The MSK-IMPACT program: Experience with comprehensive clinical testing for cancer gene alterations and oncogenic fusions in over 20,000 patients with advanced solid cancers

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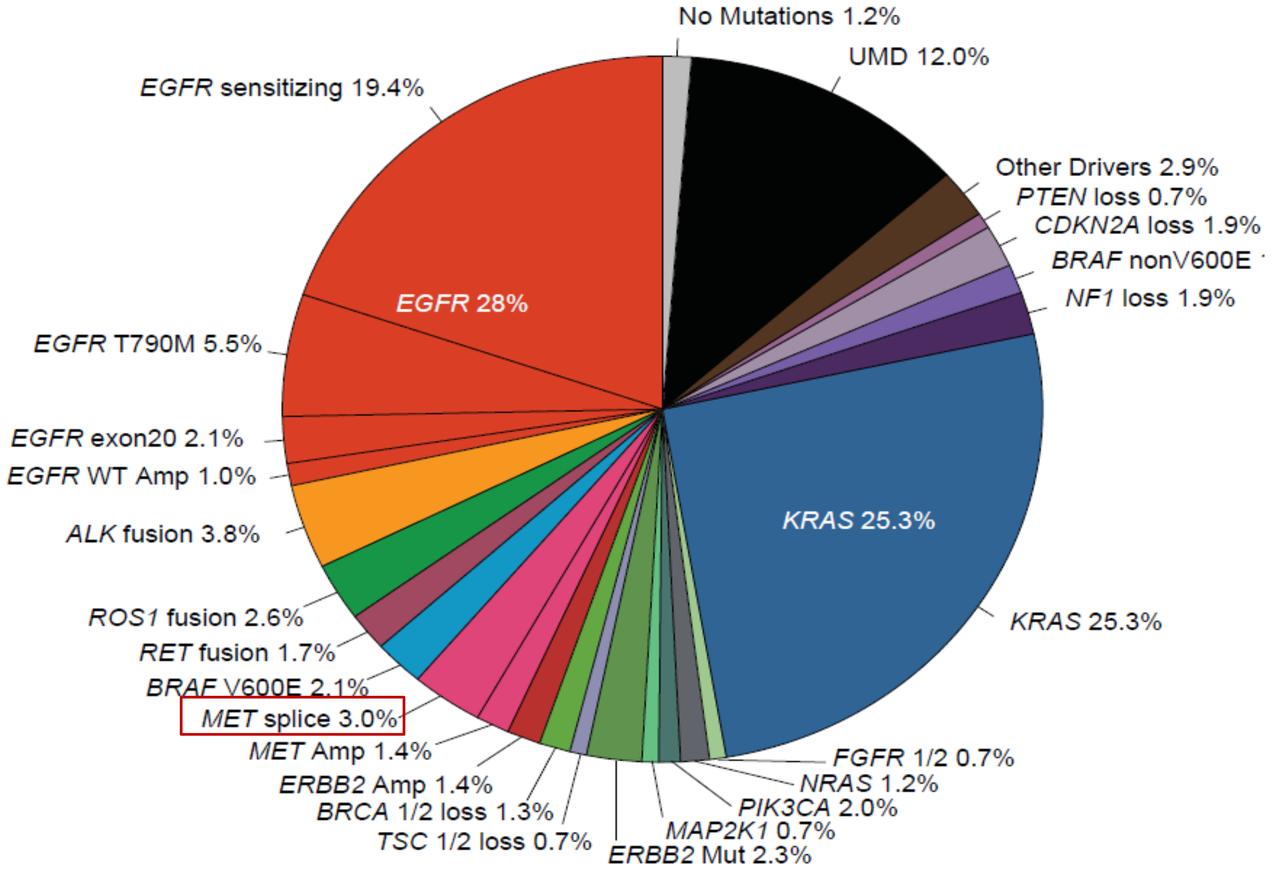
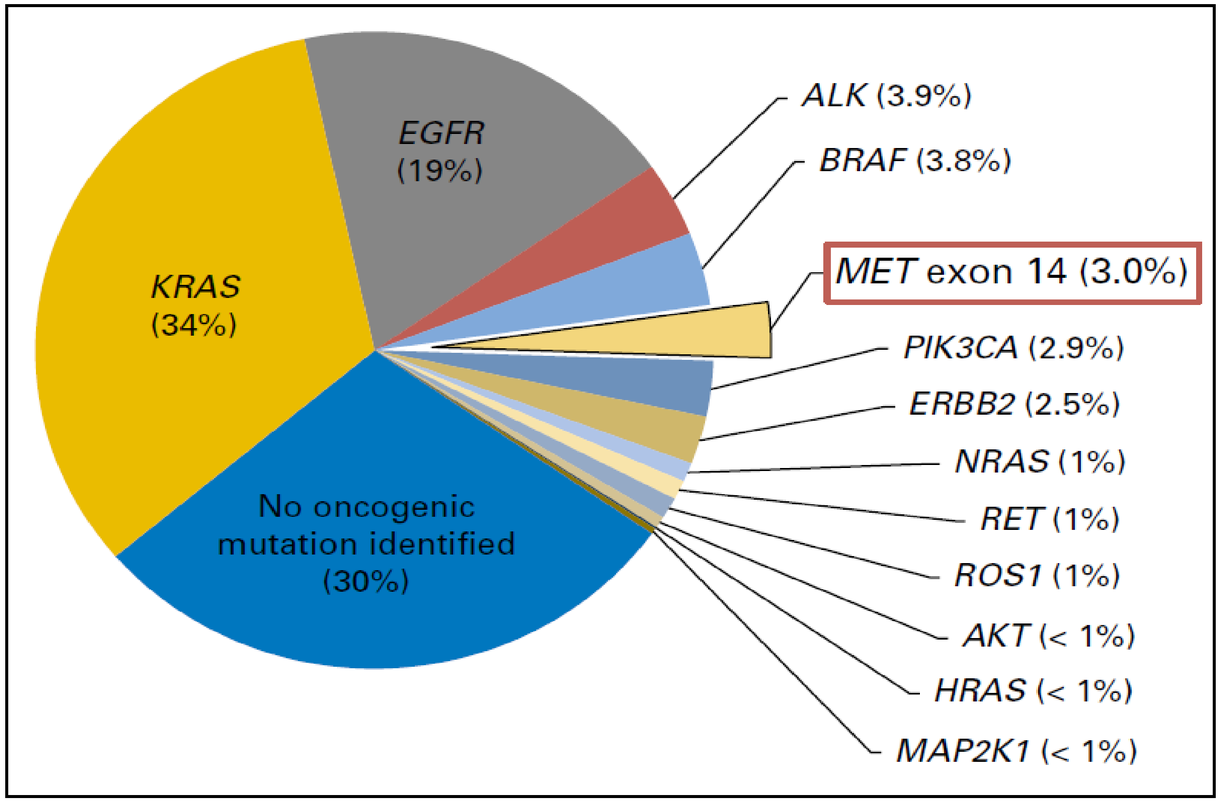
Proliferation of targetable molecular subsets
Example: lung adenocarcinoma

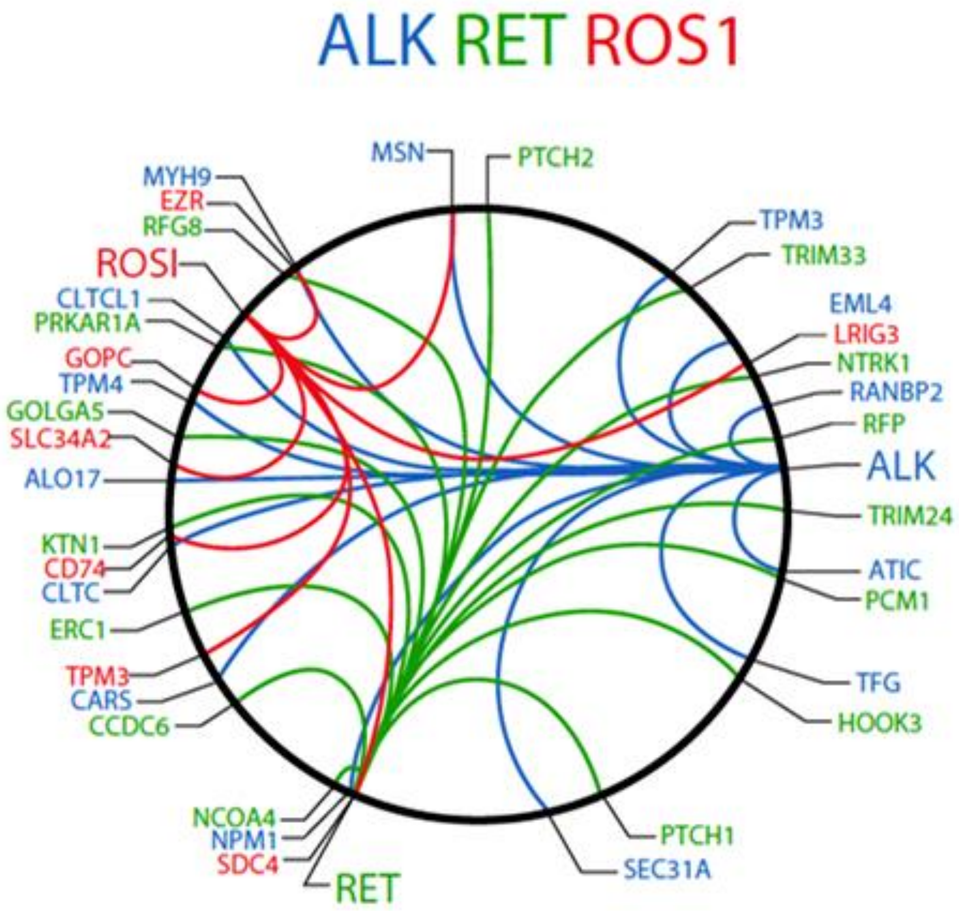


Jordan E, et al., *Cancer Discovery* (in press) 2017



Awad *et al*. *J Clin Oncol*. 2016 Mar 1;34(7):721-30

Proliferation of targetable molecular subsets
Example: lung adenocarcinoma



Proliferation of targetable fusions

Promiscuity of major fusion genes:
recombine with multiple partners

Pan Genomic Analysis

- Whole genome
- Whole exome
- Whole transcriptome

Primarily research labs

- Unbiased approach – comprehensive
- Higher cost limits sequencing depth
- Longer turnaround time (TAT) – usually not suitable for therapeutic decisions in most clinical lung cancer settings

Targeted Assays

- Single gene assays
- Low multiplex
- NGS panels:
  - Hybrid capture
  - Amplicon capture

Clinical Labs

- Higher coverage of genomic regions of interest
- Lower cost and time
- Enrichment methods to improve detection
- Provide results in clinical time frame

Ideal Features of a Clinical NGS assay for Cancer Molecular Diagnosis

- Can be performed at high volume in a resource-efficient manner
- Clinically viable time to final results (3 weeks or less)
- Performs well on typical clinical samples (small FFPE biopsies)
- Multiple types of genetic alterations studied by a single test
- Multiple types of cancer studied by a single test
- Relatively easy to update content

- **Platforms**
  - Illumina sequencing
  - Ion Torrent sequencing
- **Target enrichment**
  - Hybrid capture
  - Amplicon capture (highly multiplexed PCR)

MiSeq

HiSeq

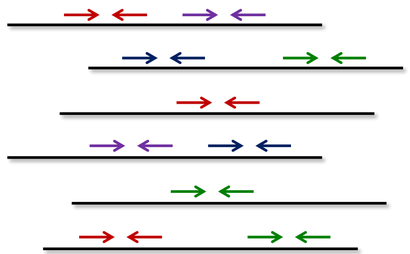
NovaSeq

Ion PGM

Ion Proton

Targeted Deep Sequencing of Key Cancer Genes

Amplicon Capture

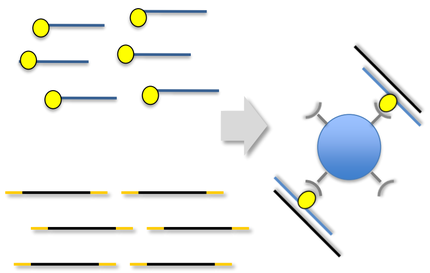


Illumina MiSeq

Ion Torrent PGM

10 – 100s of genes
Advantage: lowest input DNA requirement
Disadvantages: poorly suited to detection of copy number changes and rearrangements

Hybridization Capture



Illumina HiSeq

Ion Torrent Proton

100s to 20,000 genes
Advantage: better suited to detection of copy number changes and rearrangements , more sensitive
Disadvantages: higher input DNA requirement

The MSK-IMPACT initiative at MSKCC

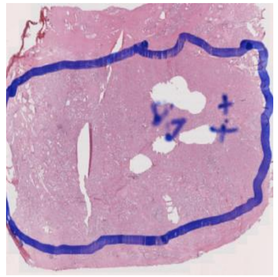
1. Overview of test design and validation, data and experience; secondary germline testing, driving basket trials; sharing data
2. Gene fusions: novel and underestimated; need for complementary targeted RNAseq

MSK-IMPACT

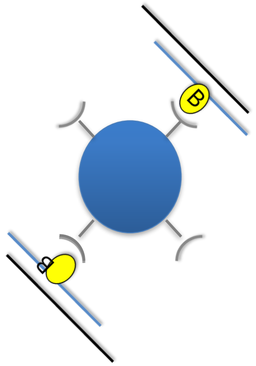
Integrated Mutation Profiling of Actionable Cancer Targets

Cheng DT, *et al.*, *J Mol Diagnostics*, March 2015 - **Method**

DNA from **FFPE Tumor** and **Normal** cells



Capture DNA for **468 cancer genes**

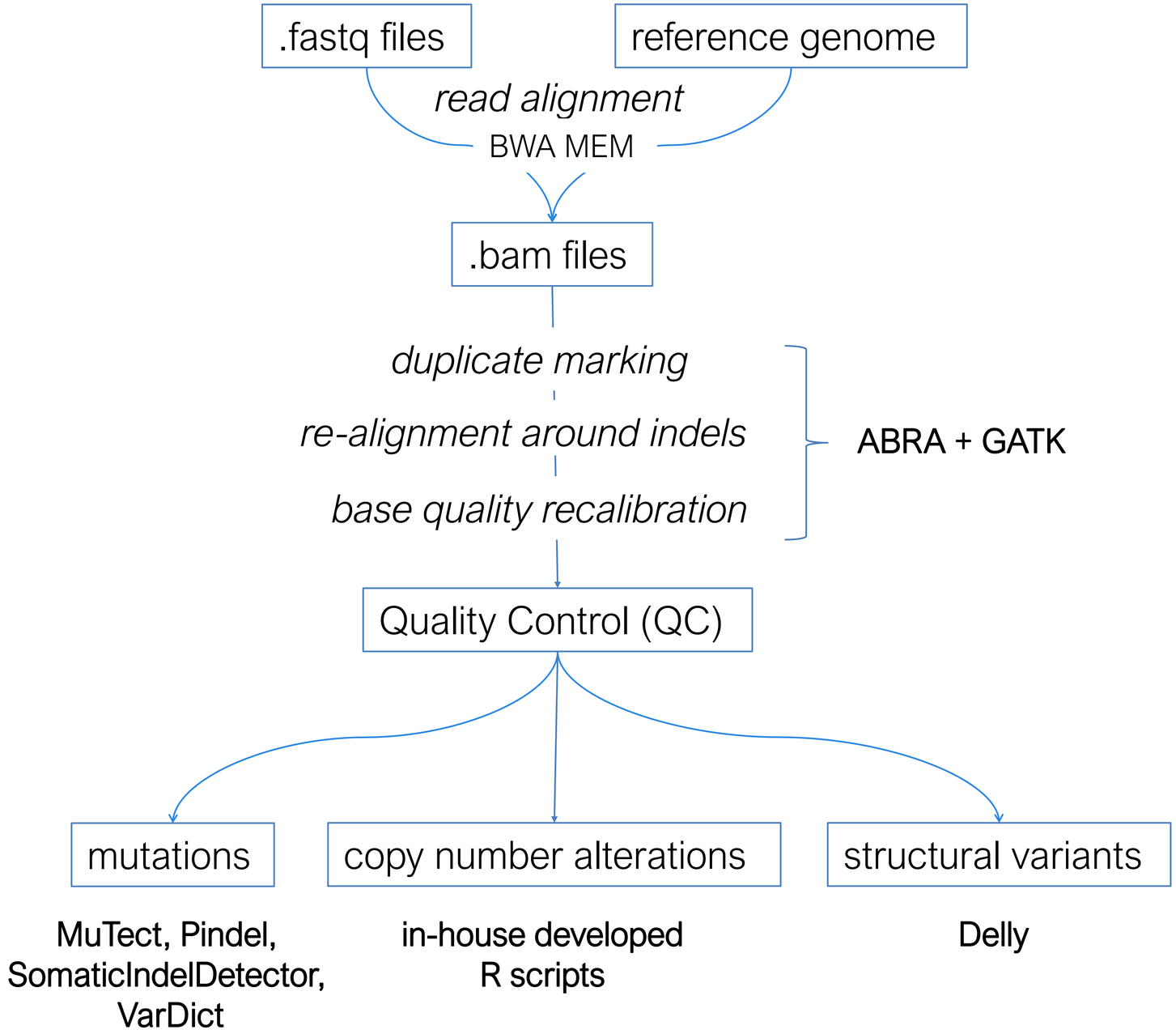


Next-gen Sequencing (500 - 1000 x)

Align to genome and analyze

<u>Somatic Mutations (Tumor-Normal Pairs)</u>:
Base Substitutions
Small Indels
Copy Number Alterations
Select Rearrangements

MSK-IMPACT: bioinformatic pipeline overview



Ahmet Zehir

MSK-IMPACT: Current content (468 genes)

ABL1	BRCA2	CUL3	FANCC	IDH1	MAPK1	NOTCH4	PRDM1	SDHAF2	TNFAIP3	H3F3A	RHEB	MSI1
AKT1	BRD4	DAXX	FAT1	IDH2	MAX	NPM1	PRKAR1A	SDHB	TNFRSF14	H3F3B	SH2B3	MSI2
AKT2	BRIP1	DCUN1D1	FBXW7	IFNGR1	MCL1	NRAS	PTCH1	SDHC	TOP1	HIST1H3A	SRSF2	NTHL1
AKT3	BTK	DDR2	FGF19	IGF1	MDC1	NSD1	PTEN	SDHD	TP53	HIST1H3C	STAT3	NUF2
ALK	CARD11	DICER1	FGF3	IGF1R	MDM2	NTRK1	PTPN11	SETD2	TP63	HIST1H3D	STAT5A	PDCD1LG2
ALOX12B	CASP8	DIS3	FGF4	IGF2	MDM4	NTRK2	PTPRD	SF3B1	TRAF7	HIST1H3E	STAT5B	PPARG
APC	CBFB	DNMT1	FGFR1	IKBKE	MED12	NTRK3	PTPRS	SH2D1A	TSC1	HIST1H3F	TCEB1	PPP4R2
AR	CBL	DNMT3A	FGFR2	IKZF1	MEF2B	PAK1	PTPRT	SHQ1	TSC2	HIST1H3G	TCF3	PRDM14
ARAF	CCND1	DNMT3B	FGFR3	IL10	MEN1	PAK7	RAC1	SMAD2	TSHR	HIST1H3H	TCF7L2	PREX2
ARID1A	CCND2	DOT1L	FGFR4	IL7R	MET	PALB2	RAD50	SMAD3	U2AF1	HIST1H3I	TRAF2	PRKCI
ARID1B	CCND3	E2F3	FH	INPP4A	MITF	PARK2	RAD51	SMAD4	VHL	HIST1H3J	VEGFA	PRKD1
ARID2	CCNE1	EED	FLCN	INPP4B	MLH1	PARP1	RAD51C	SMARCA4	VTCN1	HIST2H3C	XRCC2	PTP4A1
ARID5B	CD274	EGFL7	FLT1	INSR	MLL	PAX5	RAD51L1	SMARCB1	WT1	HIST2H3D	ZFHX3	RAC2
ASXL1	CD276	EGFR	FLT3	IRF4	MLL2	PBRM1	RAD51L3	SMARCD1	XIAP	HIST3H3	ZRSR2	RECQL
ASXL2	CD79B	EIF1AX	FLT4	IRS1	MLL3	PDCD1	RAD52	SMO	XPO1	HLA-A	AGO2	RRAGC
ATM	CDC73	EP300	FOXA1	IRS2	MPL	PDGFRA	RAD54L	SOCS1	YAP1	HOXB13	BABAM1	RRAS
ATR	CDH1	EPCAM	FOXL2	JAK1	MRE11A	PDGFRB	RAF1	SOX17	YES1	ID3	CARM1	RRAS2
ATRX	CDK12	EPHA3	FOXP1	JAK2	MSH2	PDPK1	RARA	SOX2	ACVR1	INHA	CDC42	RTEL1
AURKA	CDK4	EPHA5	FUBP1	JAK3	MSH6	PHOX2B	RASA1	SOX9	ANKRD11	INHBA	CSDE1	RXRA
AURKB	CDK6	EPHB1	GATA1	JUN	MTOR	PIK3C2G	RB1	SPEN	BCL10	MALT1	CYLD	SESN1
AXIN1	CDK8	ERBB2	GATA2	KDM5A	MUTYH	PIK3C3	RBM10	SPOP	BIRC3	MAP3K14	CYSLTR2	SESN2
AXIN2	CDKN1A	ERBB3	GATA3	KDM5C	MYC	PIK3CA	RECQL4	SRC	CALR	MAPK3	DROSHA	SESN3
AXL	CDKN1B	ERBB4	GNA11	KDM6A	MYCL1	PIK3CB	REL	STAG2	CD79A	MGA	DUSP4	SHOC2
B2M	CDKN2A	ERCC2	GNAQ	KDR	MYCN	PIK3CD	RET	STK11	CEBPA	MST1	ELF3	SLX4
BAP1	CDKN2B	ERCC3	GNAS	KEAP1	MYD88	PIK3CG	RFWD2	STK40	CENPA	MST1R	EPAS1	SMYD3
BARD1	CDKN2C	ERCC4	GREM1	KIT	MYOD1	PIK3R1	RHOA	SUFU	CSF3R	NCOA3	ERF	SOS1
BBC3	CHEK1	ERCC5	GRIN2A	KLF4	NBN	PIK3R2	RICTOR	SUZ12	CXCR4	NEGR1	EZH1	SPRED1
BCL2	CHEK2	ERG	GSK3B	KRAS	NCOR1	PIK3R3	RIT1	SYK	DNAJB1	NFKBIA	FAM58A	STK19
BCL2L1	CIC	ESR1	H3F3C	LATS1	NF1	PIM1	RNF43	TBX3	EIF4A2	NUP93	HLA-B	TAP1
BCL2L11	CREBBP	ETV1	HGF	LATS2	NF2	PLK2	ROS1	TERT	EIF4E	PGR	INPPL1	TAP2
BCL6	CRKL	ETV6	HIST1H1C	LMO1	NFE2L2	PMAIP1	RPS6KA4	TET1	EPHA7	PLCG2	KMT2B	TEK
BCOR	CRLF2	EZH2	HIST1H2BD	MAP2K1	NKX2-1	PMS1	RPS6KB2	TET2	ERRFI1	POLD1	KMT5A	TP53BP1
BLM	CSF1R	FAM123B	HIST1H3B	MAP2K2	NKX3-1	PMS2	RPTOR	TGFBR1	FOXO1	PPM1D	KNSTRN	UPF1
BMPR1A	CTCF	FAM175A	HNF1A	MAP2K4	NOTCH1	PNRC1	RUNX1	TGFBR2	FYN	PPP6C	LYN	WHSC1
BRAF	CTLA4	FAM46C	HRAS	MAP3K1	NOTCH2	POLE	RYBP	TMEM127	GLI1	RAB35	MAPKAP1	WHSC1L1

MSK-IMPACT Genes

Panel includes exons, introns, and other noncoding regions

15,265 cases

341 genes: 2,894 cases

410 genes: 9,880 cases

468 genes: 2,491 cases

Cancer Gene Exons:

6,614 protein-coding exons of 468 genes

- actionable mutations
- targets of investigational agents
- frequently mutated in cancer
- cancer susceptibility genes

Cancer Gene Introns:

70 introns of 20 recurrently rearranged genes

Cancer Gene Promoters: TERT promoter

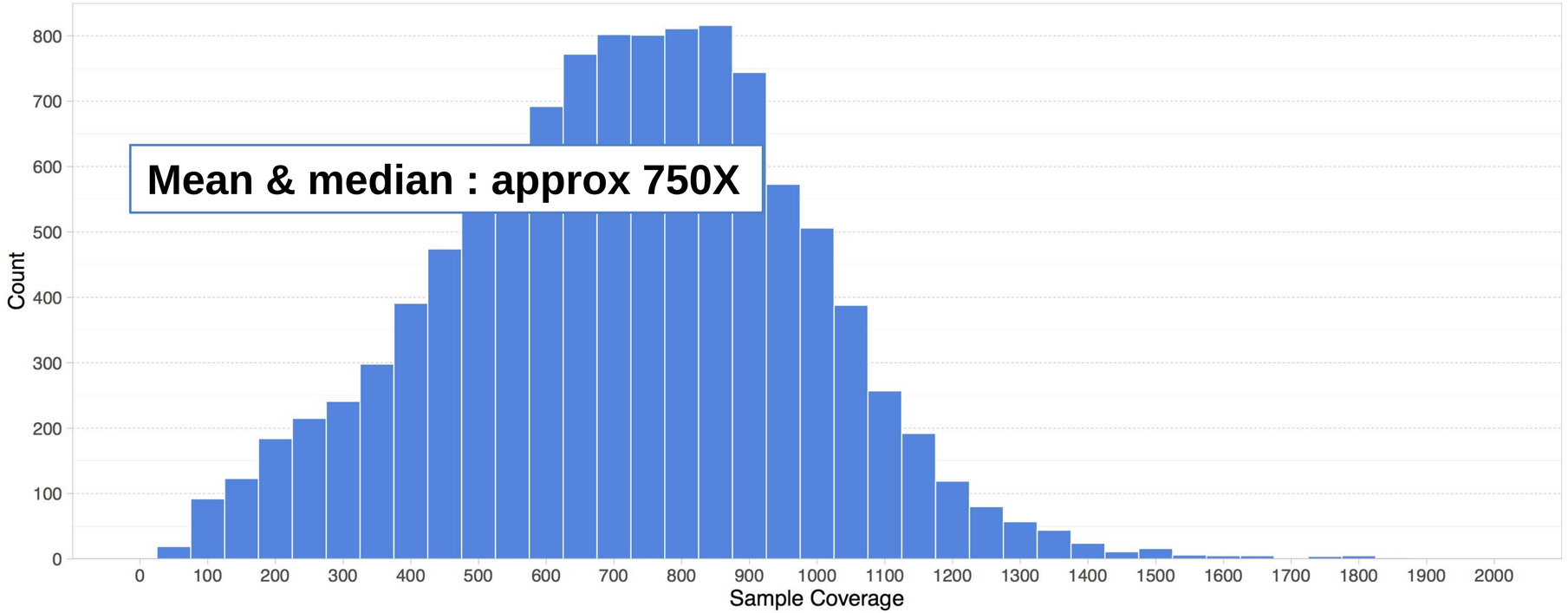
SNP Probes: >1,000 non-coding SNPs

- copy number analysis, various QC checks

Target Territory = 1.52 Mb

MSK-IMPACT: sample statistics

Sequencing Coverage Distribution



- **> 99.5%** are sequenced to >100X
- **98.6%** are sequenced to >250X
- Important for detection of low allele frequency mutations and gene rearrangements
- Important for generation of informative copy number profiles

Ryma Benayed, Ahmet Zehir

MSK-IMPACT: Validation of Clinically Actionable Targets

Accuracy

Reproducibility

Sensitivity

100% … 50% … 25% … 12.5% …

Normal vs normal analysis to set conservative thresholds that minimize false positives in the MSK-IMPACT hybrid capture assay
(sequence normal DNA twice and declare one to be tumor)

Filtering criteria based on coverage depth (DP), number of mutant reads (AD) , variant frequency (VF)

Hotspots:
DP ≥ 20, AD ≥ 8, VF ≥ 2%

Non-hotspots:
DP ≥ 20, AD ≥ 10, VF ≥ 5%

Based on normal vs normal variant calling experiments, the detection limit for low frequency variants was set at **2%** for 'hotspot' mutations and **5%** for non-'hotspot' mutations.

SNVs

"mutant" allele frequency

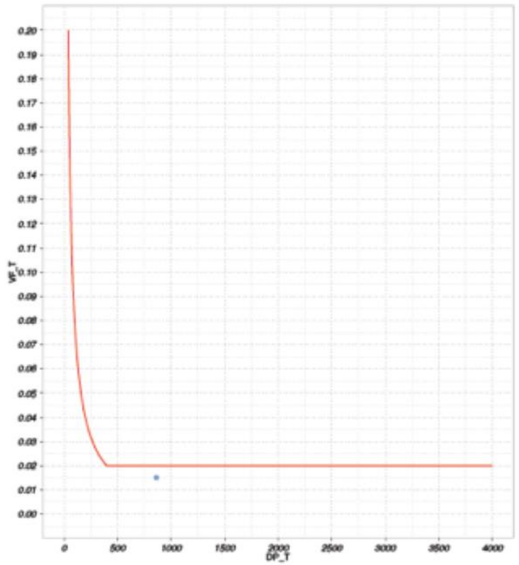
Sequencing depth at position

Indels

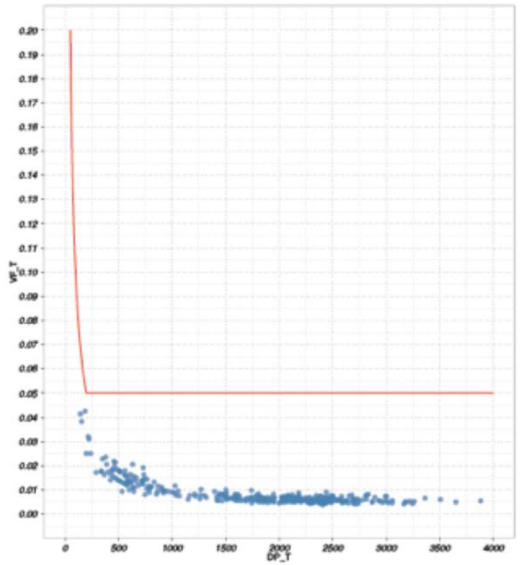
Hotspots

Non-hotspots

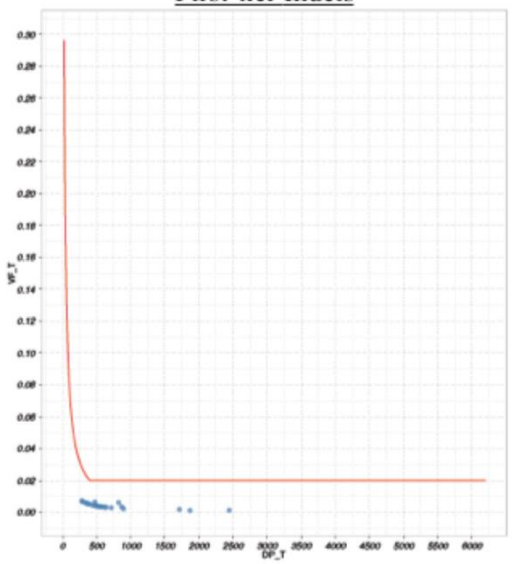
First-tier SNVs



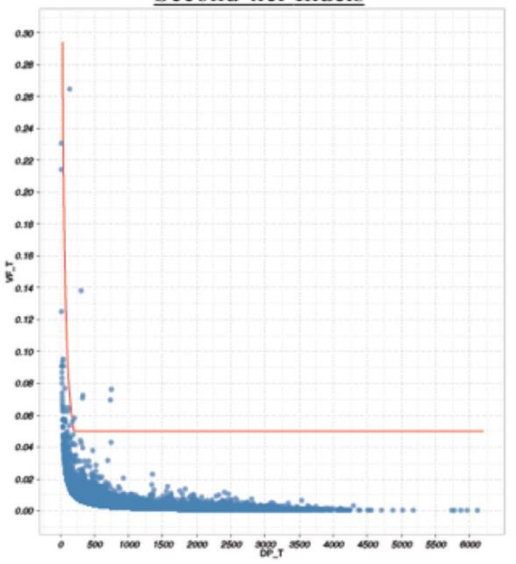
Second-tier SNVs



First-tier Indels

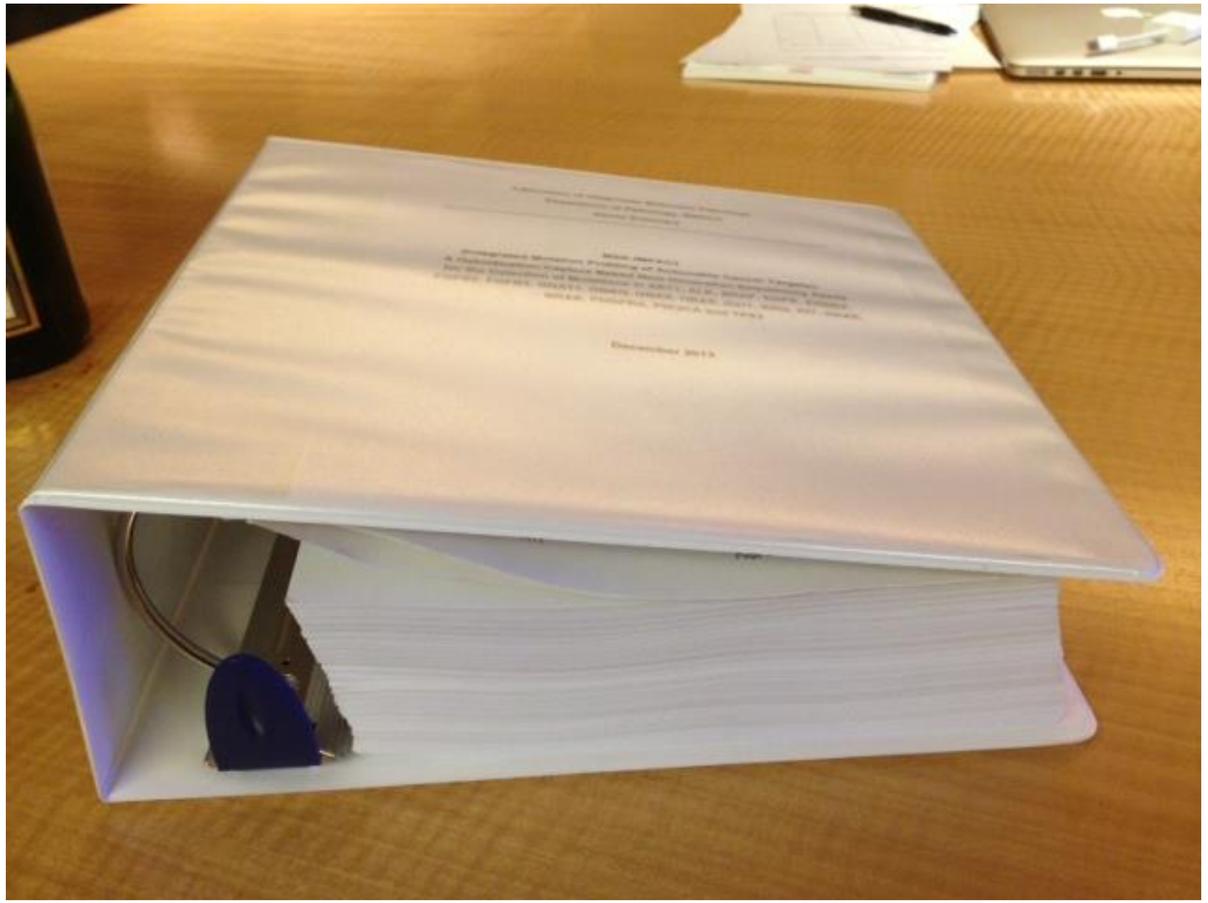


Second-tier Indels



Donavan Cheng

Validation of Clinically Actionable Targets



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Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT)

A Hybridization Capture-Based Next-Generation Sequencing Clinical Assay for Solid Tumor Molecular Oncology

Donavan T. Cheng,* Talia N. Mitchell,* Ahmet Zehir,* Ronak H. Shah,* Ryma Benayed,* Aijazuddin Syed,* Raghu Chandramohan,* Zhen Yu Liu,* Helen H. Won,* Sasinya N. Scott,* A. Rose Brannon,* Catherine O'Reilly,* Justyna Sadowska,* Jacklyn Casanova,* Angela Yannes,* Jaclyn F. Hechtman,* Jinjuan Yao,* Wei Song,* Dara S. Ross,* Alifya Oultache,* Snjezana Dogan,* Laetitia Borsu,* Meera Hameed,* Khedoudja Nafa,* Maria E. Arcila,* Marc Ladanyi,*† and Michael F. Berger*†

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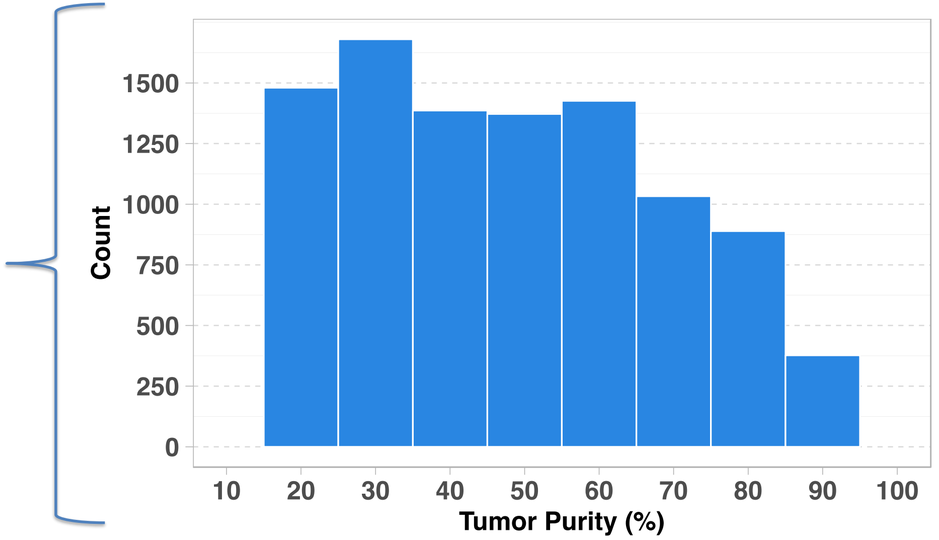
Journal of Molecular Diagnostics (March 20, 2015)

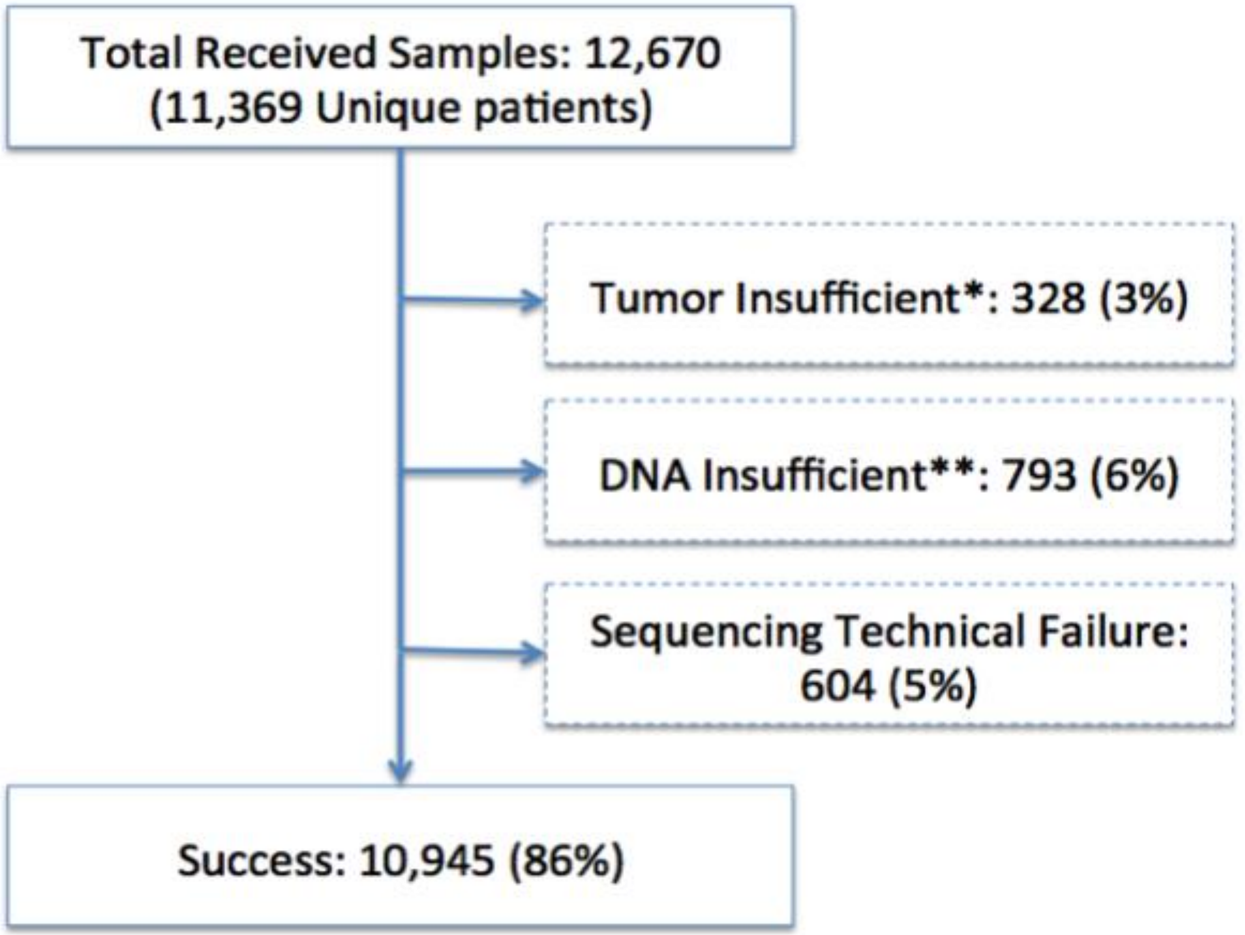
MSK-IMPACT : Specimen requirements

- Requires matched tumor and patient normal DNA
- Tumor DNA: formalin-fixed paraffin embedded tumor tissue
- Two important criteria for tumor specimen adequacy:
  - >10% of tumor cells in the material submitted for analysis
  - Sections with >20% viable tumor are preferred
    - Require minimum of 50ng of DNA (Qubit)



- Source of Normal DNA:
  - Whole Blood sample **(preferred)** : 5–10ml of peripheral blood in an EDTA tube
  - Normal tissue block: 5 - 20 unstained FFPE sections, 10 microns thick
  - Other sources: saliva, nail clippings
- Turnaround time: approx 2-3 weeks from receipt of T + N in the lab
  - 92% of cases returned in <3 weeks.

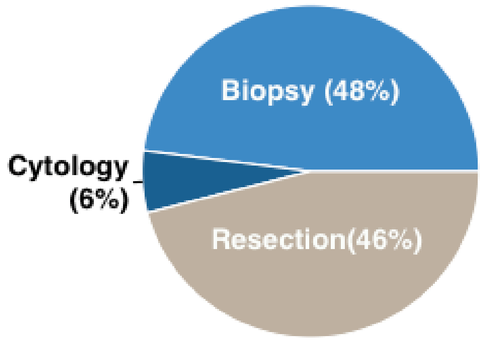
MSK-IMPACT: results obtained in >85% of submitted FFPE samples



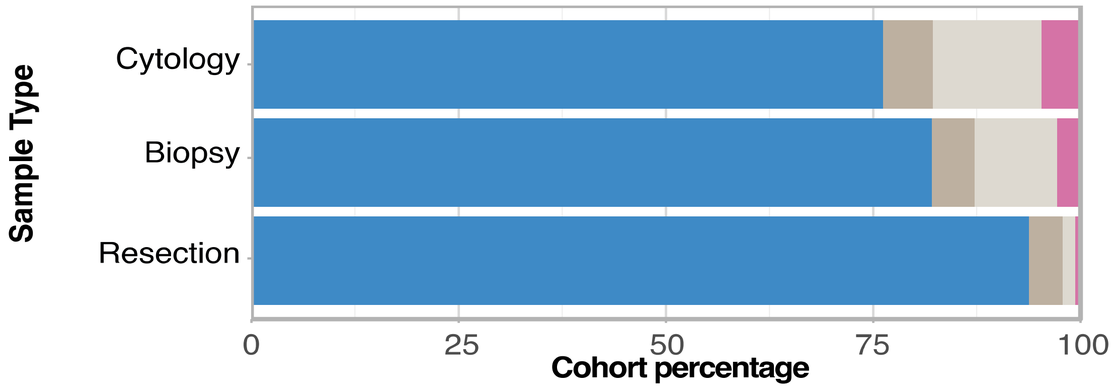
* <10% Tumor Purity
** <50ng

Ryma Benayed

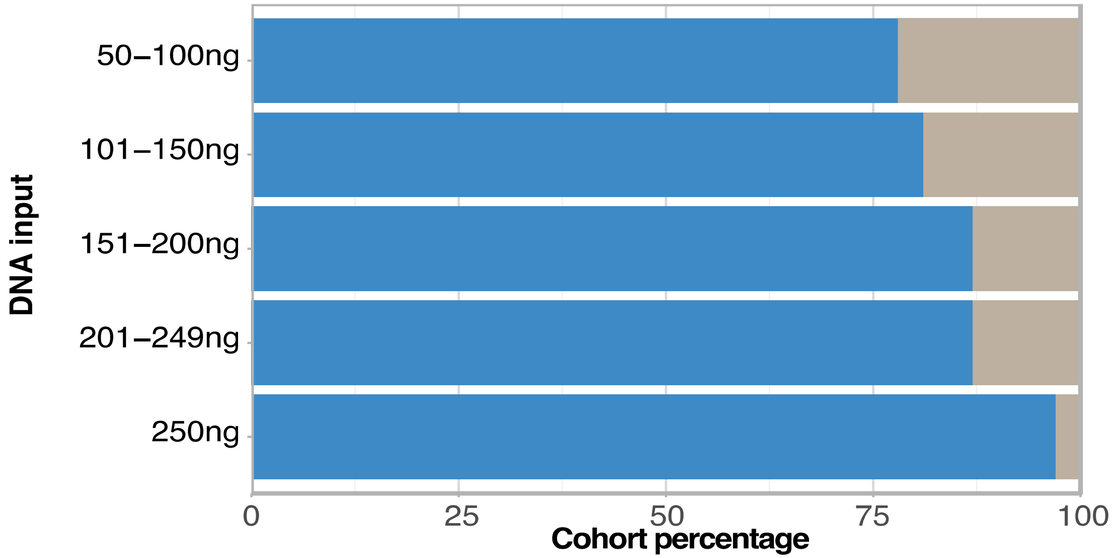
MSK-IMPACT: determinants of success



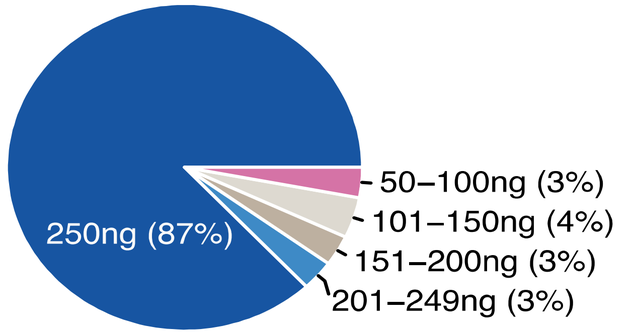
a *Association of different submitted specimen types with assay performance*

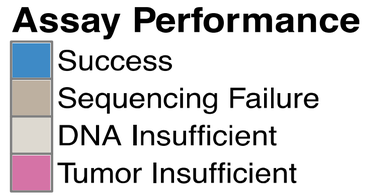


b *Assay performance as a function of genomic DNA input values*



c *Distribution of DNA input*

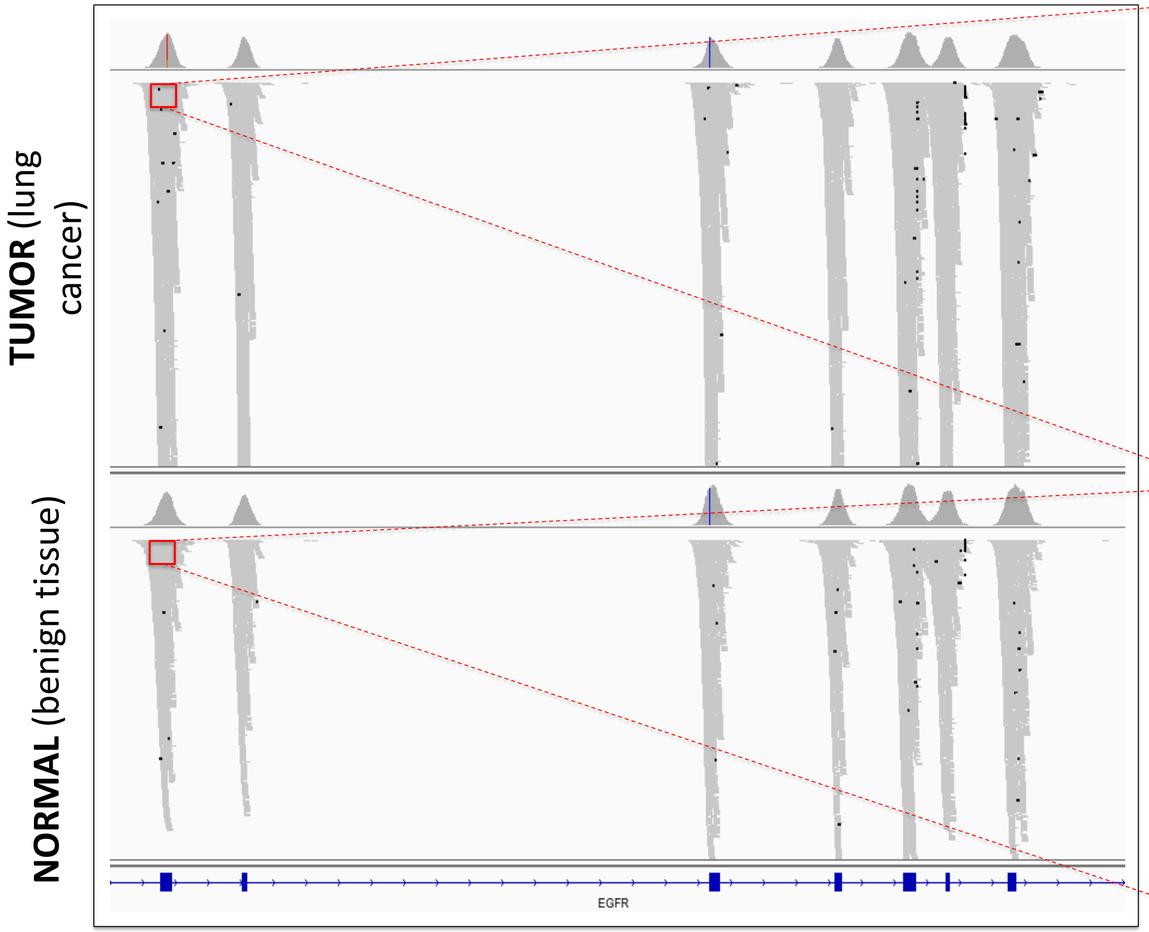




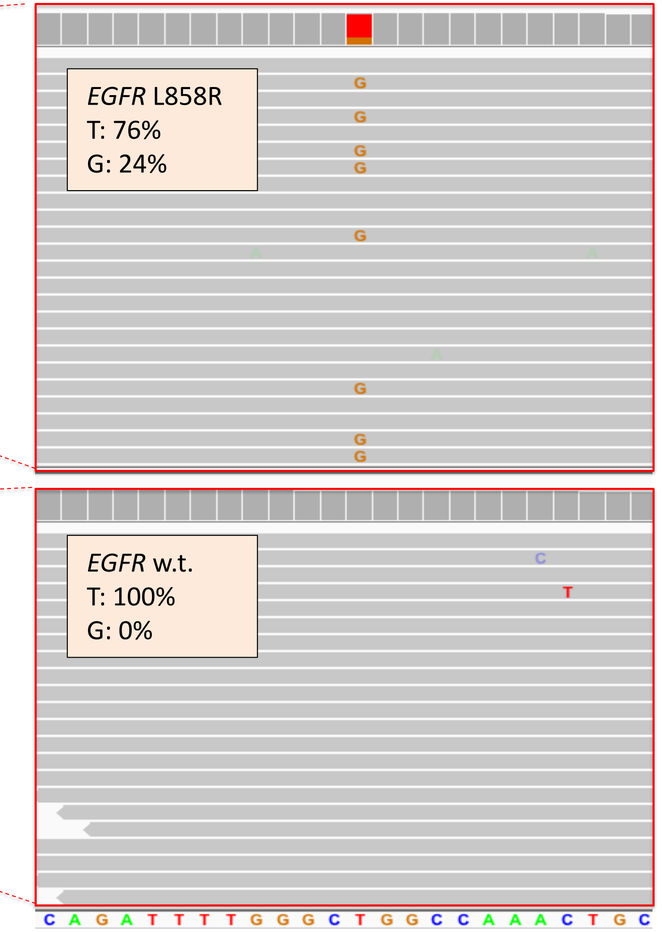
Ryma Benayed

MSK-IMPACT: detection of somatic point mutations

Coverage Is Specific to Target Exons

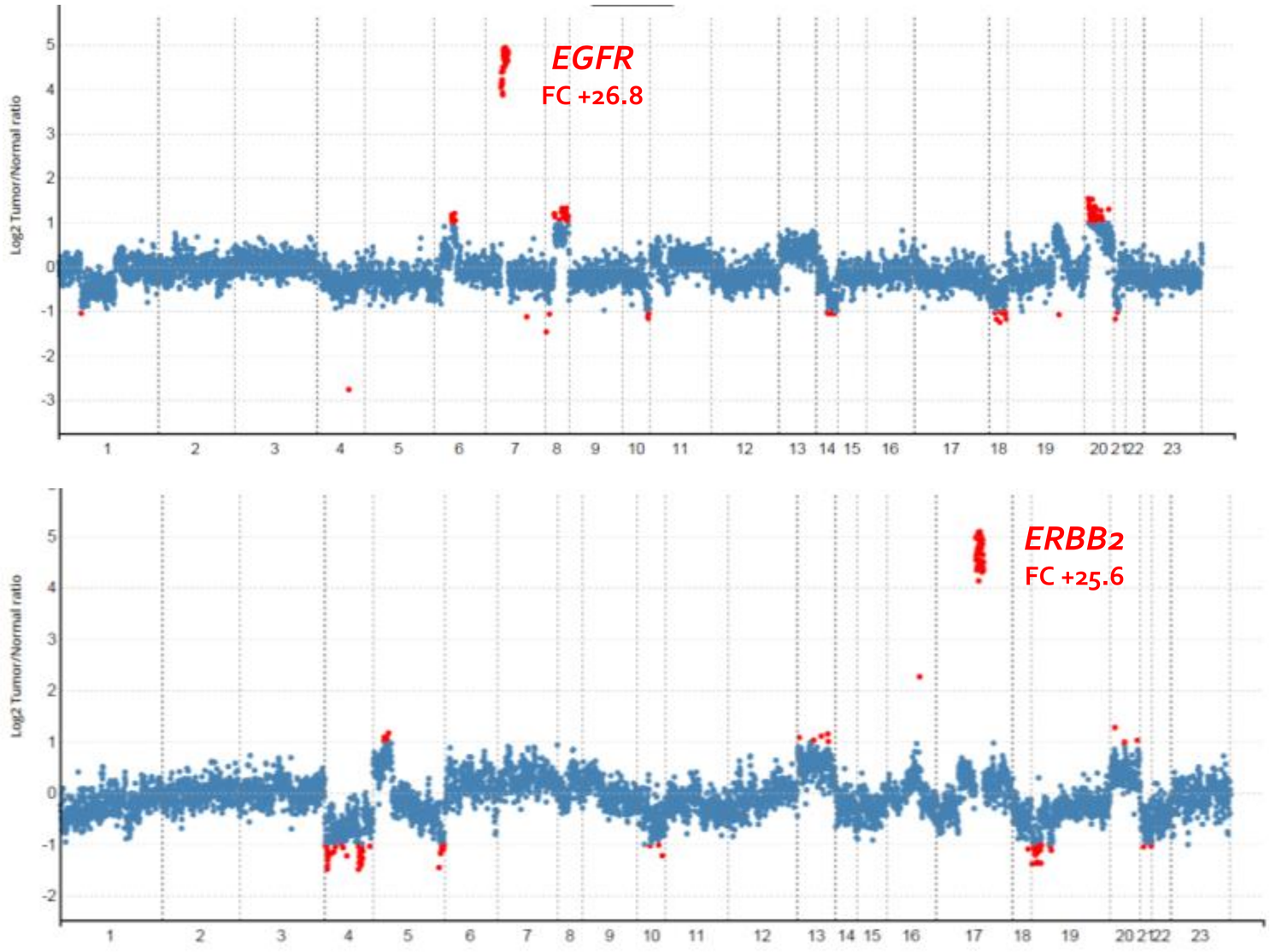


Lung adenocarcinoma case



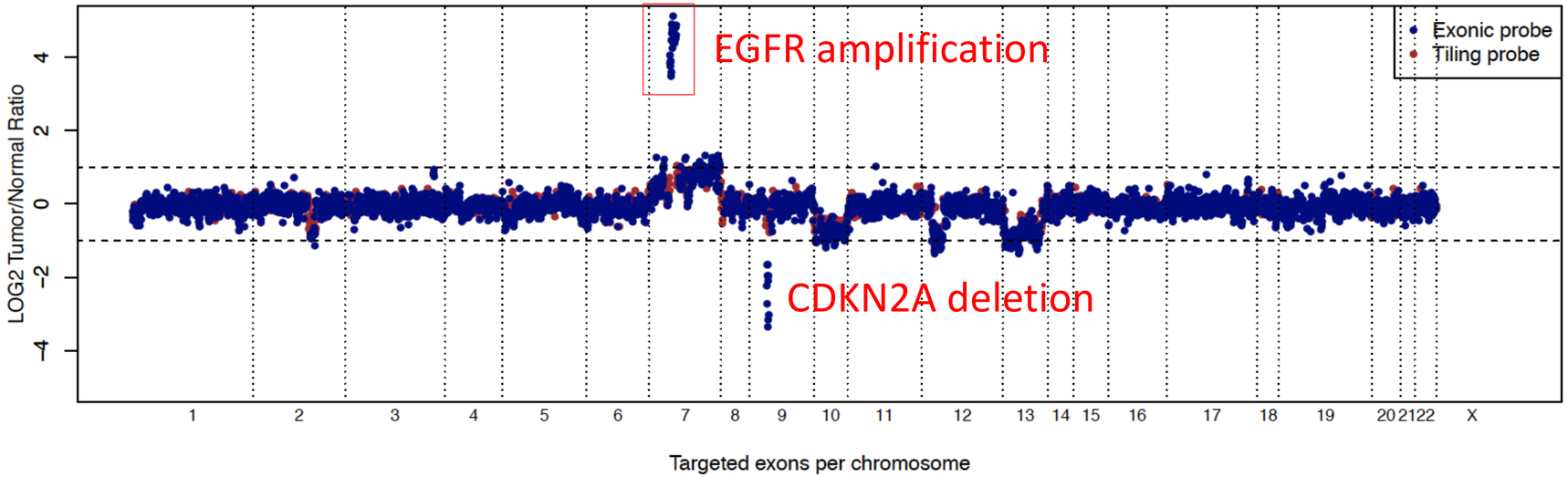
MSK-IMPACT: robust detection of copy number changes

EGFR and *ERBB2* (*HER2*) amplification in two colorectal cancers

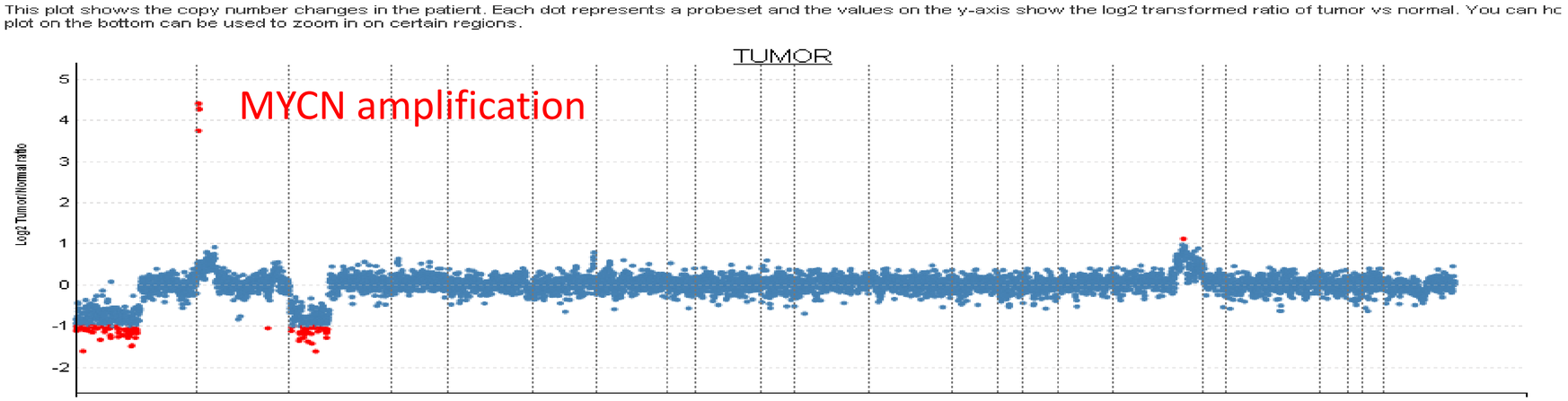


MSK-IMPACT: robust detection of copy number changes

Glioblastoma (also had EGFRvIII)

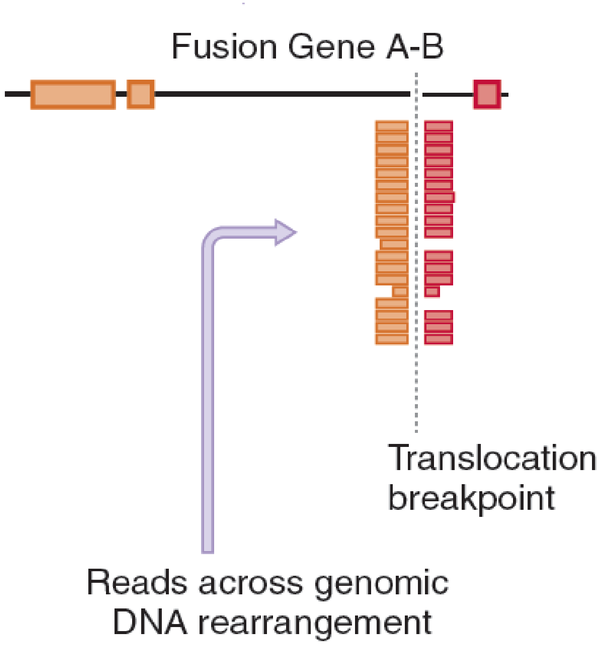


Neuroblastoma (also had ALK F1174L mutation)



MSK-IMPACT: DNA-based Detection of Cancer Fusion Genes (by intron capture sequencing of rearranged introns)

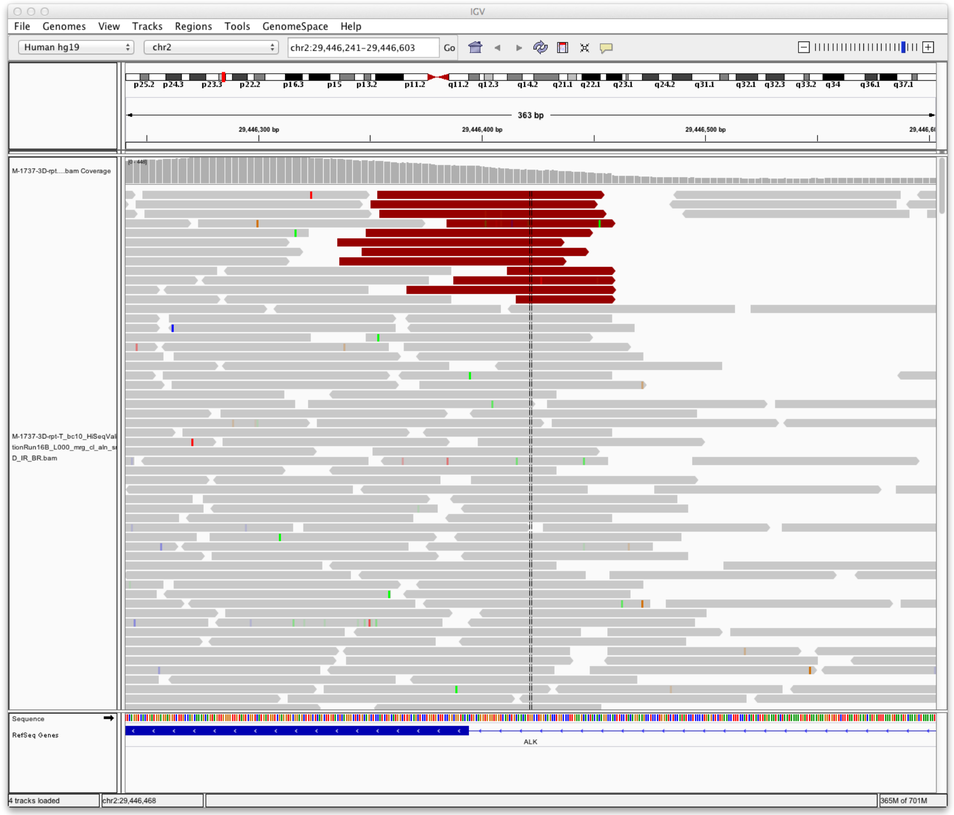
Specific introns of cancer fusion genes captured by MSK-IMPACT

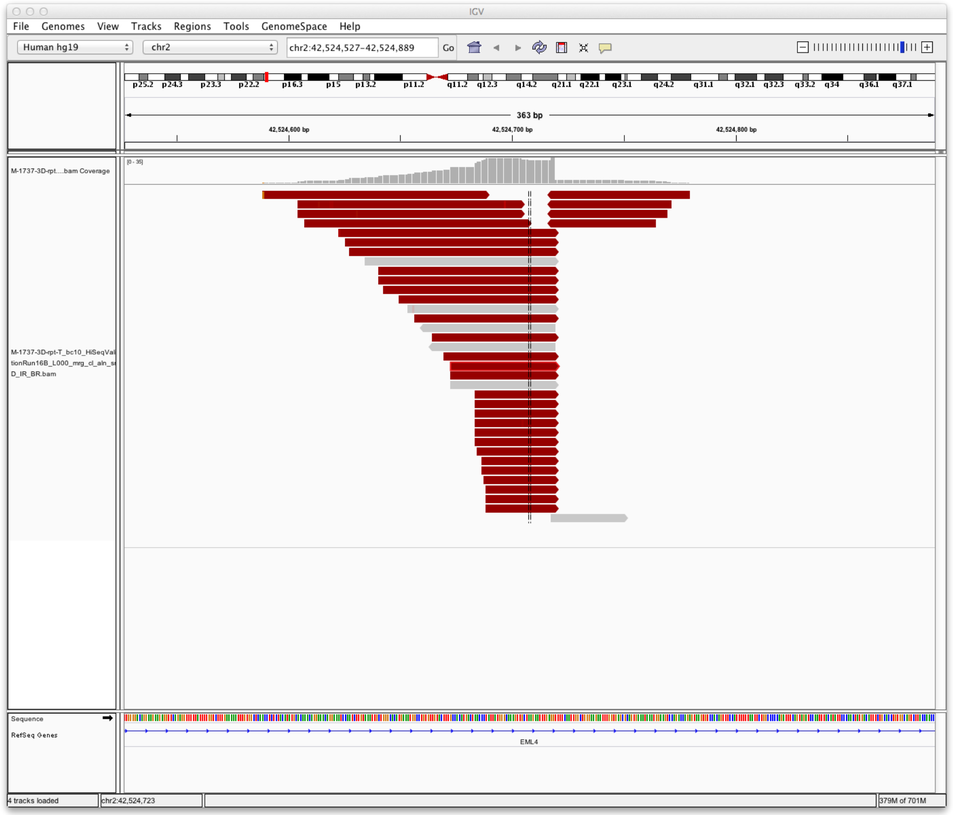


Gene	Introns	Target (kb)
ALK	17, 18, 19	3.9
BRAF	7, 8, 9, 10	18.3
CD74	4, 5, 6	2.9
DNAJB1	1, 2	1.4
EGFR	7, 17, 24, 25, 26	4.4
ETV6	4, 5	30.3
EWSR1	7, 8, 9, 10, 11, 12, 13	11.7
FGFR2	16, 17	5.3
FGFR3	15, 16, 17, 18	0.6
MET	13, 14	3.1
NAB2	2, 3, 4, 5, 6	3.5
NTRK1	3, 7, 8, 9, 10, 11, 12	5.8
NTRK2	15	6.2
NUT	1	1.9
PAX8	8, 9, 10	16.2
RELA	1,2	0.7
RET	7, 8, 9, 10, 11	4.5
ROS1	30, 31, 32, 33, 34, 35	20.4
TFE3	3, 4, 5	4.9
TMPRSS2	1, 2	13.3

MSK-IMPACT: Detecting Specific Rearrangements

Lung adenocarcinoma





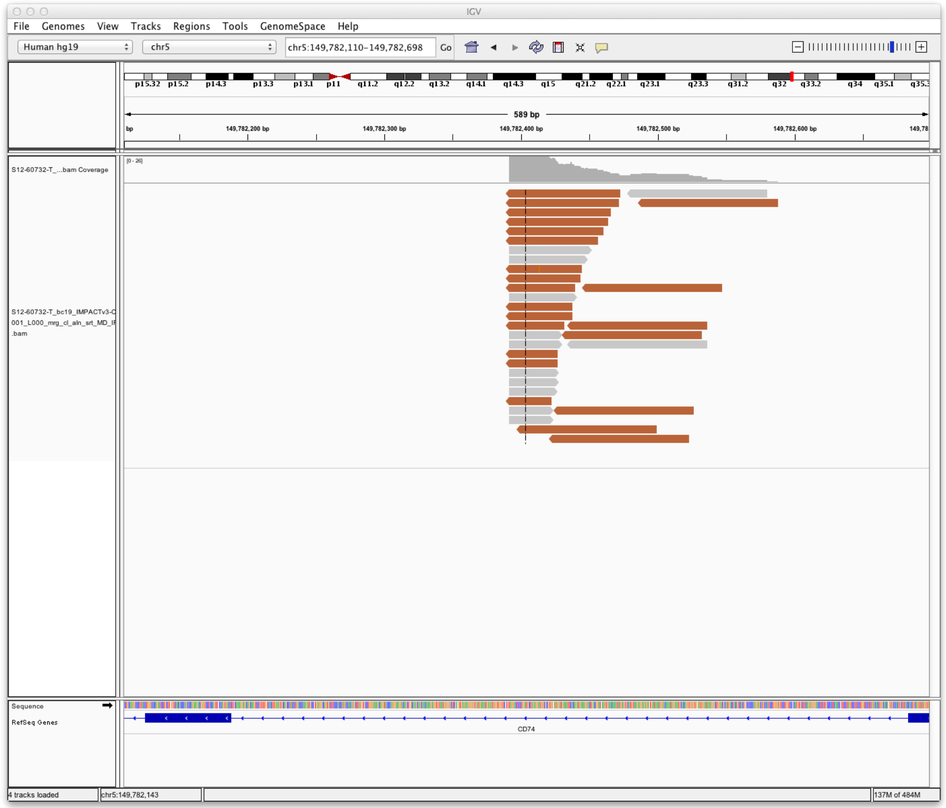
ALK

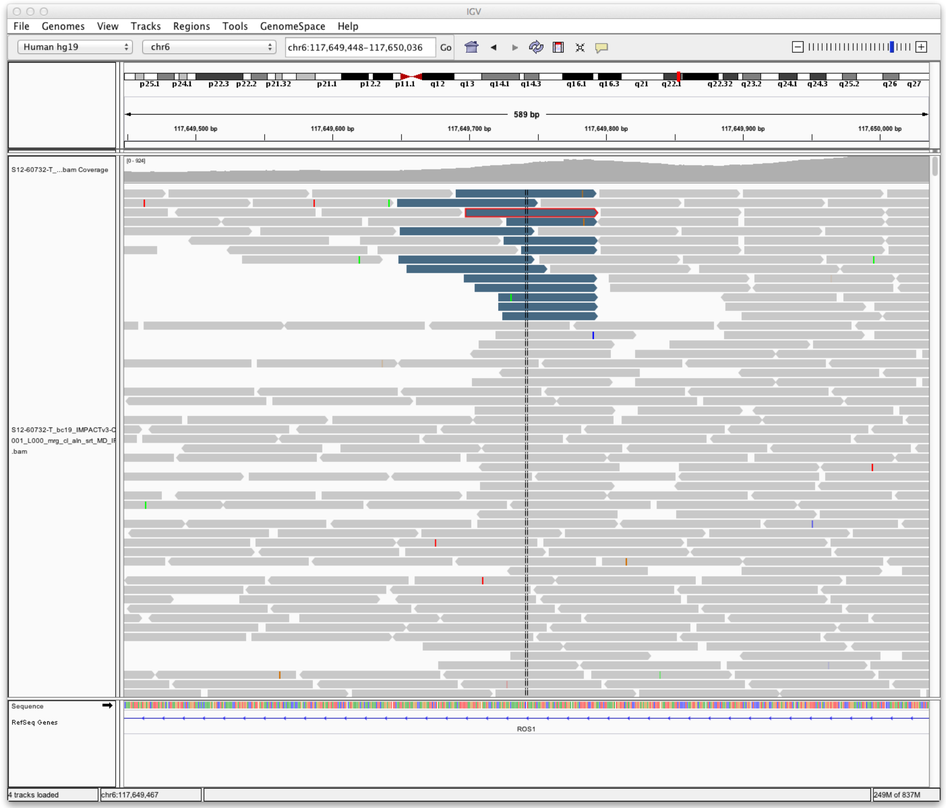
EML4

EML4 *ALK*

MSK-IMPACT: Detecting Specific Rearrangements

Lung adenocarcinoma





CD74 *ROS1*

CD74 *ROS1*

MSK-IMPACT: Institution-wide Clinical Sequencing Initiative

Run MSK-IMPACT in patients with recurrent/metastatic solid cancers

- Carcinomas, sarcomas, brain tumors
- Provide improved diagnosis and better prognostication
- Select approved targeted therapies based on mutations
- Screen (or pre-screen) patients for clinical trial eligibility, especially “basket” trials

All results are reported to the ordering clinician and placed in patient medical record

Started in 2014; surpassed 10,000 patients in May 2016

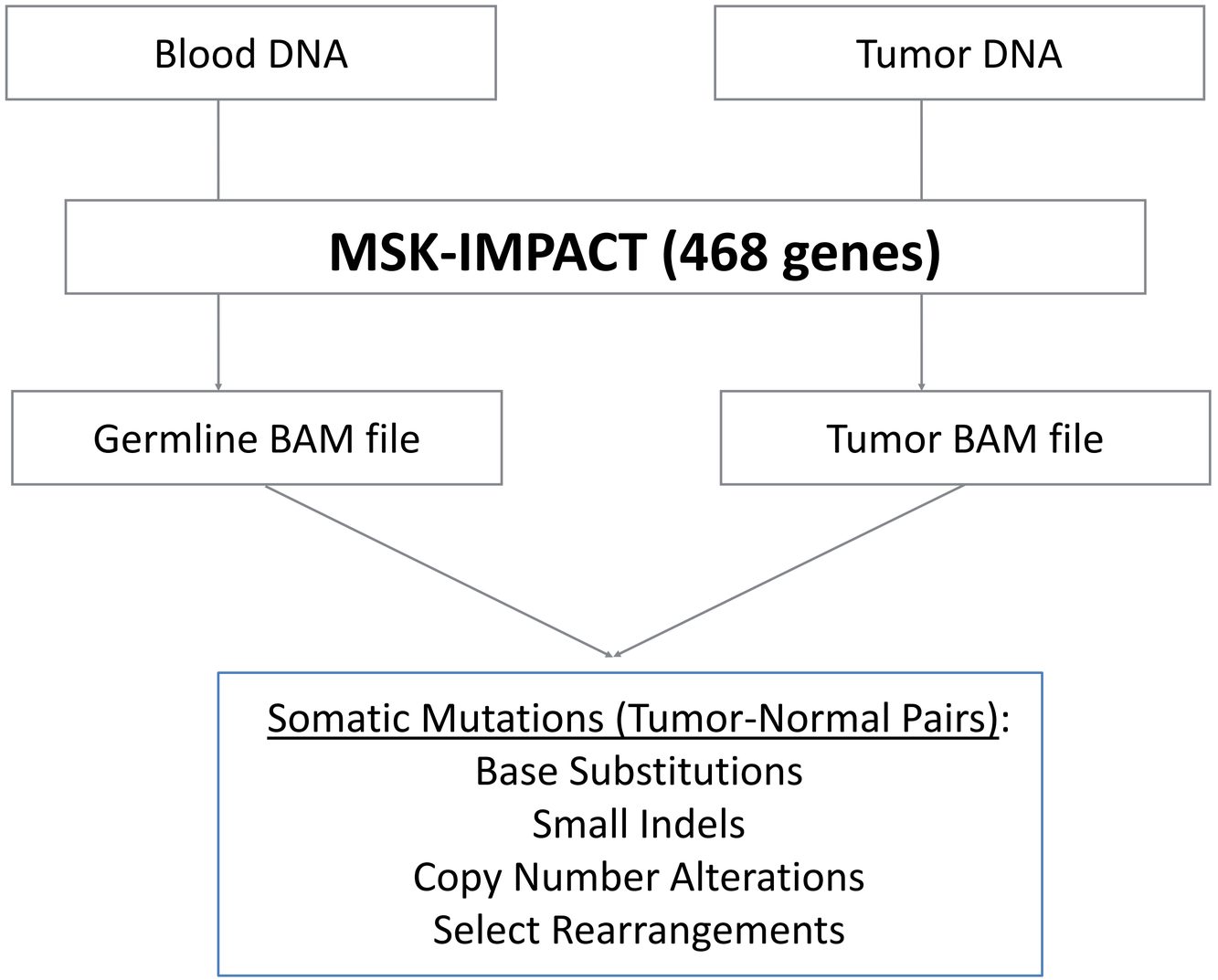
Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients

Ahmet Zehir[1,13], Ryma Benayed[1,13], Ronak H Shah[1], Aijazuddin Syed[1], Sumit Middha[1], Hyunjae R Kim[1], Preethi Srinivasan[1], Jianjiong Gao[2], Debyani Chakravarty[2], Sean M Devlin[3], Matthew D Hellmann[4], David A Barron[5], Alison M Schram[4], Meera Hameed[1], Snjezana Dogan[1], Dara S Ross[1], Jaclyn F Hechtman[1], Deborah F DeLair[1], JinJuan Yao[1], Diana L Mandelker[1], Donavan T Cheng[1,12], Raghu Chandramohan[1,12], Abhinita S Mohanty[1], Ryan N Ptashkin[1], Gowtham Jayakumaran[1], Meera Prasad[1], Mustafa H Syed[1], Anoop Balakrishnan Rema[1], Zhen Y Liu[1], Khedoudja Nafa[1], Laetitia Borsu[1], Justyna Sadowska[1], Jacklyn Casanova[1], Ruben Bacares[1], Iwona J Kiecka[1], Anna Razumova[1], Julie B Son[1], Lisa Stewart[1], Tessara Baldi[1], Kerry A Mullaney[1], Hikmat Al-Ahmadie[1], Efsevia Vakiani[1], Adam A Abeshouse[3], Alexander V Penson[3,6], Philip Jonsson[3,6], Niedzica Camacho[1], Matthew T Chang[3,6], Helen H Won[1], Benjamin E Gross[2], Ritika Kundra[2], Zachary J Heins[2], Hsiao-Wei Chen[2], Sarah Phillips[2], Hongxin Zhang[2], Jiaojiao Wang[2], Angelica Ochoa[2], Jonathan Wills[7], Michael Eubank[7], Stacy B Thomas[7], Stuart M Gardos[7], Dalicia N Reales[8], Jesse Galle[8], Robert Durany[8], Roy Cambria[8], Wassim Abida[4], Andrea Cercek[4], Darren R Feldman[4], Mrinal M Gounder[4], A Ari Hakimi[9], James J Harding[4], Gopa Iyer[4], Yelena Y Janjigian[4], Emmet J Jordan[4], Ciara M Kelly[4], Maeve A Lowery[4], Luc G T Morris[9], Antonio M Omuro[10], Nitya Raj[4], Pedram Razavi[4], Alexander N Shoushtari[4], Neerav Shukla[11], Tara E Soumerai[4], Anna M Varghese[4], Rona Yaeger[4], Jonathan Coleman[8], Bernard Bochner[8], Gregory J Riely[4], Leonard B Saltz[4], Howard I Scher[4], Paul J Sabbatini[4], Mark E Robson[4], David S Klimstra[1], Barry S Taylor[2,3,6], Jose Baselga[4,6], Nikolaus Schultz[2,3,6], David M Hyman[4], Maria E Arcila[1], David B Solit[2,4,6], Marc Ladanyi[1,6] & Michael F Berger[1,2,6]

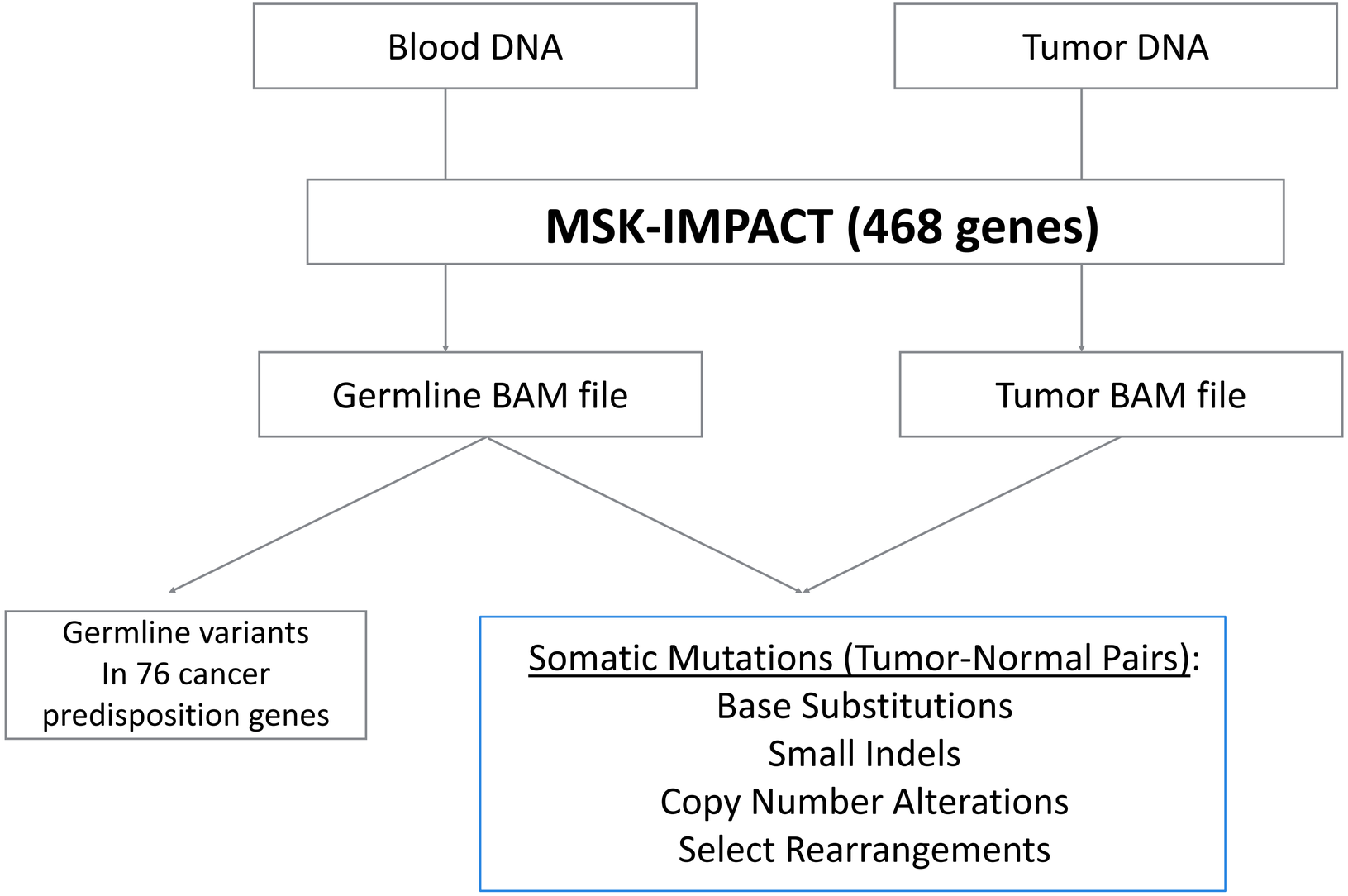
NATURE MEDICINE VOLUME 23 | NUMBER 6 | JUNE 2017

Now > 20,000 patients; running approx 800/month on 5 HiSeq 2500 sequencers

MSK-IMPACT: use of Germline BAM file to filter out germline variants



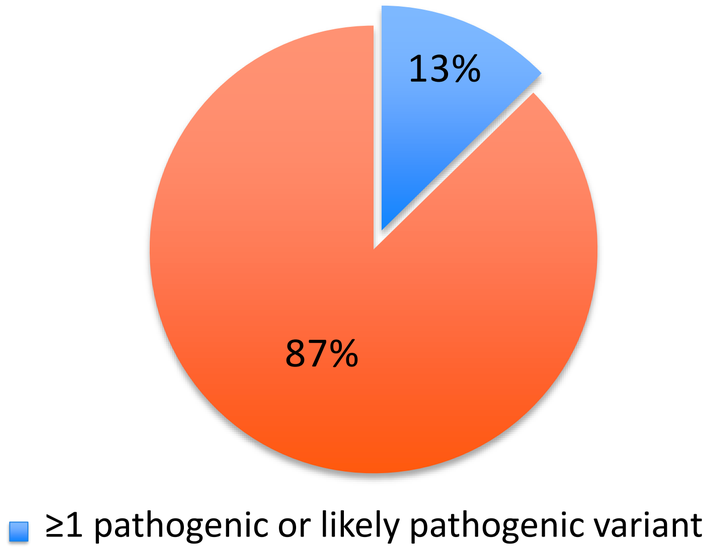
Secondary Germline MSK-IMPACT



Secondary Germline MSK-IMPACT: Prevalence of Pathogenic/Likely Pathogenic Variants

- Anonymized analysis of first 1566 cases (MSK-IMPACT)
- Considered all cancer susceptibility genes
- Variants curated by Ingenuity and manual review

- About 13% of MSK-IMPACT cases contain pathogenic/ presumed pathogenic germline variants in cancer susceptibility genes , of which about 40% are linked to the current cancer



Schrader KT, *et al.*, *JAMA Oncology*, Nov 2015

→ Germline cancer susceptibility variants are not rare in cancer patients undergoing tumor mutation profiling

- Analyzing the germline in tumor-normal analysis is superior to phenotype-directed testing to identify cancer susceptibility variants in cancer patients.

Memorial Sloan Kettering Cancer Center

Secondary Germline MSK-IMPACT: Prevalence of Pathogenic/Likely Pathogenic Variants

- report on first 1040 patients who signed additional MSK-IMPACT consent for germline analysis
- 65 patients (6.3%) who would not have been tested based on clinical guidelines had moderate or high penetrance mutations in a cancer susceptibility gene

(Germline)

JAMA | Preliminary Communication

Mutation Detection in Patients With Advanced Cancer by Universal Sequencing of Cancer-Related Genes in Tumor and Normal DNA vs Guideline-Based Germline Testing

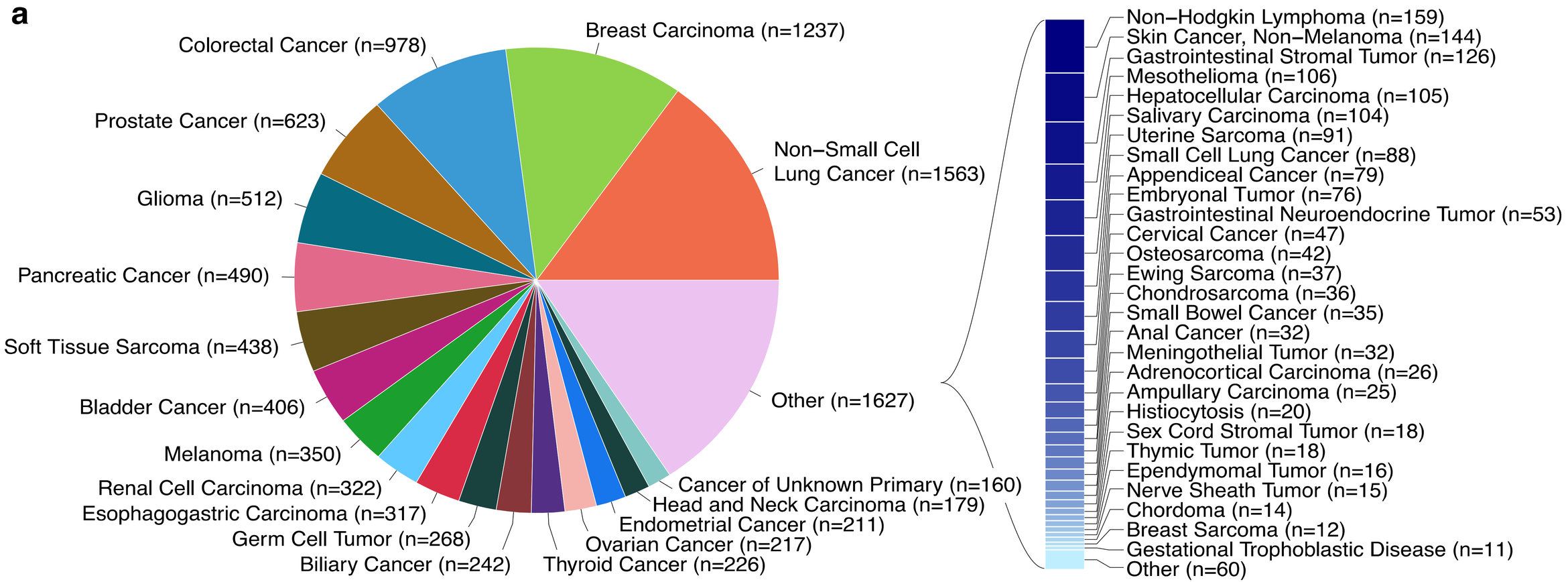
Diana Mandelker, MD, PhD; Liying Zhang, MD, PhD; Yelena Kemel, MS, ScM; Zsofia K. Stadler, MD; Vijai Joseph, PhD; Ahmet Zehir, PhD; Nisha Pradhan, BA; Angela Arnold, MS; Michael F. Walsh, MD; Yirong Li, PhD; Anoop R. Balakrishnan, MS; Aijazuddin Syed, MS; Meera Prasad, MS; Khedoudja Nafa, PharmD, PhD; Maria I. Carlo, MD; Karen A. Cadoo, MD; Meg Sheehan, MS; Megan H. Fleischut, MS; Erin Salo-Mullen, MS, MPH; Magan Trottier, MS, MSc; Steven M. Lipkin, MD, PhD; Anne Lincoln, MS; Semanti Mukherjee, PhD; Vignesh Ravichandran, MS; Roy Cambria, BS; Jesse Galle, BA; Wassim Abida, MD, PhD; Marcia E. Arcila, MD; Ryma Benayed, PhD; Ronak Shah, MS; Kenneth Yu, MD; Dean F. Bajorin, MD; Jonathan A. Coleman, MD; Steven D. Leach, MD; Maeve A. Lowery, MD; Julio Garcia-Aguilar, MD, PhD; Philip W. Kantoff, MD; Charles L. Sawyers, MD; Maura N. Dickler, MD; Leonard Saltz, MD; Robert J. Motzer, MD; Eileen M. O'Reilly, MD; Howard I. Scher, MD; Jose Baselga, MD, PhD; David S. Klimstra, MD; David B. Solit, MD; David M. Hyman, MD; Michael F. Berger, PhD; Marc Ladanyi, MD; Mark E. Robson, MD; Kenneth Offit, MD, MPH

JAMA September 5, 2017 Volume 318, Number 9

MSK-IMPACT: Distribution of Tumor Types

→ Large case numbers provide statistical power to analyses for new drivers.

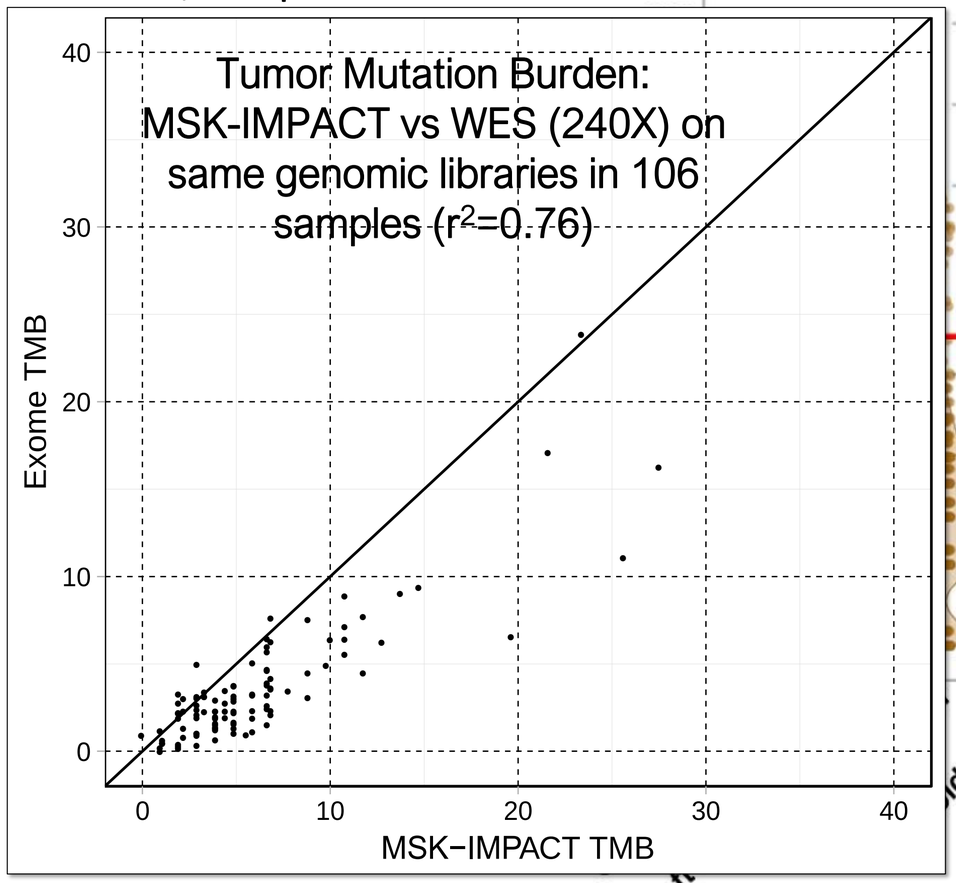
n = 12,670 tumors from 11,369 patients

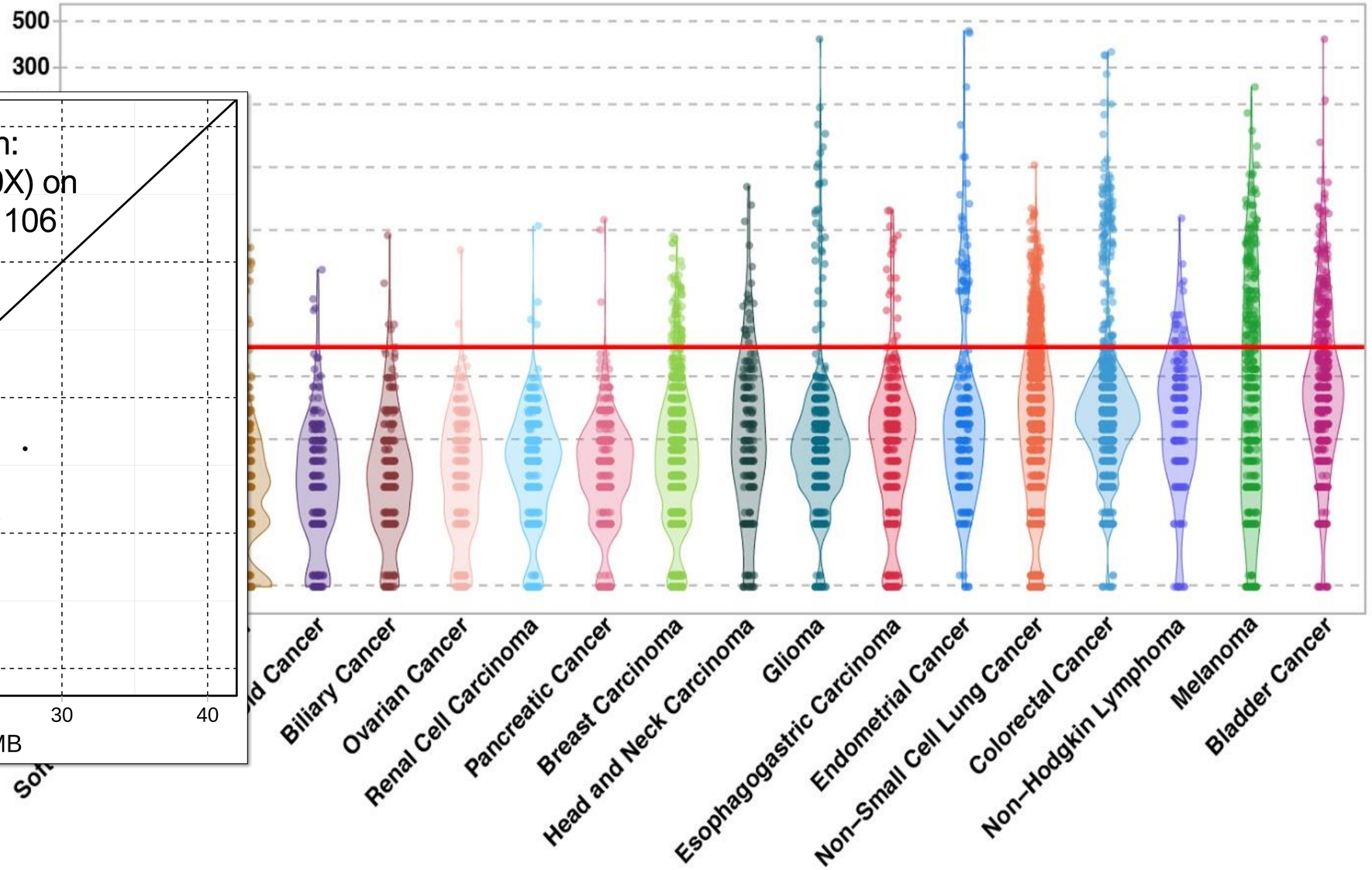


Zehir A, et al. Mutational Landscape of Metastatic Cancer Revealed from Prospective Clinical Sequencing of 10,000 Patients. *Nature Medicine,* June 2017

MSK-IMPACT: Mutation Counts by Major Tumor Types

n = 12,670 tumors
from 11,369 patients





Zehir A, et al. Mutational Landscape of Metastatic Cancer Revealed from Prospective Clinical Sequencing of 10,000 Patients. *Nature Medicine,* June 2017

MSK-IMPACT: Detecting microsatellite instability by MSIsensor analysis

BIOINFORMATICS APPLICATIONS NOTE
Vol. 30 no. 7 2014, pages 1015–1016
doi:10.1093/bioinformatics/btt755

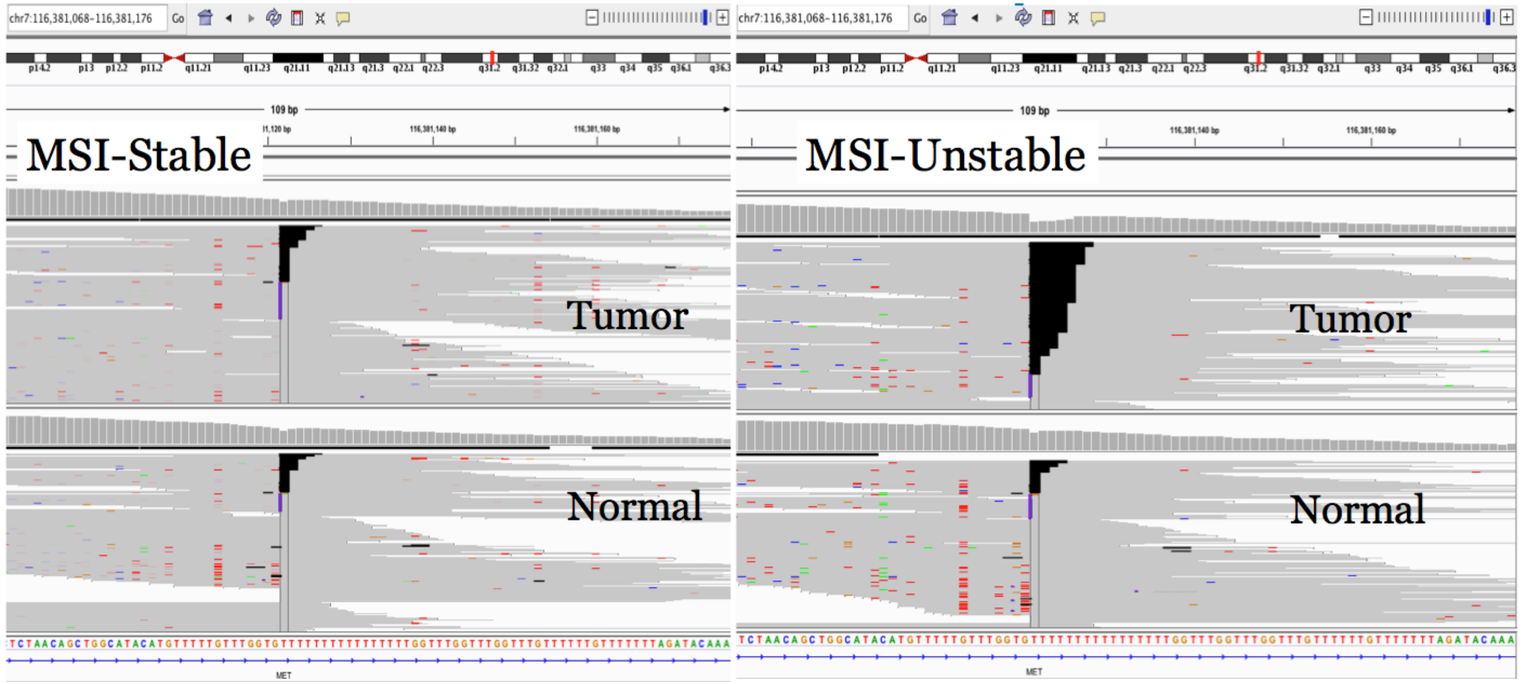
Sequence analysis
Advance Access publication December 25, 2013

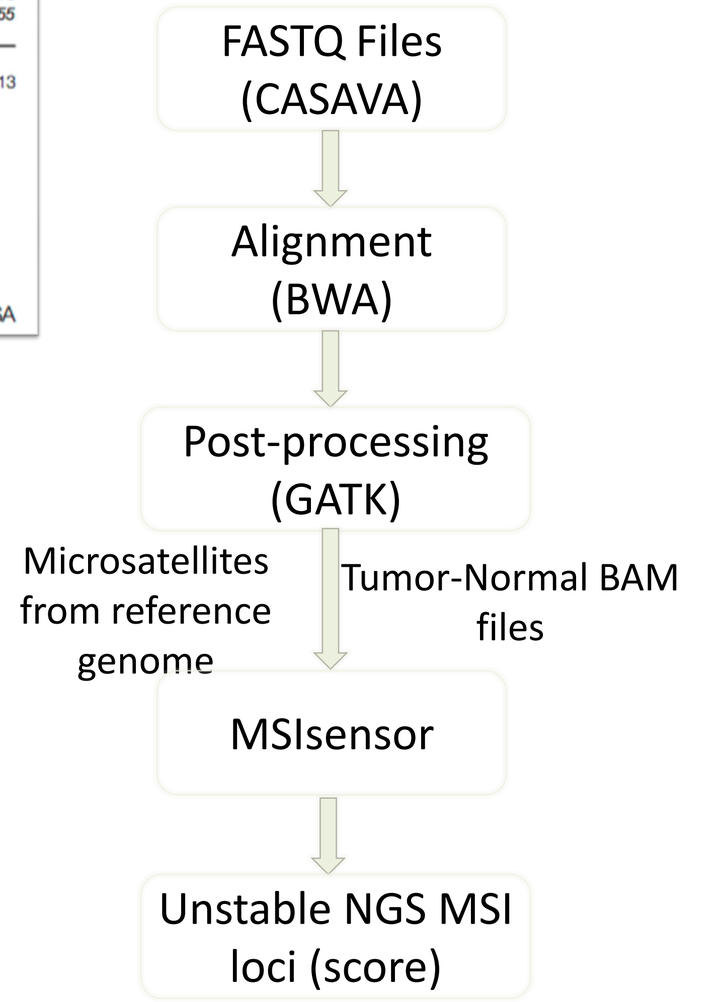
MSIsensor: microsatellite instability detection using paired tumor-normal sequence data

Beifang Niu[1,†], Kai Ye[1,2,*,†], Qunyuan Zhang[1,2], Charles Lu[1], Mingchao Xie[1], Michael D. McLellan[1], Michael C. Wendl[1] and Li Ding[1,2,3,4,*]

[1]Departments of Genetics and Mathematics, The Genome Institute, [2]Department of Genetics, Division of Statistical Genomics, [3]Department of Medicine and [4]Siteman Cancer Center, Washington University in St. Louis, MO 63108, USA

IGV screenshot of MSIsensor on a microsatellite locus in an MSS tumor (left) and an MSI-H (right) tumor





MSK-IMPACT panel contains approx 1500 microsatellite loci

Sumit Middha, Ahmet Zehir, Jackie Hechtman

JCO™ Precision Oncology October 4, 2017

Sumit Middha
Liying Zhang
Khedoudja Nafa
Gowtham Jayakumaran
Donna Wong
Hyunjae R. Kim
Justyna Sadowska
Michael F. Berger
Deborah F. Delair
Jinru Shia
Zsofia Stadler
David S. Klimstra
Marc Ladanyi
Ahmet Zehir
Jaclyn F. Hechtman

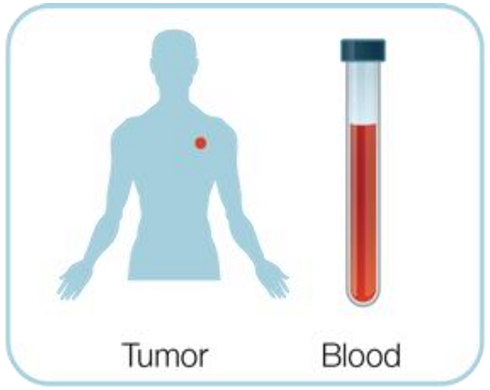
original report

Reliable Pan-Cancer Microsatellite Instability Assessment by Using Targeted Next-Generation Sequencing Data

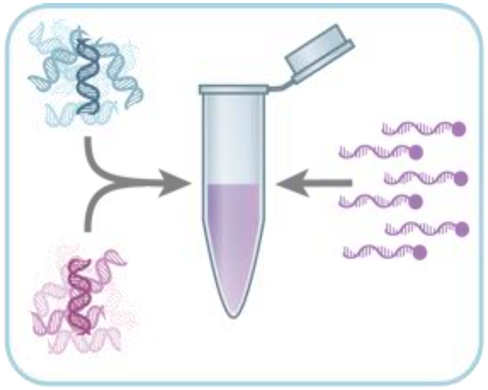
Conclusion **MSI status can be reliably inferred by MSIsensor from large-panel targeted NGS data. Concurrent MSI testing by NGS is resource efficient, is potentially more sensitive for MMR-D than MSI PCR, and allows identification of MSI-H across various cancers not typically screened, as highlighted by the finding that 35% (68 of 193) of all MSI-H tumors were non-CRC/UEC.**

MSK-IMPACT: Integrated Mutation Profiling of Actionable Cancer Targets

1. Patient Consent

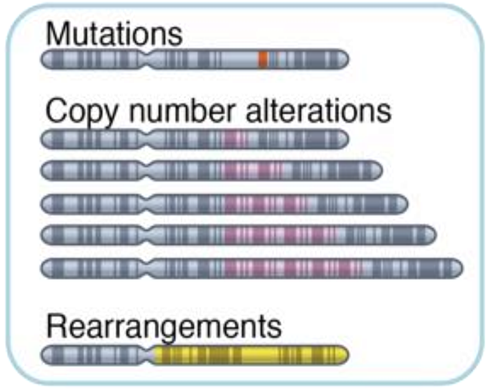


2. Sample Accessioning



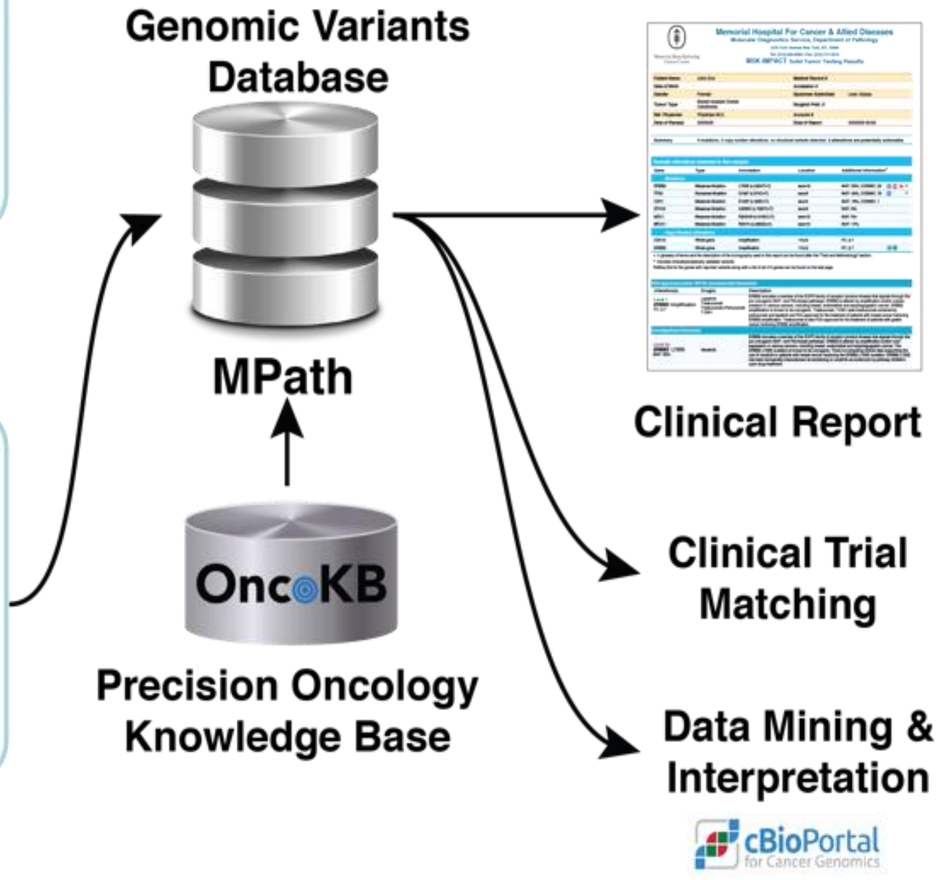
3. Sample Preparation

4. Sequencing



5. Bioinformatics Analysis

6. Case Review and Sign Out



Precision medicine at Memorial Sloan Kettering Cancer Center: clinical next-generation sequencing enabling next-generation targeted therapy trials

Drug Discovery Today

Hyman D, et al. August 2015

Mike Berger, Ahmet Zehir

MSK-IMPACT: clinical annotation of results

OncoKB

Chakravarti D, et al. **OncoKB: A Precision Oncology Knowledge Base.** *JCO Precision Oncol,* May 2017

MSK-IMPACT: Institution-wide Clinical Sequencing Initiative

Memorial Sloan Kettering Cancer Center

Memorial Hospital For Cancer & Allied Diseases

Molecular Diagnostics Service, Department of Pathology

1275 York Avenue New York, NY, 10065

Tel: (212) 639-8280 | Fax: (212) 717-3515

MSK-IMPACT Testing Report

Patient Name	Redacted	**Medical Record #**	Redacted
Date of Birth	Redacted	**Accession #**	Redacted
Gender	Redacted	**Specimen Submitted**	N/A
Tumor Type	High-Grade Serous Ovarian Cancer	**Surgical Path. #**	Redacted
Ref. Physician	Redacted	**Account #**	Redacted
Date of Receipt	Redacted	**Date of Report**	Redacted
Date of Procedure	Redacted		

Summary	1 mutation, 20 copy number alterations, no structural variants detected. 2 alterations have OncoKB interpretations.
Comments	MSK-IMPACT tiling probes also show a high level gain at 3q13, in a region that includes CD47, CBLB, and ALCAM (genes that are not represented on the MSK-IMPACT panel). Copy number profile is suggestive of a fragmented genome..

Somatic alterations detected in this sample:

Gene	Type	Alteration	Location	Additional Information		
Mutations						
TP53	Frameshift Deletion	L93Rfs*30 *(c.278del)*	exon 4	MAF: 63.3%		α
Copy Number Alterations						
FGFR1	Whole gene	Amplification	8p11.22	FC: 2.3	3B	
MYC	Whole gene	Amplification	8q24.21	FC: 2.0		
KRAS	Whole gene	Amplification	12p12.1	FC: 2.0		
CCNE1	Whole gene	Amplification	19q12	FC: 2.7		
PIK3R1	Whole gene	Deletion	5q13.1	FC: -2.1		
BRCA1	Whole gene	Deletion	17q21.31	FC: -2.0	1	
RAD21	Whole gene	Amplification	8q24.11	FC: 2.0		
AGO2	Whole gene	Amplification	8q24.3	FC: 2.0		
RECQL4	Whole gene	Amplification	8q24.3	FC: 2.0		
PIK3C2G	Whole gene	Amplification	12p12.3	FC: 2.0		
RECQL	Whole gene	Amplification	12p12.1	FC: 2.0		
CYLD	Whole gene	Amplification	16q12.1	FC: 2.2		
WHSC1L1	Whole gene	Amplification	8p11.23	FC: 2.3		
CALR	Whole gene	Amplification	19p13.2	FC: 2.9		
DNAJB1	Whole gene	Amplification	19p13.12	FC: 2.9		
STAT5B	Whole gene	Deletion	17q21.2	FC: -2.0		
STAT5A	Whole gene	Deletion	17q21.2	FC: -2.0		

STAT3	Whole gene	Deletion	17q21.2	FC: -2.0
EZH1	Whole gene	Deletion	17q21.2	FC: -2.0
MAP3K14	Whole gene	Deletion	17q21.31	FC: -2.0

+: A glossary of terms and icons used in this report can be found after the "Test and Methodology" section .
α: Denotes clinically/analytically validated variants.
RefSeq IDs for the genes with reported variants along with a list of all 468 genes can be found on the last page

FDA Approved and/or NCCN recommended biomarker:

Alteration(s)	Drugs(s)	Annotation
Level 1 **BRCA1** Deletion FC: -2.0	Rucaparib	BRCA1 is a tumor suppressor involved in the DNA damage response. Select germline mutations of BRCA1 are associated with a significantly increased lifetime risk of developing breast and ovarian cancer. BRCA1 deletion is likely oncogenic. PARP-inhibitors olaparib and rucaparib are FDA-approved for the treatment of patients with BRCA1-mutant ovarian cancer who have received prior lines of therapy. While olaparib is only FDA-approved in the germline setting and is considered standard care for the treatment of BRCA1-mutant ovarian cancers in the somatic setting, rucaparib is FDA-approved in both the germline and somatic setting for these patients.

Investigational biomarker:

Level 3B **FGFR1** Amplification FC: 2.3	AZD4547, Debio1347	FGFR1, a receptor tyrosine kinase, is altered by mutation, chromosomal rearrangement or amplification in a diverse range of cancers, including lung and breast cancers. FGFR1 amplification is known to be oncogenic. While there is promising clinical data supporting the use of FGFR-inhibitors such as AZD4547 in patients with high-level FGFR1-amplified squamous cell lung cancer, their clinical utility in patients with FGFR1-amplified high-grade serous ovarian cancer is unknown.

Technical Assessments

Tumor Coverage	632X	**Test Version**	468 genes
Status	Matched Sample	**Run Number**	2017-111

Coverage assessment: Unless specified, all exons tested had minimum depth of coverage of 100X.

Mutation assessment: Mutation assessment: Mutations are called against the patient's matched normal sample. This assay reports somatic variants confirmed to be absent in the matched normal.

Copy number assessment: The criteria for gene amplification and deletions are as follows: if the fold change is greater than 2, it is reported as amplification. If the fold change is -2 or below, it is reported as a deletion. The degree of copy number change is influenced by tumor content and the ability to detect copy number changes is progressively compromised in samples with less than 50% tumor. For samples with low tumor content, the absence of detectable copy number changes should be interpreted with caution.

Test and Methodology

MSK-IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets) was used to identify specific mutations in 468 genes (Cheng DT *et al.*, 2015, JMD). The following table shows the exons that have been clinically validated:

Gene	RefSeq ID	Exon(Amino Acid range)
AKT1	NM_001014431	exon3 (16-59), exon3 (16-59)
ALK	NM_004304	exon23 (1172-1215), exon25 (1248-1279), exon23 (1172-1215), exon25 (1248-1279)
BRAF	NM_004333	exon11 (439-478), exon15 (581-620), exon11 (439-478), exon15 (581-620)
EGFR	NM_005228	exon18 (688-728), exon19 (729-761), exon20 (762-823), exon21 (824-875), exon18 (688-728), exon19 (729-761), exon20 (762-823), exon21 (824-875)
ERBB2	NM_004448	exon8 (301-341), exon19 (737-769), exon20 (770-831), exon8 (301-341), exon19 (737-769), exon20 (770-831)
FGFR2	NM_000141	exon7 (250-313), exon9 (362-429), exon12 (521-558), exon7 (250-313), exon9 (362-429), exon12 (521-558)
FGFR3	NM_000142	exon7 (247-310), exon9 (359-422), exon18 (759-807), exon7 (247-310), exon9 (359-422), exon18 (759-807)

Select Basket Studies @ MSKCC

Histology-independent or multi-histology trial design

- Pre-defined qualifying genomic alteration(s)
- Uniform therapy (single drug or combination)
- Multiple disease-specific cohorts each with independent statistical analysis
- Centralized prospective single target molecular screening uncommon

→ Assumes that ongoing broad clinical genotyping effort can prepopulate genetically informed basket trials

Gene(s)	Alterations	Drug
BRAF	V600	Vemurafenib
FGFR1, FGFR2, FGFR3	Fusions, Amplification, Select Missence Mutations	Debio1347
PIK3CA	Missence Mutations	Taselisib
ERBB2, ERBB3	Select Missence Mutations, Fusions	Neratinib
AKT1	Select Missence Mutations	AZD5363
NTRK1, NTRK2, NTRK3	Fusions	LOXO-101
ALK1, ROS1, NTRK1, NTRK2, NTRK3	Fusions, Select Missence Mutations	Entrectinib
TSC1, TSC2, mTOR	Truncating Mutations (TSC1/2), Select mTOR mutants	Everolimus
MDM2	Amplifications (High Copy Number)	AMG232
SMARCA4	Truncating Mutations	Tazemetostat
RET	Fusions, Select Missence Mutations	RXDX-105
IDH1	Select Missence Mutations	AG-120

David Hyman

MSK-IMPACT Extramural Data Sharing:

AACR
American Association for Cancer Research
FINDING CURES TOGETHER℠

PROJECT GENIE
Genomics Evidence Neoplasia Information Exchange

- The Center for Personalized Cancer Treatment, The Netherlands
- Dana-Farber Cancer Institute
- Institut Gustave Roussy, France
- Johns Hopkins University's Sidney Kimmel Comprehensive Cancer Center
- Memorial Sloan Kettering Cancer Center
- Princess Margaret Cancer Centre, Canada
- Vanderbilt-Ingram Cancer Center

CPCT Center for Personalized Cancer Treatment

Johns Hopkins University

DANA-FARBER CANCER INSTITUTE

GUSTAVE ROUSSY CANCER CAMPUS GRAND PARIS

Memorial Sloan Kettering Cancer Center

UHN Princess Margaret Cancer Centre

VANDERBILT UNIVERSITY

Aggregate and Link International Clinical Oncology Datasets into a Single, Shared Database

The clinical cancer genomics meta-database

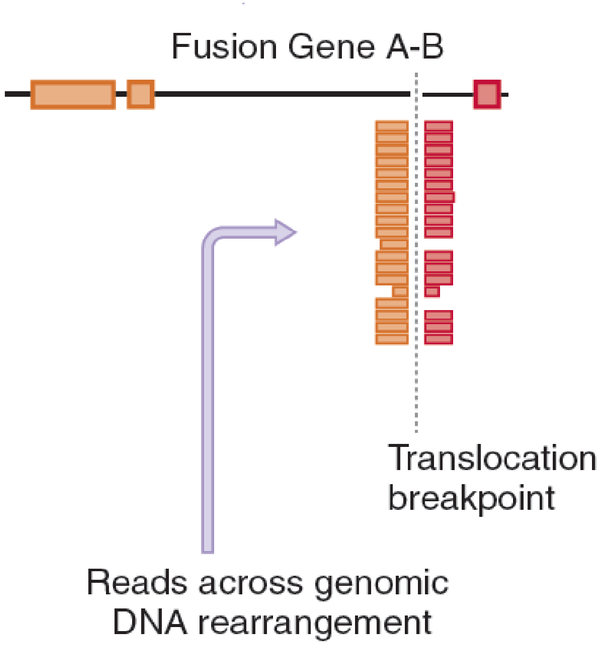
The longitudinal clinical outcomes database

The MSK-IMPACT initiative at MSKCC

1. Overview of test design and validation, data and experience; secondary germline testing, driving basket trials; sharing data
2. Gene fusions: novel and underestimated; need for complementary targeted RNAseq

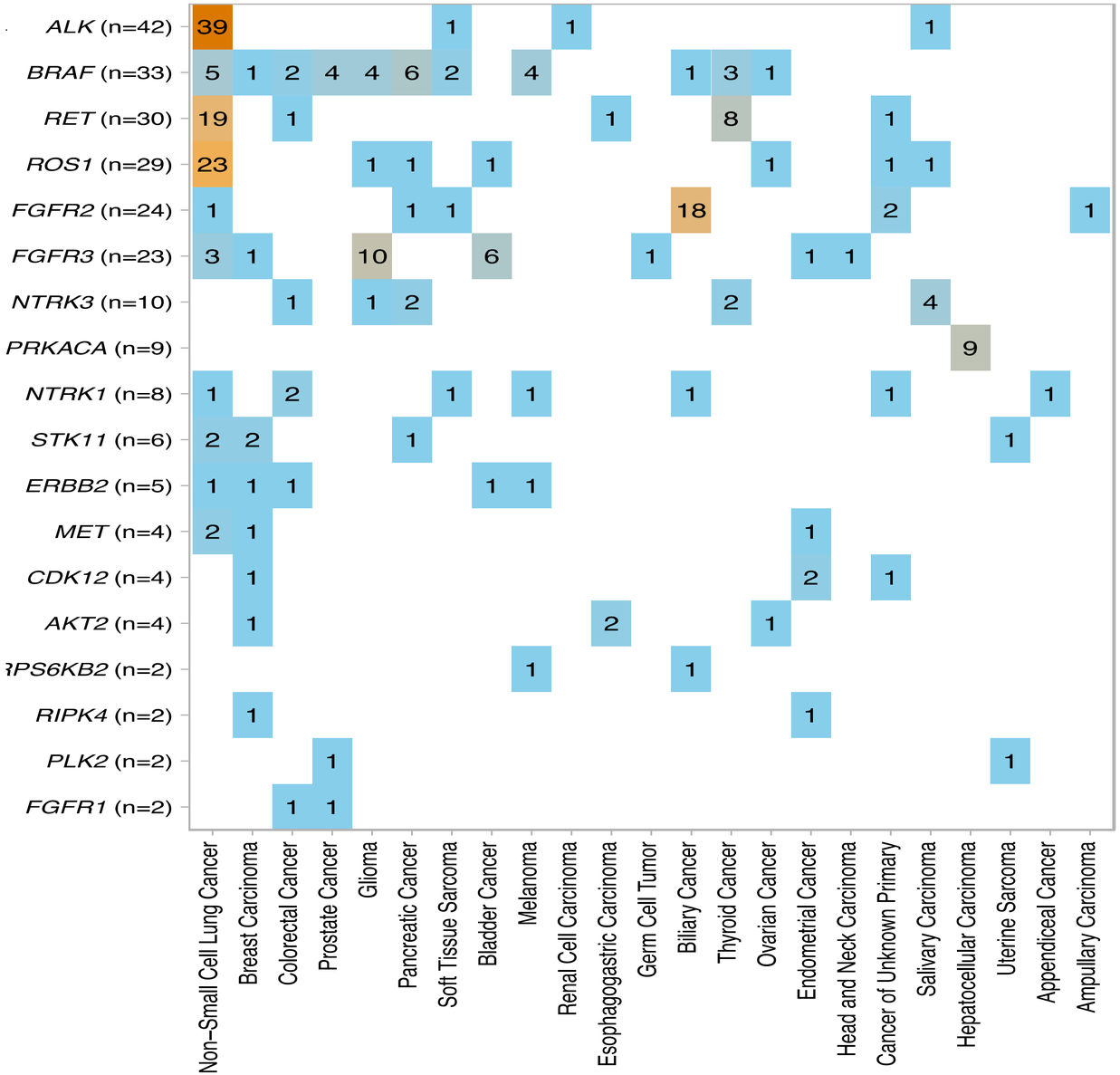
MSK-IMPACT: DNA-based Detection of Cancer Fusion Genes (by intron capture sequencing of rearranged introns)

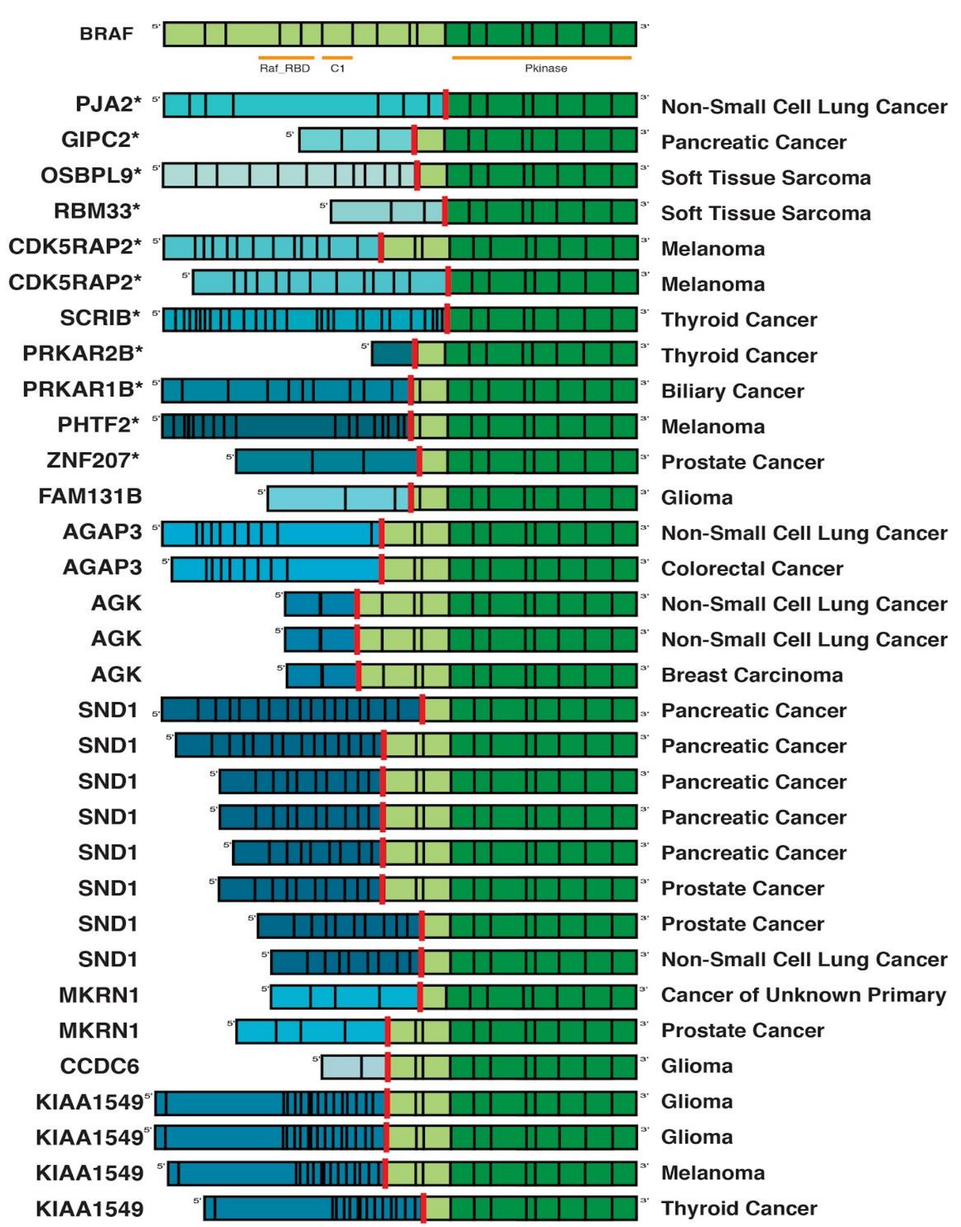
Specific introns of cancer fusion genes captured by MSK-IMPACT



Gene	Introns	Target (kb)
ALK	17, 18, 19	3.9
BRAF	7, 8, 9, 10	18.3
CD74	4, 5, 6	2.9
DNAJB1	1, 2	1.4
EGFR	7, 17, 24, 25, 26	4.4
ETV6	4, 5	30.3
EWSR1	7, 8, 9, 10, 11, 12, 13	11.7
FGFR2	16, 17	5.3
FGFR3	15, 16, 17, 18	0.6
MET	13, 14	3.1
NAB2	2, 3, 4, 5, 6	3.5
NTRK1	3, 7, 8, 9, 10, 11, 12	5.8
NTRK2	15	6.2
NUT	1	1.9
PAX8	8, 9, 10	16.2
RELA	1,2	0.7
RET	7, 8, 9, 10, 11	4.5
ROS1	30, 31, 32, 33, 34, 35	20.4
TFE3	3, 4, 5	4.9
TMPRSS2	1, 2	13.3

MSK-IMPACT: DNA-based Detection of Cancer Fusion Genes





Zehir A, et al. Mutational Landscape of Metastatic Cancer Revealed from Prospective Clinical Sequencing of 10,000 Patients. *Nat Med,* June 2017

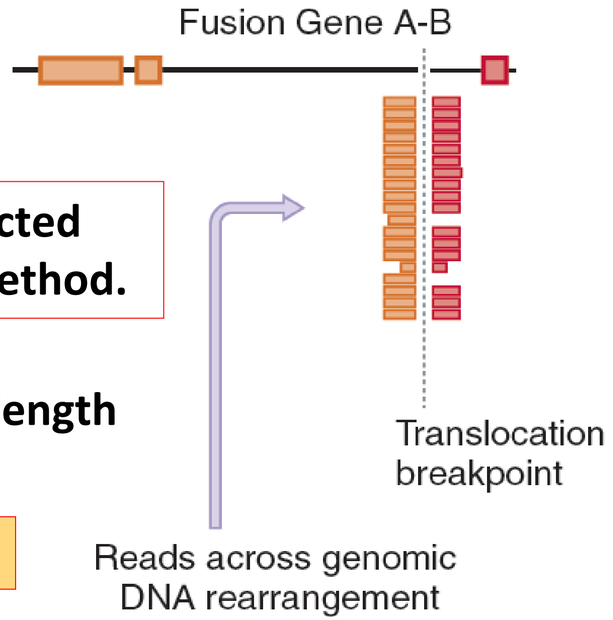
MSK-IMPACT: DNA-based Detection of Cancer Fusion Genes (by intron capture sequencing of rearranged introns)

Problem: not all fusions can be detected by this genomic DNA capture-based method.

Main limitation : intron length

Solution: targeted RNAseq assay

Specific introns of cancer fusion genes captured by MSK-IMPACT

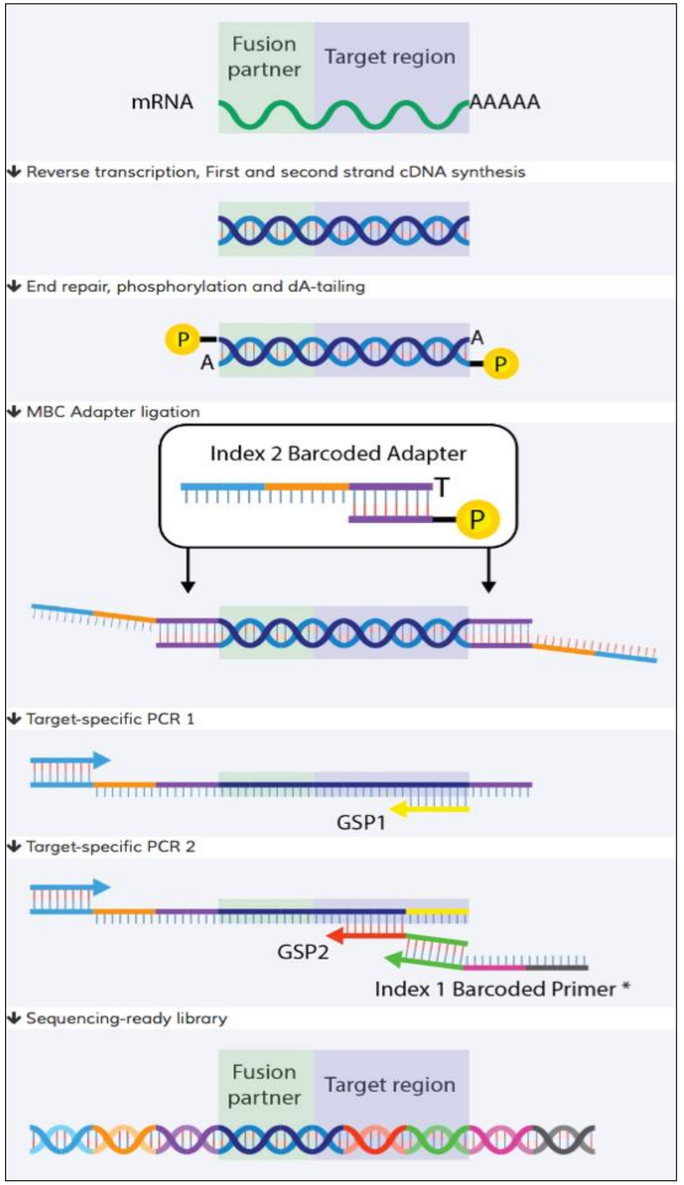


Gene	Introns	Target (kb)
ALK	17, 18, 19	3.9
BRAF	7, 8, 9, 10	18.3
CD74	4, 5, 6	2.9
DNAJB1	1, 2	1.4
EGFR	7, 17, 24, 25, 26	4.4
ETV6	4, 5	30.3
EWSR1	7, 8, 9, 10, 11, 12, 13	11.7
FGFR2	16, 17	5.3
FGFR3	15, 16, 17, 18	0.6
MET	13, 14	3.1
NAB2	2, 3, 4, 5, 6	3.5
NTRK1	3, 7, 8, 9, 10, 11, 12	5.8
NTRK2	15	6.2
NUT	1	1.9
PAX8	8, 9, 10	16.2
RELA	1,2	0.7
RET	7, 8, 9, 10, 11	4.5
ROS1	30, 31, 32, 33, 34, 35	20.4
TFE3	3, 4, 5	4.9
TMPRSS2	1, 2	13.3

Anchored multiplex PCR for targeted next-generation sequencing

Zongli Zheng[1,2], Matthew Liebers[1], Boryana Zhelyazkova[1], Yi Cao[1], Divya Panditi[1], Kerry D Lynch[1], Juxiang Chen[1,3], Hayley E Robinson[1], Hyo Sup Shim[1,4], Juliann Chmielecki[5], William Pao[5], Jeffrey A Engelman[6], A John Iafrate[1,6] & Long Phi Le[1,6]

NATURE MEDICINE VOLUME 20 | NUMBER 12 | DECEMBER 2014



Targeted RNAseq (Archer FusionPlex panels)

- captures any fusion partners of genes of interest
- uses one gene-specific primer + one universal primer
- fusion detection sensitivity is good <5% tumor content
  - -minimum RNA input is 50ng.
  - -samples with 1< RIN<2: min input is 250ng
  - -works well on most FFPE RNA samples
- paired-end sequencing (2x150) on Illumina MiSeq (or NextSeq)

MSK Archer Custom Solid Tumor Targeted RNAseq Panel

ALK	EPC1	FUS	MET	NRG1	PPARG	TCF12
BCOR	ERBB2	GLI1	MGEA5	NTRK1	PRKACA	TFE3
BRAF	ERG	GRB7	MKL2	NTRK2	RET	TFEB
CAMTA1	ETV6	HMGA2	MYB	NTRK3	ROS1	TFG
CCNB3	EWSR1	JAK3	NCOA1	PDGFB	RSPO2	TMPRSS2
CIC	FGFR2	JAZF1	NCOA2	PDGFRA	RSPO3	USP6
COL6A3	FGFR3	KIT	NOTCH1	PHF1	SS18	YWHAE
DNAJB1	FOSB	MAML2	NOTCH2	PIK3CA	STAT6	
EGFR	FOXO1	MEAF6	NR4A3	PLAG1	TAF15	

→ Allows robust detection in FFPE RNA of any fusion involving these genes, regardless of the fusion partner.

MSK-IMPACT + Archer for efficient fusion detection in clinical cancer samples

Gene
ALK
BRAF
CD74
DNAJB1
EGFR
ETV6
EWSR1
FGFR2
FGFR3
MET
NAB2
NTRK1
NTRK2
NUT
PAX8
RELA
RET
ROS1
TFE3
TMPRSS2

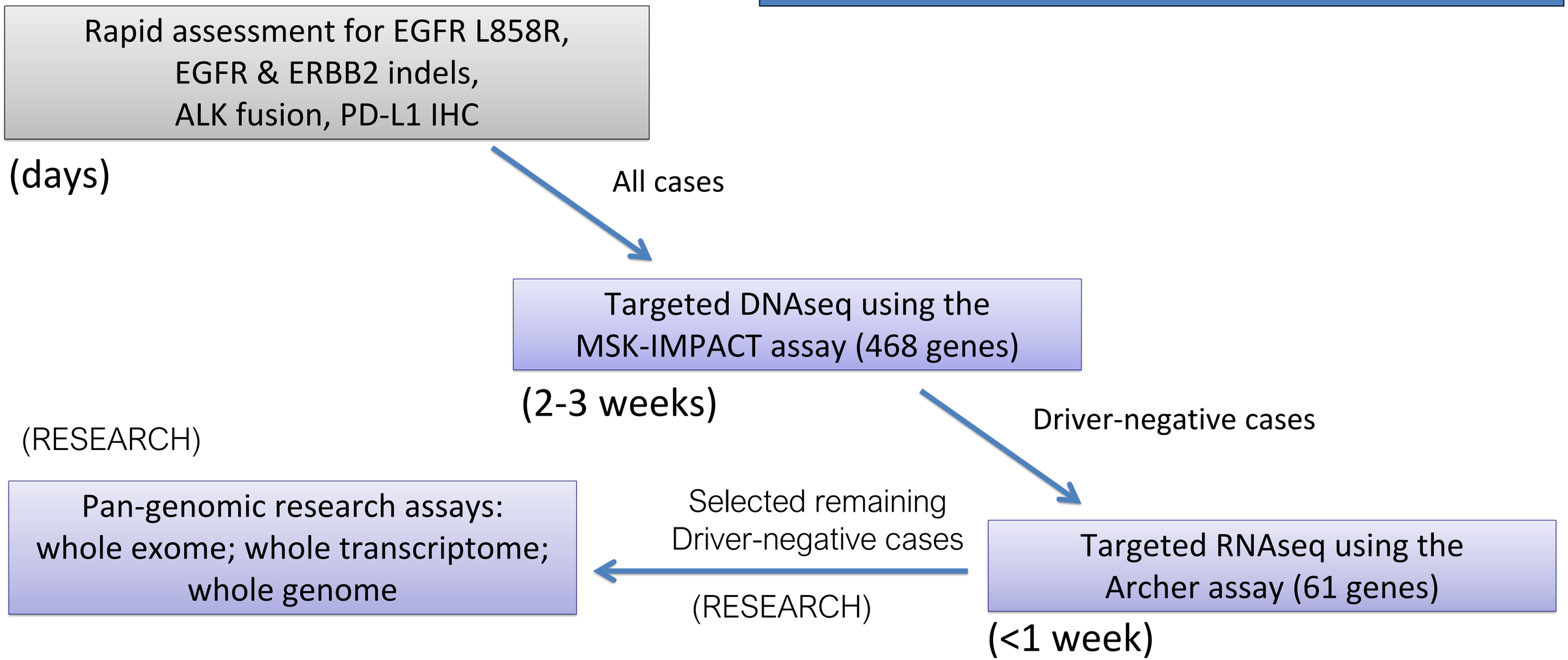
DNA-based fusion detection

ALK	EPC1	FUS	MET	NRG1	PPARG	TCF12
BCOR	ERBB2	GLI1	MGEA5	NTRK1	PRKACA	TFE3
BRAF	ERG	GRB7	MKL2	NTRK2	RET	TFEB
CAMTA1	ETV6	HMGA2	MYB	NTRK3	ROS1	TFG
CCNB3	EWSR1	JAK3	NCOA1	PDGFB	RSPO2	TMPRSS2
CIC	FGFR2	JAZF1	NCOA2	PDGFRA	RSPO3	USP6
COL6A3	FGFR3	KIT	NOTCH1	PHF1	SS18	YWHAE
DNAJB1	FOSB	MAML2	NOTCH2	PIK3CA	STAT6	
EGFR	FOXO1	MEAF6	NR4A3	PLAG1	TAF15	

RNA-based fusion detection

RED: cancer fusion genes covered by only one panel

Lung adenocarcinoma clinical testing workflow – MSKCC 2017



The MSK-IMPACT initiative at MSKCC

1. Overview of test design and validation, data and experience; secondary germline testing, driving basket trials; sharing data
2. Gene fusions: novel and underestimated; need for complementary targeted RNAseq

Clinical MSK-IMPACT Leadership Team

Marc Ladanyi

Michael Berger

Maria Arcila

Ahmet Zehir

Ryma Benayed

Center for Molecular Oncology

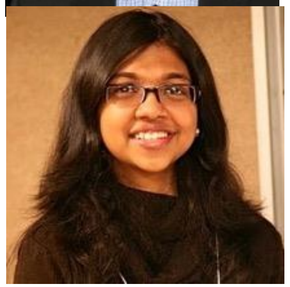
David Solit

Memorial Sloan Kettering Cancer Center™

Clinical MSK-IMPACT Bioinformatics Team

Ahmet Zehir

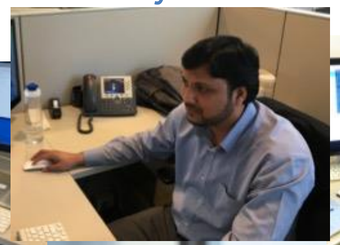
Aijaz Syed

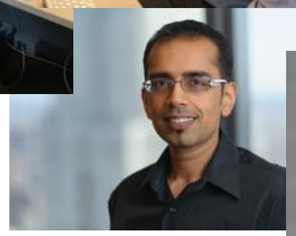


Abhinita Mohantya

Meera Prasad

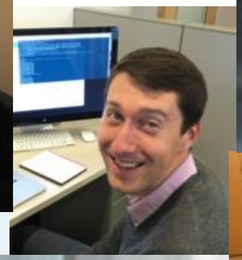
Mustafa Syed

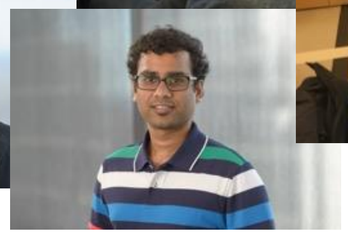




Sumit Middha

John Ziegler





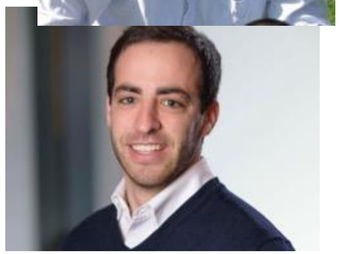
Raghu Chandramohan

Anoop Balakrishnan

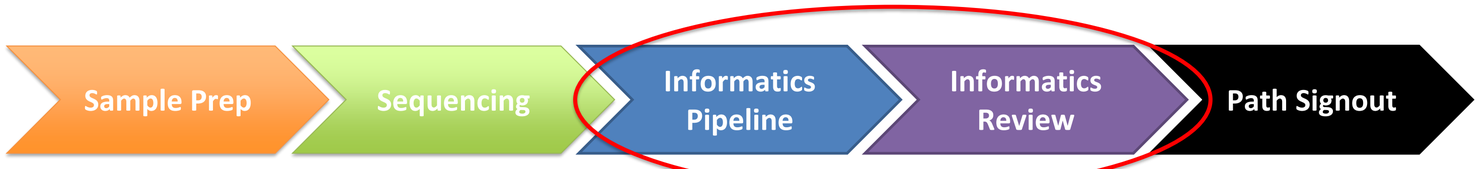
Ronak Shah

Gowtham Jayakumaran

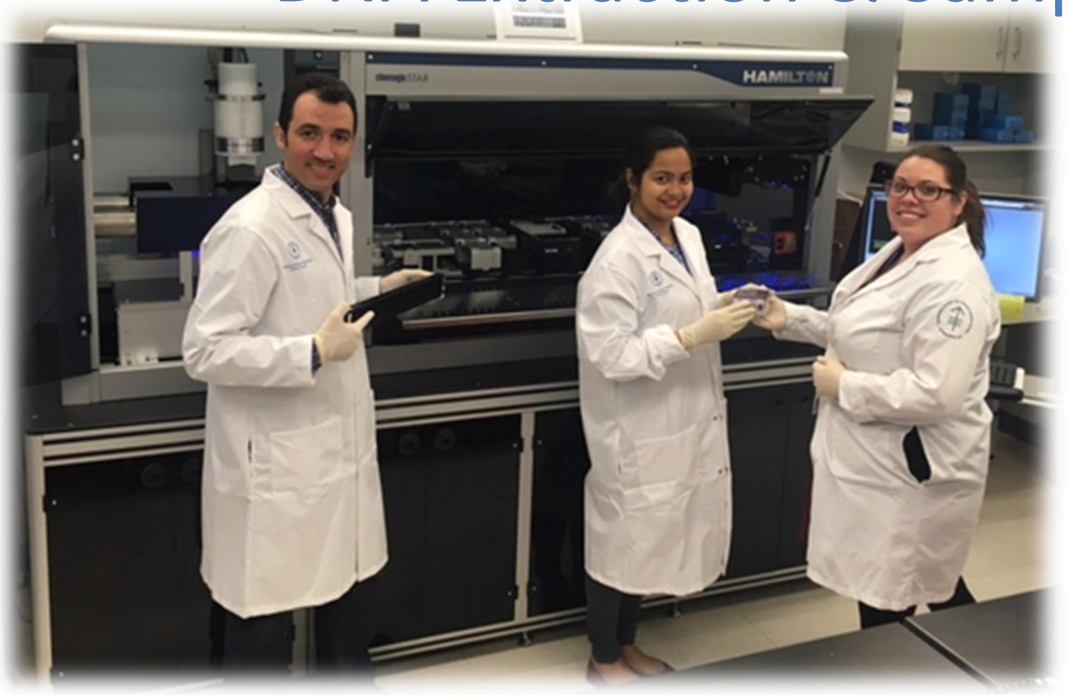
Liu (Tony) Zhen



Ryan Ptashkin



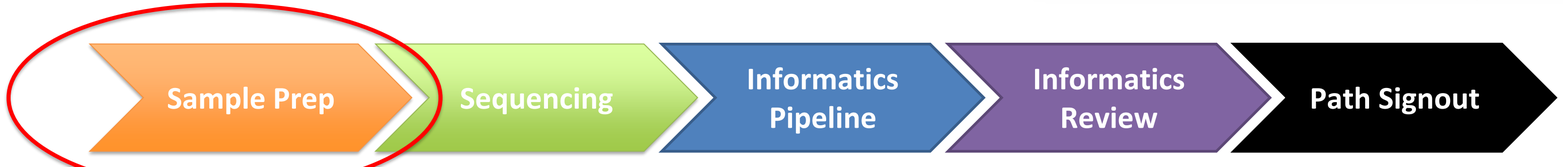
Clinical MSK-IMPACT
DNA Extraction & Sample Management Team



Tessa Baldi & Brian Murphy
with Maria Arcila

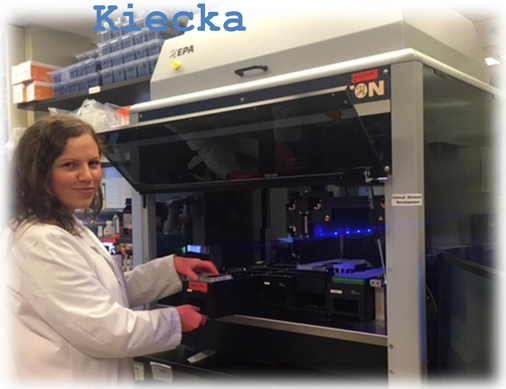
Nelio Chaves

Elise Gallahger
Mustafa
Syed

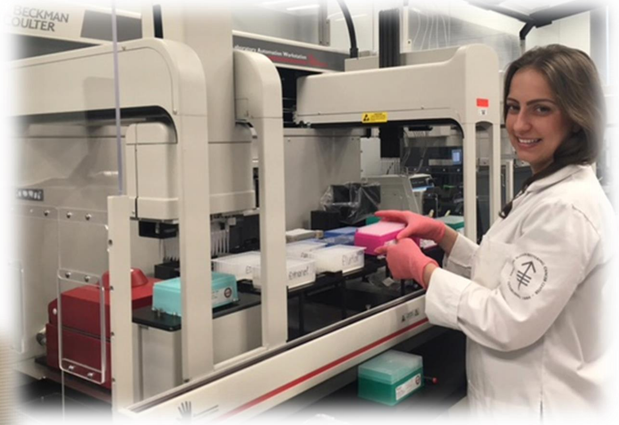


Clinical MSK-IMPACT Sequencing Team

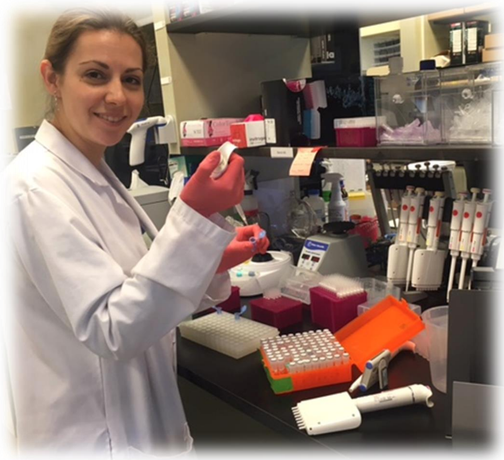
Iwona Kiecka



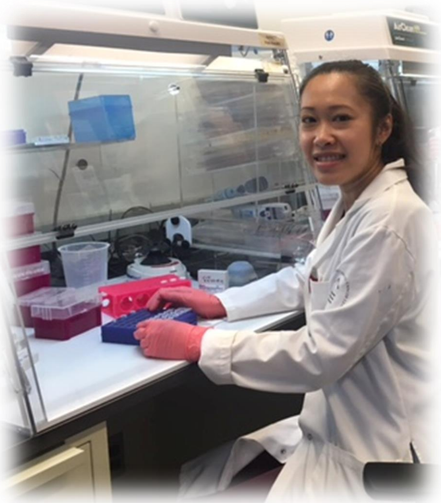
Jackie Casanova



Anna Razumova



Julie Son



Justyna Sadowska

Ruben Bacares

Sample Prep → Sequencing → Informatics Pipeline → Informatics Review → Path Signout

Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients

Ahmet Zehir[1,13], Ryma Benayed[1,13], Ronak H Shah[1], Aijazuddin Syed[1], Sumit Middha[1], Hyunjae R Kim[1], Preethi Srinivasan[1], Jianjiong Gao[2], Debyani Chakravarty[2], Sean M Devlin[3], Matthew D Hellmann[4], David A Barron[5], Alison M Schram[4], Meera Hameed[1], Snjezana Dogan[1], Dara S Ross[1], Jaclyn F Hechtman[1], Deborah F DeLair[1], JinJuan Yao[1], Diana L Mandelker[1], Donavan T Cheng[1,12], Raghu Chandramohan[1,12], Abhinita S Mohanty[1], Ryan N Ptashkin[1], Gowtham Jayakumaran[1], Meera Prasad[1], Mustafa H Syed[1], Anoop Balakrishnan Rema[1], Zhen Y Liu[1], Khedoudja Nafa[1], Laetitia Borsu[1], Justyna Sadowska[1], Jacklyn Casanova[1], Ruben Bacares[1], Iwona J Kiecka[1], Anna Razumova[1], Julie B Son[1], Lisa Stewart[1], Tessara Baldi[1], Kerry A Mullaney[1], Hikmat Al-Ahmadie[1], Efsevia Vakiani[1], Adam A Abeshouse[3], Alexander V Penson[3,6], Philip Jonsson[3,6], Niedzica Camacho[1], Matthew T Chang[3,6], Helen H Won[1], Benjamin E Gross[2], Ritika Kundra[2], Zachary J Heins[2], Hsiao-Wei Chen[2], Sarah Phillips[2], Hongxin Zhang[2], Jiaojiao Wang[2], Angelica Ochoa[2], Jonathan Wills[7], Michael Eubank[7], Stacy B Thomas[7], Stuart M Gardos[7], Dalicia N Reales[8], Jesse Galle[8], Robert Durany[8], Roy Cambria[8], Wassim Abida[4], Andrea Cercek[4], Darren R Feldman[4], Mrinal M Gounder[4], A Ari Hakimi[9], James J Harding[4], Gopa Iyer[4], Yelena Y Janjigian[4], Emmet J Jordan[4], Ciara M Kelly[4], Maeve A Lowery[4], Luc G T Morris[9], Antonio M Omuro[10], Nitya Raj[4], Pedram Razavi[4], Alexander N Shoushtari[4], Neerav Shukla[11], Tara E Soumerai[4], Anna M Varghese[4], Rona Yaeger[4], Jonathan Coleman[8], Bernard Bochner[8], Gregory J Riely[4], Leonard B Saltz[4], Howard I Scher[4], Paul J Sabbatini[4], Mark E Robson[4], David S Klimstra[1], Barry S Taylor[2,3,6], Jose Baselga[4,6], Nikolaus Schultz[2,3,6], David M Hyman[4], Maria E Arcila[1], David B Solit[2,4,6], Marc Ladanyi[1,6] & Michael F Berger[1,2,6]

NATURE MEDICINE VOLUME 23 | NUMBER 6 | JUNE 2017

Analysis of first 10,000 patients (all solid cancer types)
→ now > 20,000 patients

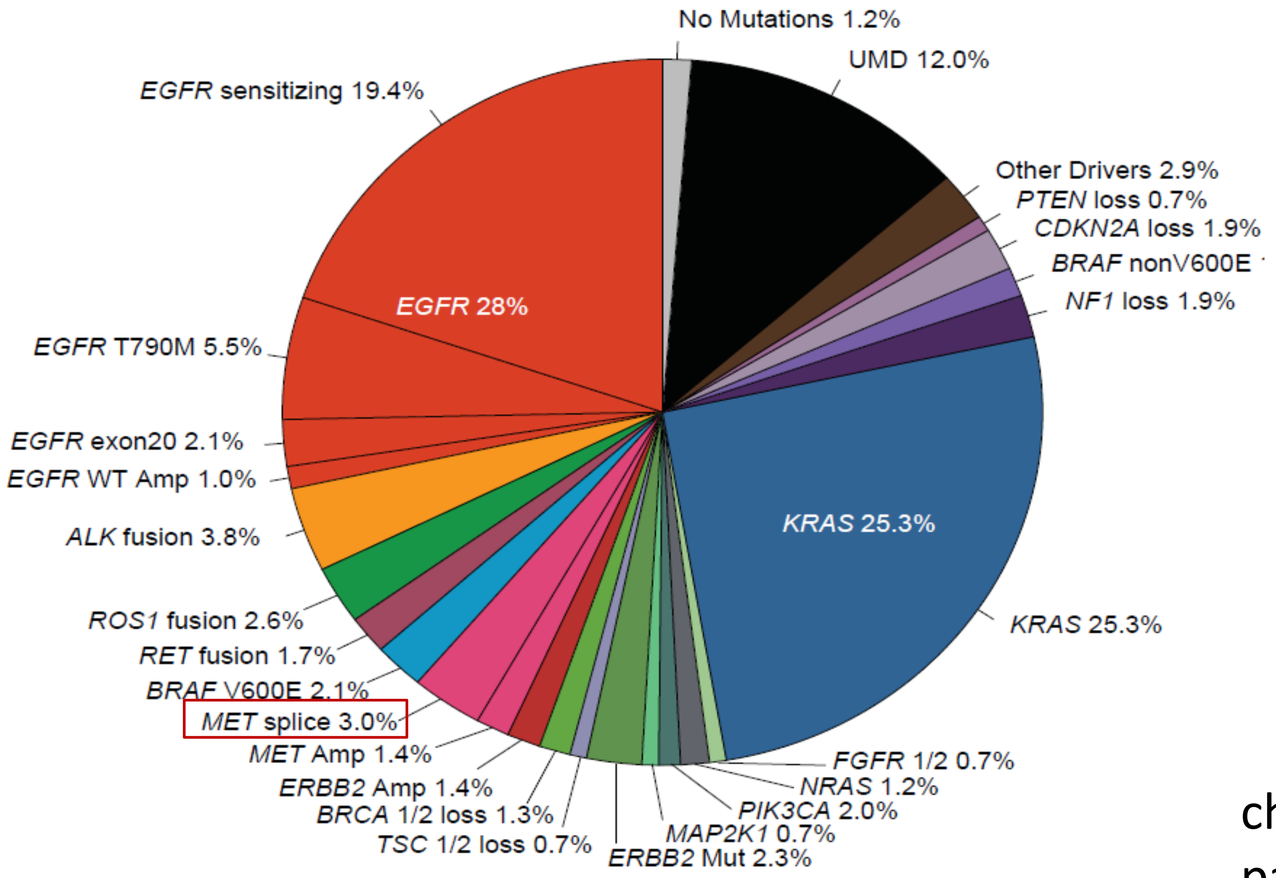
Prospective Comprehensive Molecular Characterization of Lung Adenocarcinomas for Efficient Patient Matching to Approved and Emerging Therapies

Emmet J. Jordan[1], Hyunjae R. Kim[2], Maria E. Arcila[2], David Barron[3], Debyani Chakravarty[4], JianJiong Gao[4], Matthew T. Chang[5,6], Andy Ni[6], Ritika Kundra[4], Philip Jonsson[5,6], Gowtham Jayakumaran[2], Sizhi Paul Gao[5], Hannah C. Johnsen[5], Aphrothiti J. Hanrahan[5], Ahmet Zehir[2], Natasha Rekhtman[2], Michelle S. Ginsberg[7], Bob T. Li[8], Helena A. Yu[8], Paul K. Paik[8], Alexander Drilon[8], Matthew D. Hellmann[8], Dalicia N. Reales[5], Ryma Benayed[2], Valerie W. Rusch[9], Mark G. Kris[8], Jamie E. Chaft[8], José Baselga[1,5], Barry S. Taylor[4,5,6], Nikolaus Schultz[4,6], Charles M. Rudin[8], David M. Hyman[1], Michael F. Berger[2,4], David B. Solit[1,4,5], Marc Ladanyi[2,5], and Gregory J. Riely[8]

JUNE 2017 CANCER DISCOVERY

Analysis of first 860 lung adenocarcinoma patients
→ now > 3,900 NSCLC patients

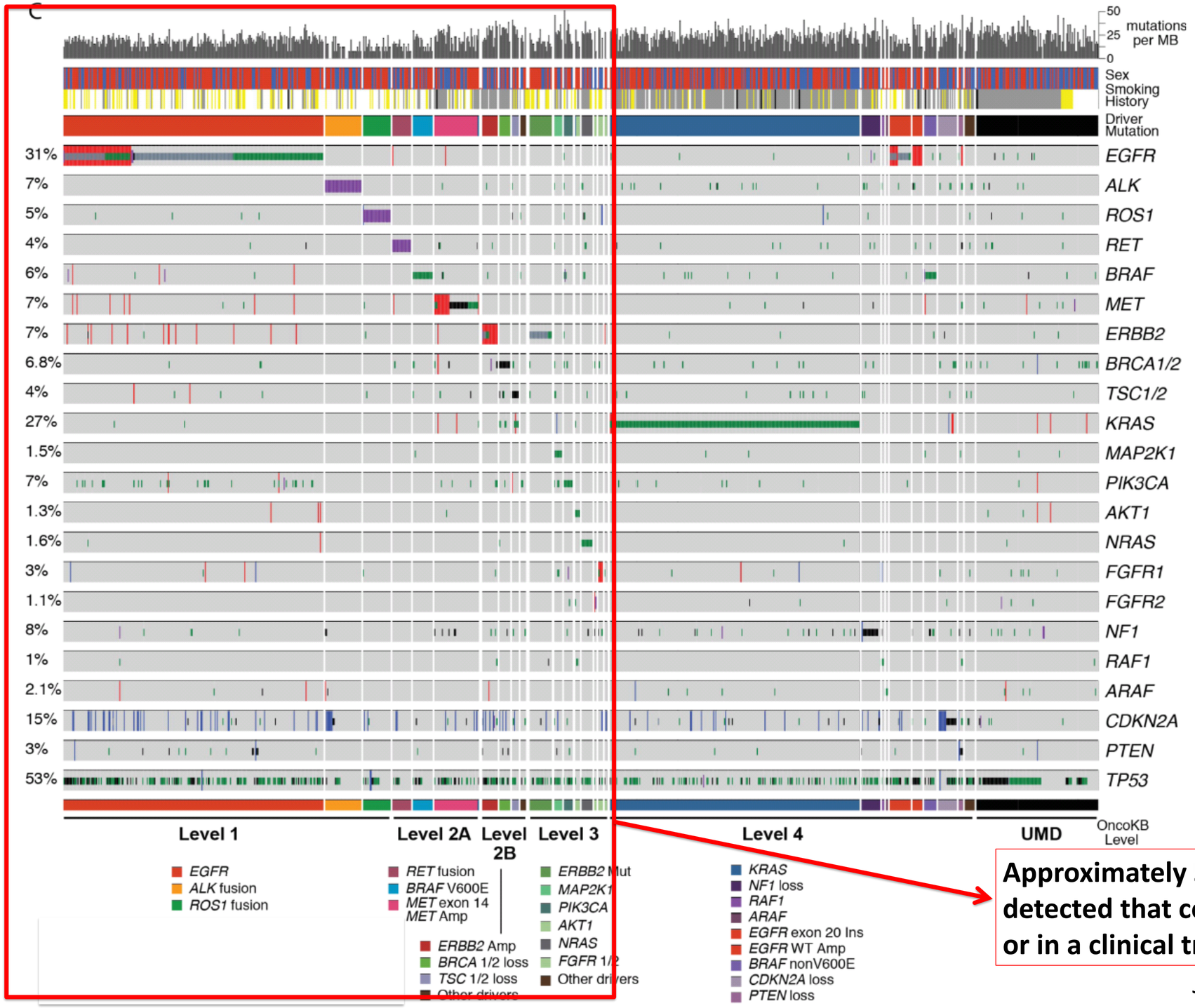
Analysis of the first 860 lung adenocarcinomas studied by MSK-IMPACT



Characteristics	N=860 (%)	Samples (n=915)
Age		
18-50	122 (14.2)	134
51-75	615 (71.5)	652
>75	123 (14.3)	129
Sex		
Men	354 (41.2)	369
Women	506 (58.8)	546
Smoking status		
Never	277 (32.2)	302
Former light (≤15 pack year)	153 (17.8)	167
Former heavy (>15 pack year)	420 (48.8)	436
Current heavy	10 (1.2)	10

Jordan EJ, et al. Prospective comprehensive molecular characterization of lung adenocarcinomas for efficient patient matching to approved and emerging therapies. *Cancer Discovery* 2017 Mar 23

MSK-IMPACT data in patients with advanced lung adenoCa



Sorted by decreasing strength of evidence for targeted therapy

n = 860

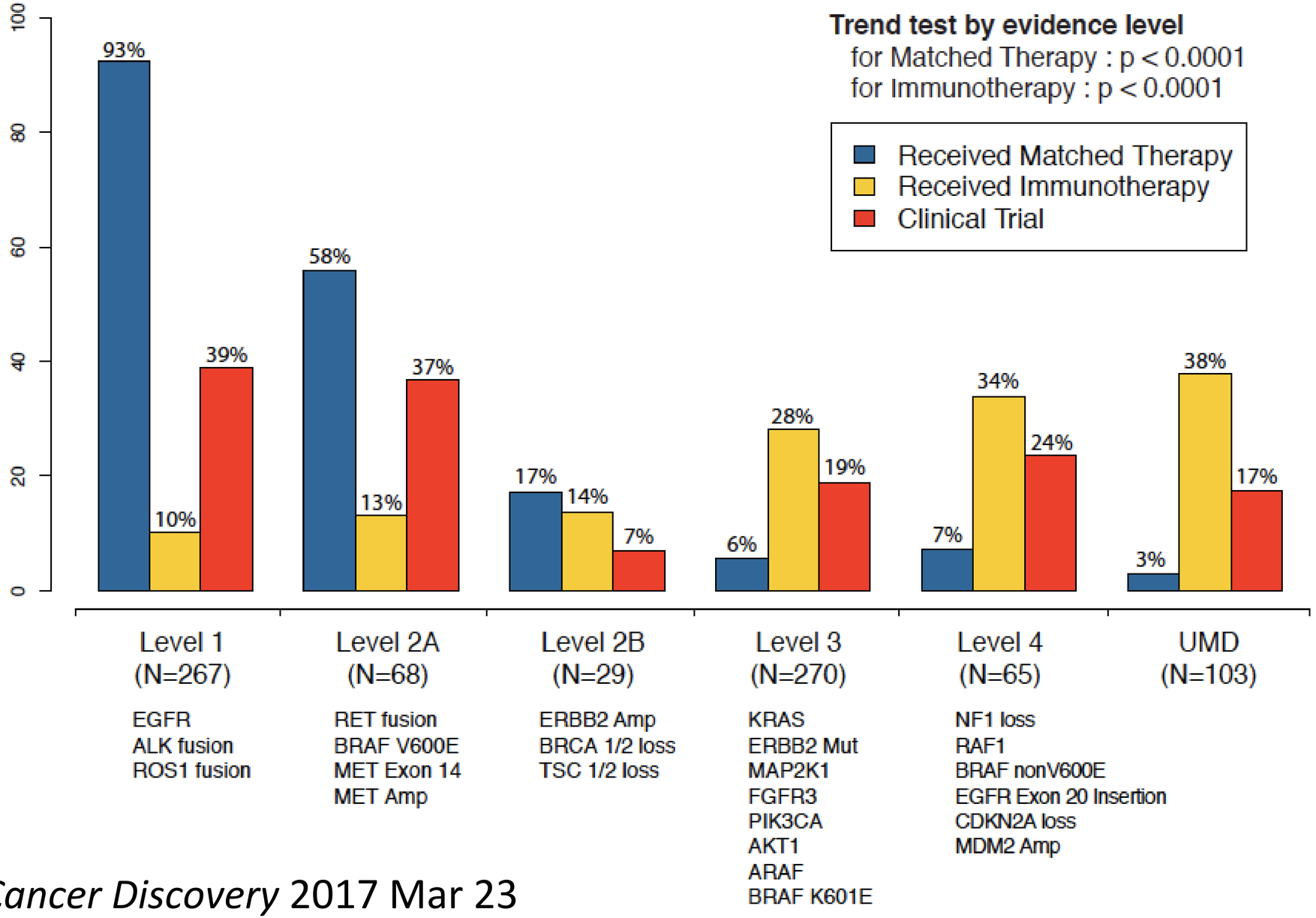
Smoking History
- Current heavy
- Former Heavy
- Former Light
- Never

Genetic Alterations
- Amplification
- Deletion
- Missense Mutation
- Inframe Mutation
- Truncating Mutation
- Fusion

Approximately 50% of patients had an alteration detected that could be targeted with an approved drug or in a clinical trial

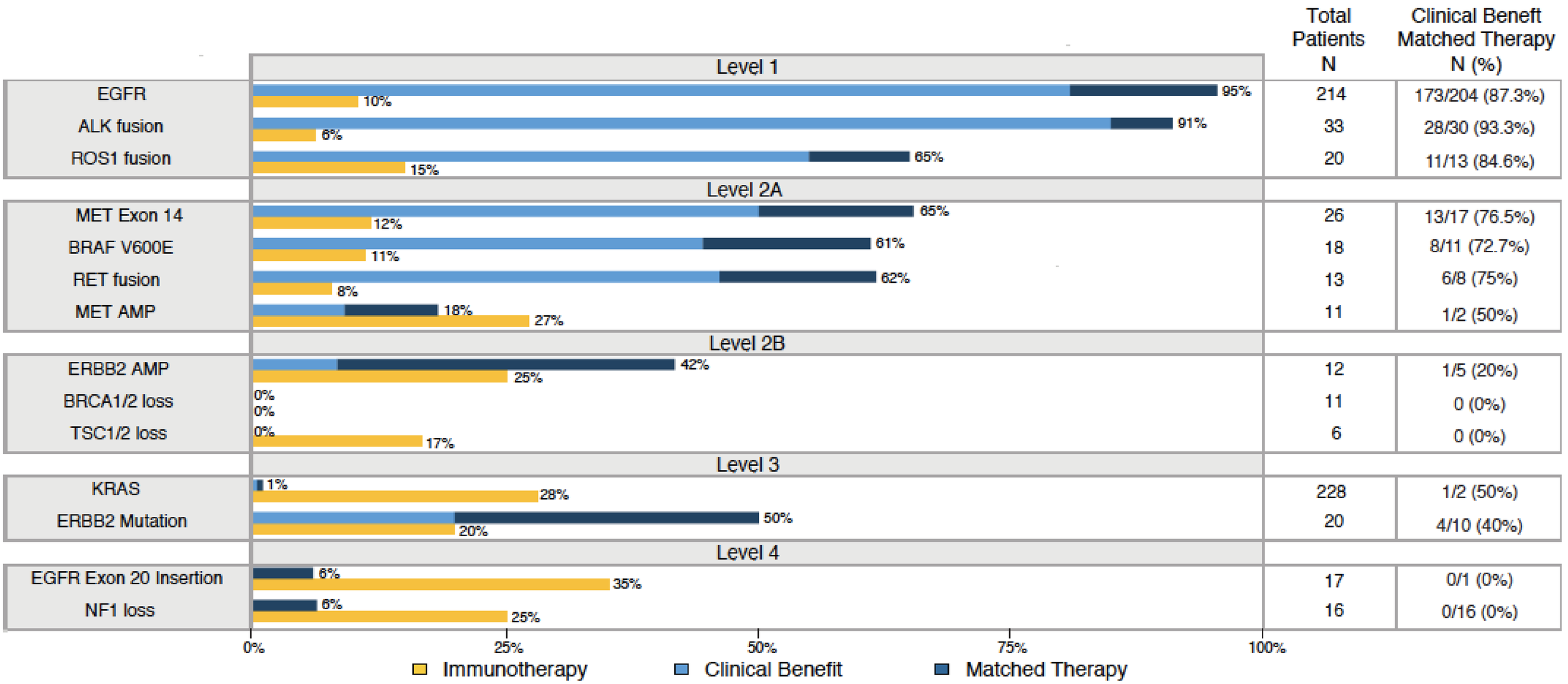
Jordan EJ, et al. *Cancer Discovery* 2017 Mar 23

MSK-IMPACT testing of lung adenocarcinomas: how many received matched therapy ? How many enrolled on a clinical trial ?



Jordan EJ, et al. *Cancer Discovery* 2017 Mar 23

MSK-IMPACT testing of lung adenocarcinomas: how many benefited from the matched therapy received based on mutation data?



Jordan EJ, et al. *Cancer Discovery* 2017 Mar 23