	109
TYMS	p.Glu429Ala/c.1286A>C	rs1801131	102
	p.Alanine222Val/c.665C>T	rs1801133	110
TYMS	c.447_452delTTAAAG	rs151264360	132

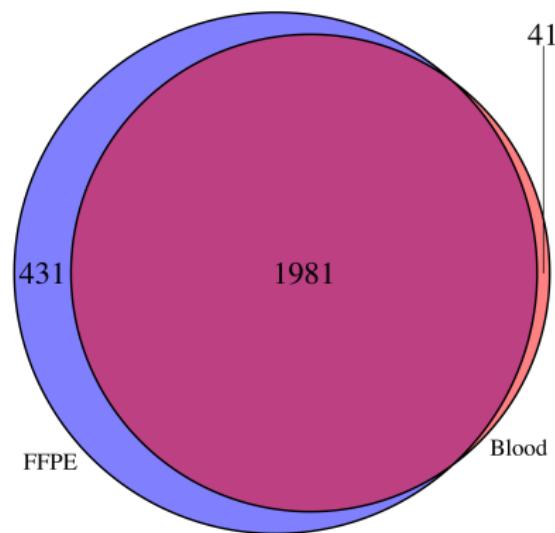
[†]Out of 213 patients.

DPYD c.1906G>A, rs3918290

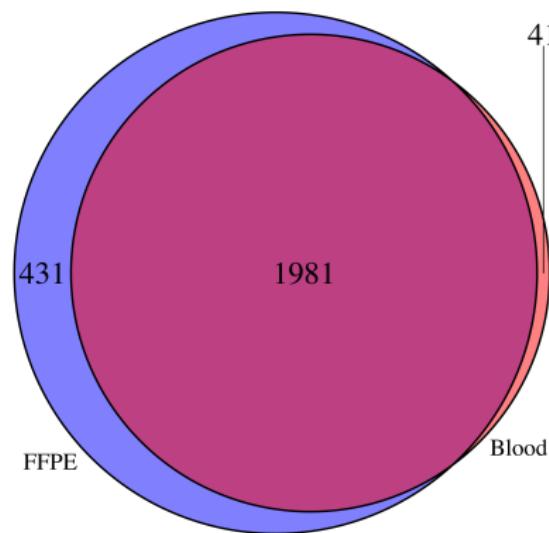


- Exon 14 is skipped, producing an inactive enzyme with no uracil-binding site.
- Strong clinical evidence indicating association with severe fluoropyrimidine-related toxicity.

98.0% of germline variants identified in blood are retained in FFPE tumours



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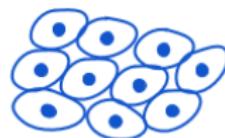
FFPE tumour DNA can be a reliable source
for germline variant calling.

Overview

- ① Background
- ② Research Question
- ③ Thesis Aims
- ④ Aim 1: Formalin-induced DNA damage could be mitigated by using shorter amplicons and avoiding older FFPE blocks.
- ⑤ Aim 2: FFPE tumour DNA is a reliable source for germline variant calling.
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- ⑦ Conclusions

Variant allele frequency (VAF)

Tumour content

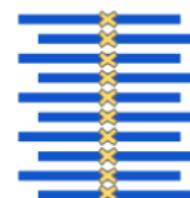


100%

Sequencing



Heterozygous variant
VAF = 50%



Homozygous variant
VAF = 100%

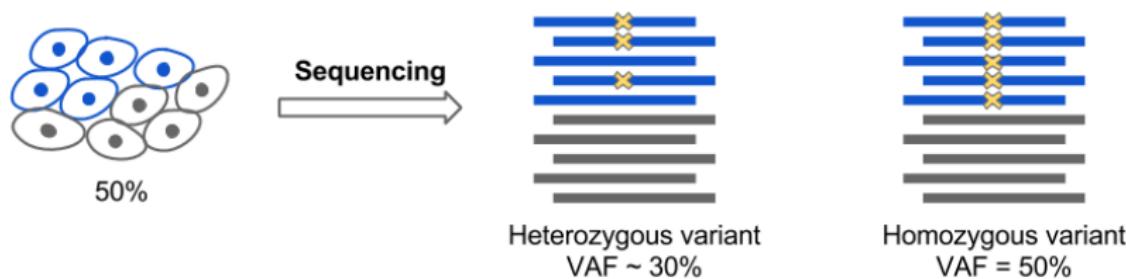
VAF in tumour specimens can deviate from diploid zygosity

DNA damage induced by formalin (e.g. fragmentation and sequence artifacts)



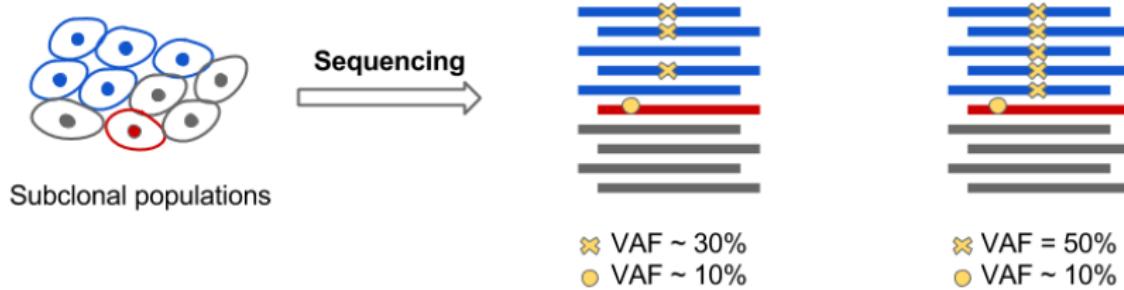
Somatic VAF in tumour specimens can deviate from diploid zygosity

Mixture of tumour and normal cells

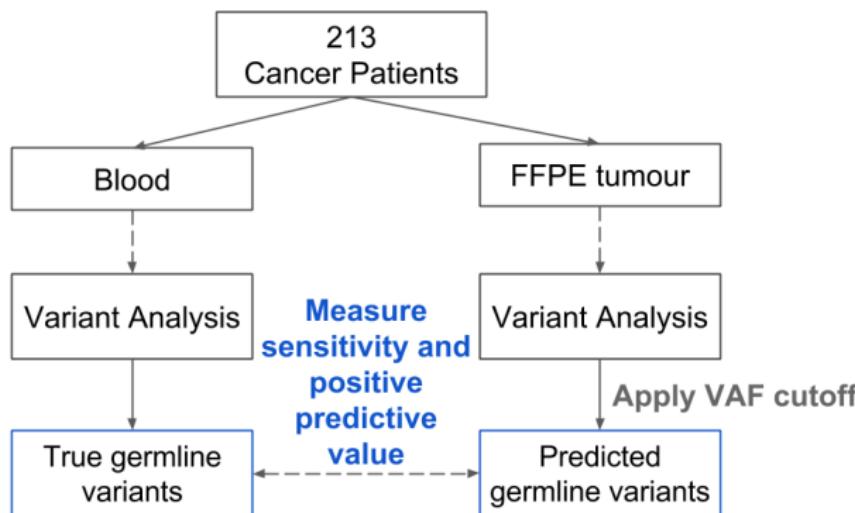


Somatic VAF in tumour specimens can deviate from diploid zygosity

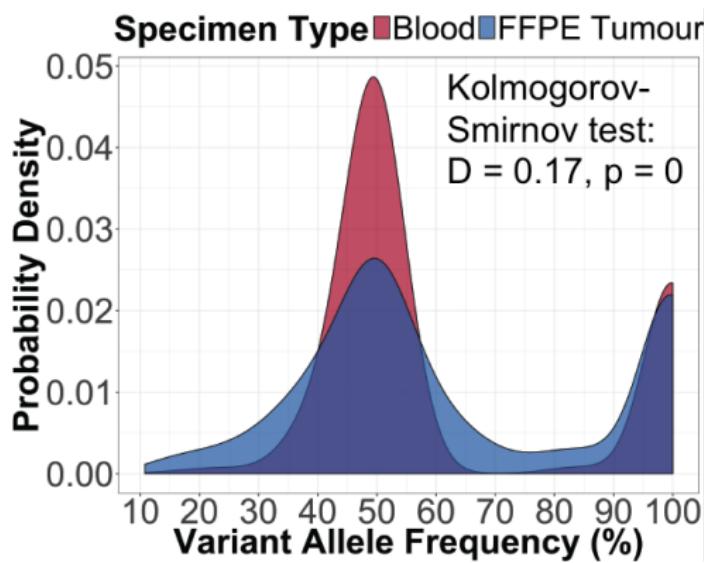
Tumour heterogeneity



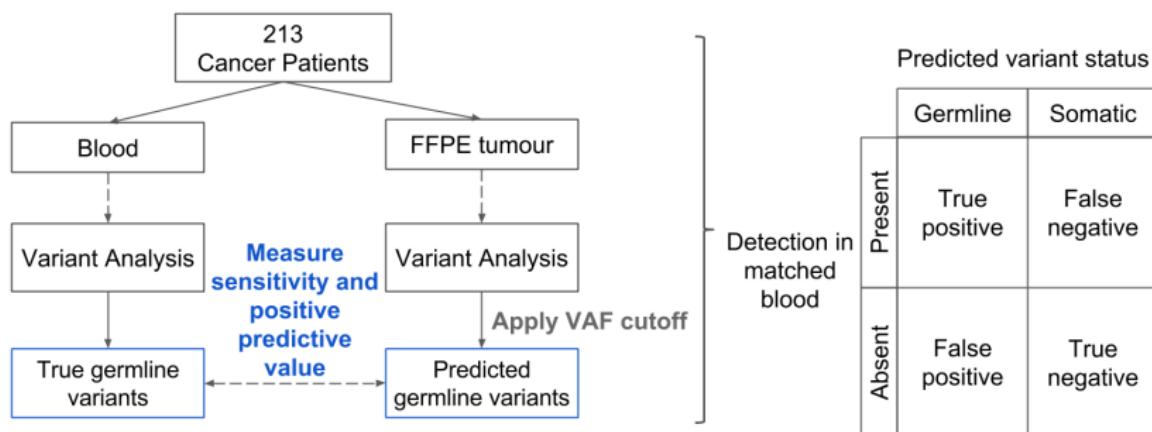
Aim 3: Evaluate the use of VAF thresholds to separate germline from somatic variants in FFPE tumours



VAF distributions of germline variants are different between blood and FFPE tumours



Measure sensitivity of identifying germline variants in FFPE tumours

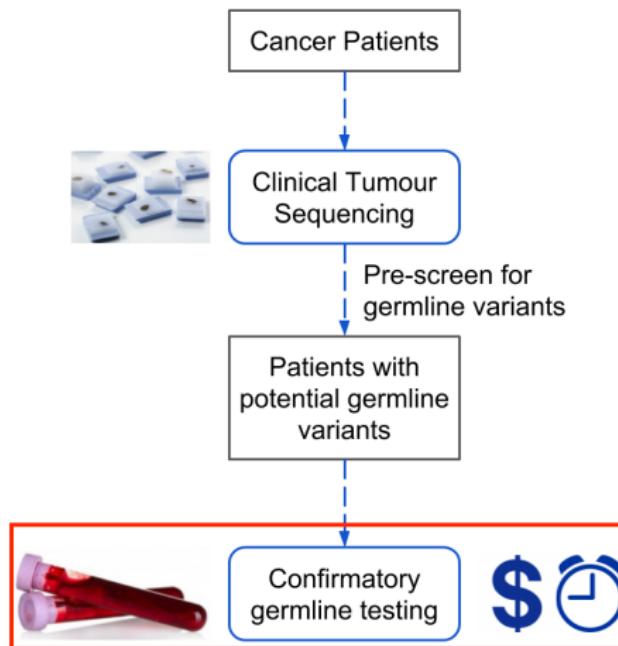


$$\text{Sensitivity} = \text{True positives} / (\text{True positives} + \text{False negatives})$$

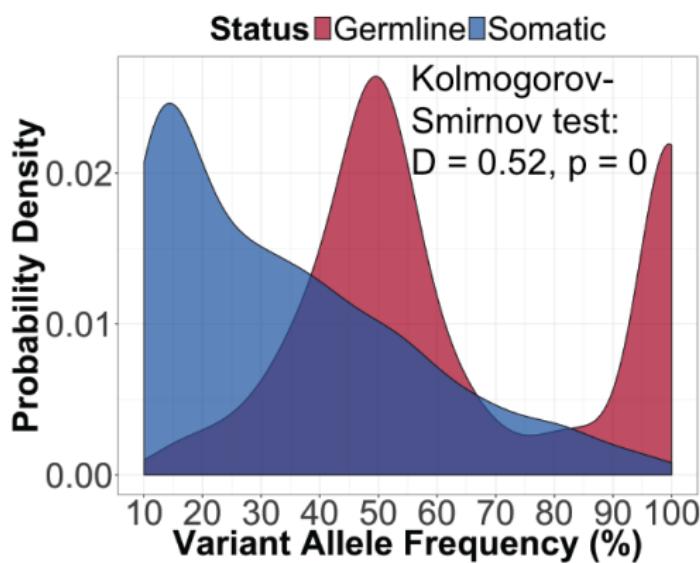
A VAF cutoff of 15% would correctly identify 99% of germline alterations in FFPE tumours

VAF (%)	False Negative	True Positive	Sensitivity	95% CI
10	0	1981	1.0	1.0–1.0
15	13	1968	0.99	0.99–1.0
20	46	1935	0.98	0.97–0.98
25	77	1904	0.96	0.95–0.97
30	117	1864	0.94	0.93–0.95
35	192	1789	0.90	0.89–0.92
40	313	1668	0.84	0.83–0.86
45	458	1523	0.77	0.75–0.79

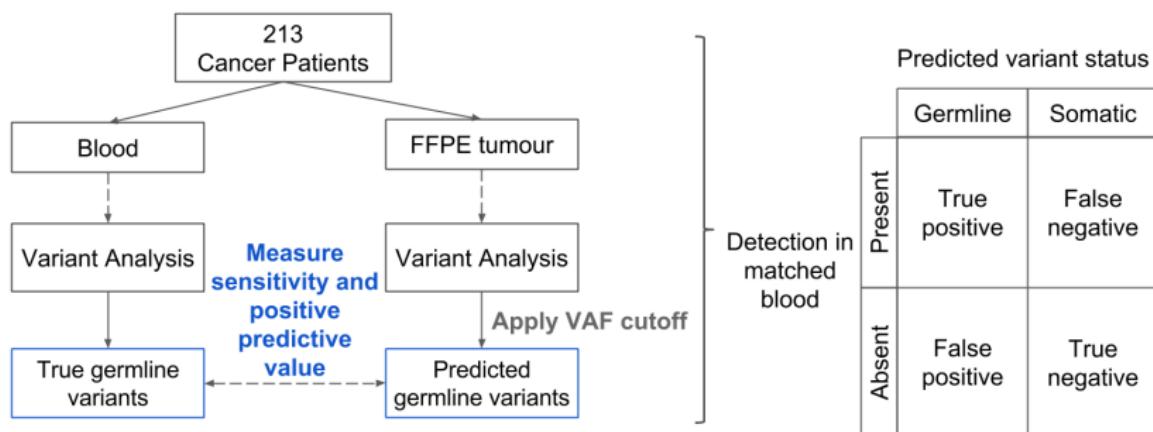
Referral of somatic variants to confirmatory germline testing should be minimized



VAF distributions are different between germline and somatic variants in FFPE tumours



Measure PPVs of identifying germline variants in FFPE tumours for follow-up testing



$$\text{PPV} = \text{True positives} / (\text{True positives} + \text{False positives})$$

A VAF cutoff of 15% would submit 14% of somatic mutations to follow-up germline testing

VAF (%)	False Positive	True Positive	Total Calls	PPV	95% CI
10	431	1981	2412	0.82	0.81–0.84
15	319	1968	2287	0.86	0.85–0.87
20	273	1935	2208	0.88	0.86–0.89
25	245	1904	2149	0.89	0.87–0.90
30	203	1864	2067	0.90	0.89–0.91
35	178	1789	1967	0.91	0.90–0.92
40	146	1668	1814	0.92	0.91–0.93
45	118	1523	1641	0.93	0.91–0.94

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Conclusions

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- ② 98.0% of germline alterations identified in the blood were retained in the tumours, suggesting that FFPE tumour DNA can be a reliable source for germline variant calling.
- ③ A VAF cut-off of 15% would correctly identify 99% of germline alterations in FFPE tumours, but erroneously submit 14% of somatic mutations for follow-up germline testing.

Conclusions

- ④ This underscores the high sensitivity and positive predictive value of using VAF to discriminate between germline and somatic variants.

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Collectively, these results demonstrate that clinical tumour amplicon sequencing could also be used to provide cost-efficient first-line germline testing.

Acknowledgements

Dr. Aly Karsan

Dr. Jennifer Grants

Dr. Jeremy Parker

Dr. Kieran O'Neill

Dr. Marion van den Bosch

Dr. Rawa Ibrahim

Dr. Sergio Martinez-Hoyer

Angela Mo

Aparna Gopal

Deborah Deng

Helen Lin

Jenny Li

Kristy Dockstader

Patrick Coulombe

Rod Docking

Sukhbir Manku

Committee members:

Dr. Martin Hirst

Dr. Ryan Morin

Centre for Clinical Genomics

Canada's Michael Smith Genome Sciences Centre

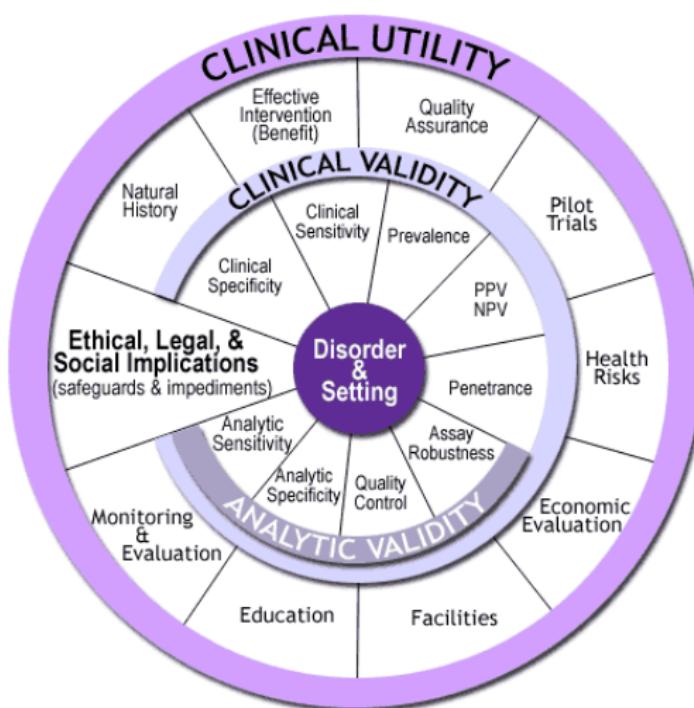
Funding sources:

Canadian Institutes of Health Research

UBC Centre for Blood Research

Patients from The OncoPanel Pilot study (H14-01212)

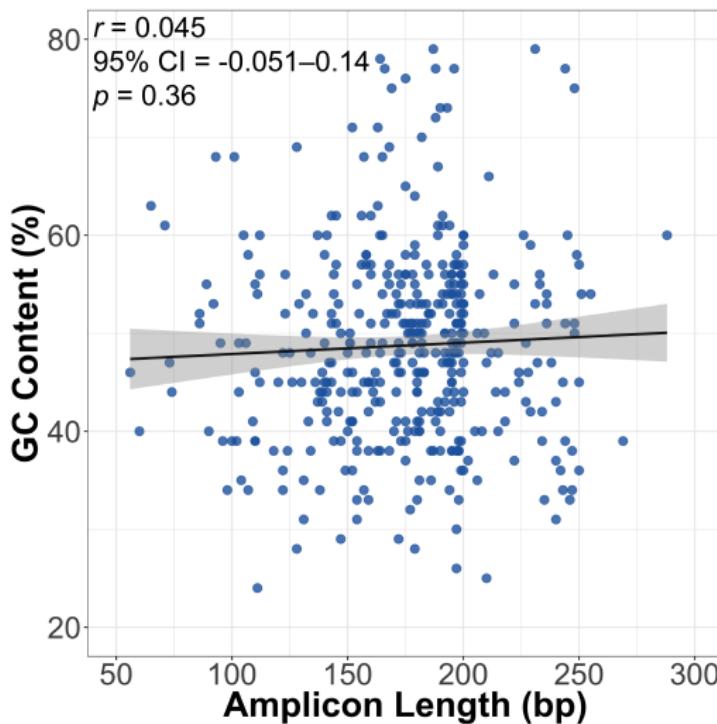
Process for evaluation of genetic tests



Percentage of target bases is significantly different at all coverage thresholds

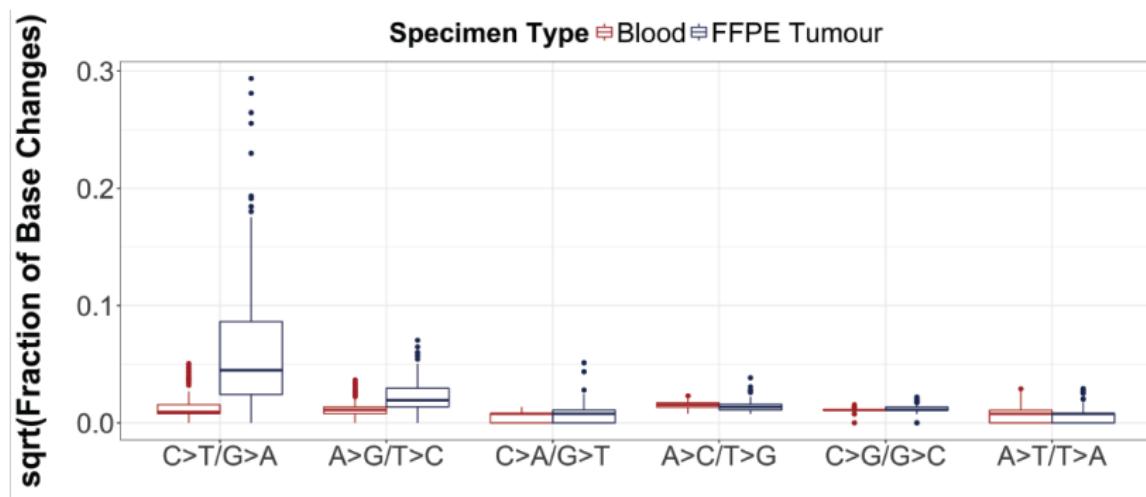
Threshold	Blood		FFPE Tumour		$p (< 0.05^*)$	r
	Median (%)	Range (%)	Median (%)	Range (%)		
$\geq 0x$	100.0	100.0–100.0	100.0	97.0–100.0	1.0	0.068
$\geq 100x$	100.0	100.0–100.0	100.0	37.0–100.0	$2.3 \times 10^{-4}^*$	0.25
$\geq 200x$	100.0	100.0–100.0	100.0	29.0–100.0	$2.9 \times 10^{-11}^*$	0.41
$\geq 300x$	100.0	98.0–100.0	99.0	24.0–100.0	$4.1 \times 10^{-18}^*$	0.55
$\geq 400x$	99.0	94.0–100.0	97.0	17.0–100.0	$5.0 \times 10^{-28}^*$	0.68
$\geq 500x$	97.0	84.0–99.0	89.5	13.0–99.0	$2.1 \times 10^{-38}^*$	0.77
$\geq 600x$	92.0	77.0–97.0	87.0	9.0–96.0	$1.5 \times 10^{-32}^*$	0.72
$\geq 700x$	84.0	70.0–91.0	80.0	6.0–91.0	$5.7 \times 10^{-25}^*$	0.65
$\geq 800x$	77.0	63.0–84.0	73.0	5.0–83.0	$4.7 \times 10^{-27}^*$	0.67
$\geq 900x$	73.0	54.0–78.0	66.0	4.0–77.0	$4.6 \times 10^{-40}^*$	0.78
$\geq 1000x$	68.5	41.0–73.0	59.0	3.0–74.0	$3.6 \times 10^{-42}^*$	0.79

Amplicon length and GC content

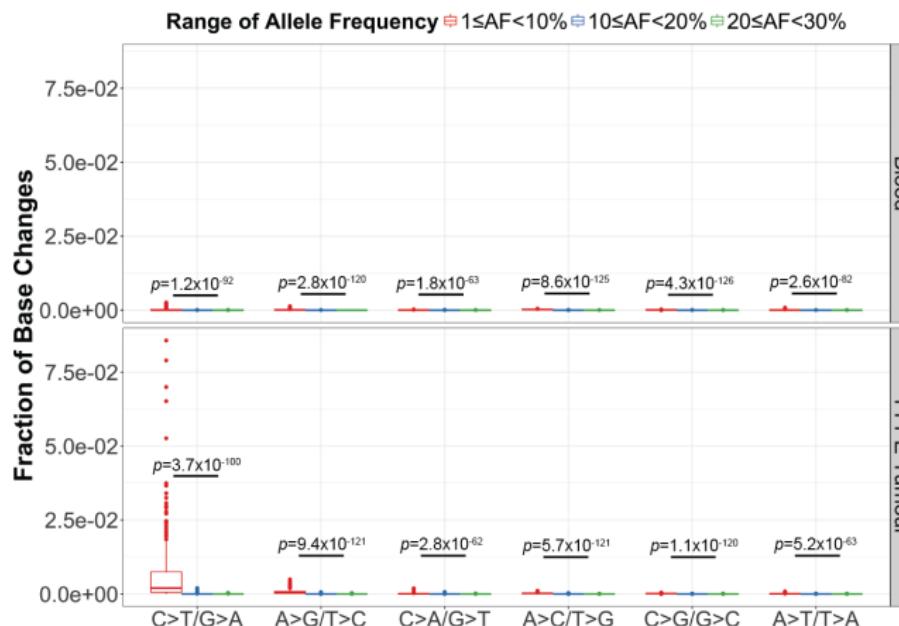


Pearson's correlation, $p < 0.05$

FFPE specimens demonstrate increased C>T/G>A sequence artifacts

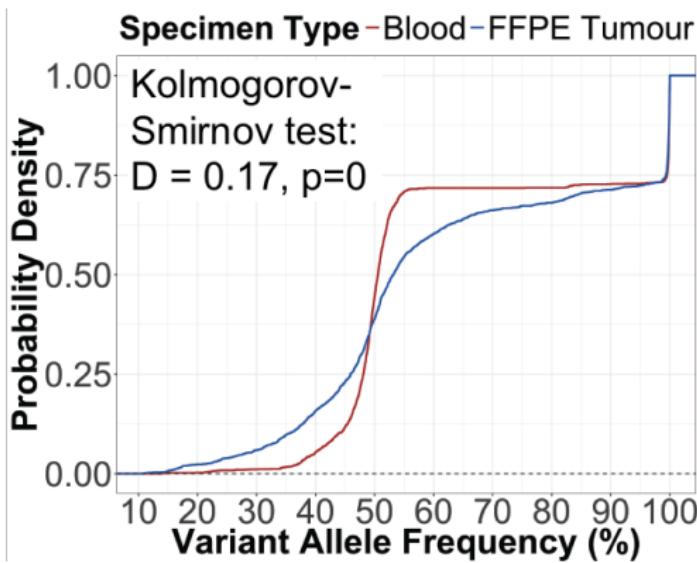


FFPE specimens demonstrate increased C>T/G>A sequence artifacts at low-allelic fraction



Kruskal-Wallis test, $p < 0.0001$

VAF distributions of germline variants are different between blood and FFPE tumours



VAF distributions are different between germline and somatic variants in FFPE tumours

