The Role of Next Generation Sequencing in Solid Tumor Mutation Testing

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Objectives

- Describe some of the advantages and disadvantages of Next Generation Sequencing (NGS) testing in oncology
- Understand how the choice of validation samples can define the limits of the test, and how this relates to sequence variant interpretation
- Discuss some of the challenges in interpretation and classification of sequence variants
- Summarize some of the resources available for help with variant interpretation and classification
- Consider proposed criteria that may help discern the pathogenicity of variants
- Review clinical cases that demonstrate the challenges of classifying and interpreting variants.

Problem: Unfamiliar Variants

- NGS provides more sequence coverage than the typical single gene assay performed in clinical laboratories
  - More genes
  - Larger regions of genes – even in “hotspot” panels
  - Unfamiliar sequence variants
    - In genes
    - In tumor type

- ***No formal guidelines on variant classification***
  - potential consequences of interpretations = choice of systemic tx

UNCERTAINTY IS AN UNCOMFORTABLE POSITION, BUT CERTAINTY IS AN ABSURD ONE

Voltaire

Advantages of NGS for Oncology

- Can be more sensitive than Sanger sequencing & other common approaches
  - GIST, melanoma, lung carcinoma
    - *KIT*, *PDGFRA, EGFR* indels
- Can be cost effective for certain tumors
  - Melanoma – *BRAF, NRAS, KIT*
  - Lung adenocarcinoma – *EGFR, KRAS, ERBB2, BRAF*, other
  - Colorectal carcinoma – *KRAS, NRAS, BRAF, PTEN, PIK3CA*
- Preservation of tissue from small biopsies – one extraction, many genes
- Efficient – can promote timely clinical decision-making by avoiding sequential testing
- Discovery – unanticipated actionable targets
- Potential detection of a variety of mutation types in one test
  - Point mutations, indels, rearrangements, copy # gains/losses

Disadvantages of NGS for Oncology Testing

- Requires significant informatics and software support for variant calling and annotating
- Requires significant interpretive time and effort
- Relatively new field with few guidelines for testing, analysis, and reporting

Important Components of Development & Validation

***Quality*: challenge with variety of mutations & those most difficult to accurately detect**

- tumors with known prognostic / actionable mutations
  - **point mutations*: KRAS*, NRAS, *BRAF*, *PDGFRA*, *PIK3CA* , *IDH1/2*, EGFR, etc.**
  - **indels (up to ?): *KIT*, *EGFR*, *PDGFRA***

- **FFPE – test variable amounts of input (resections –> small biopsies)**
- **FNA – scrape tumor cells off EtOH-fixed slides**

***Quantity*: challenge with samples with known low frequency variants**

- **samples with known low allele frequency mutations (≤ 5%)**
- **small samples with few tumor cells**

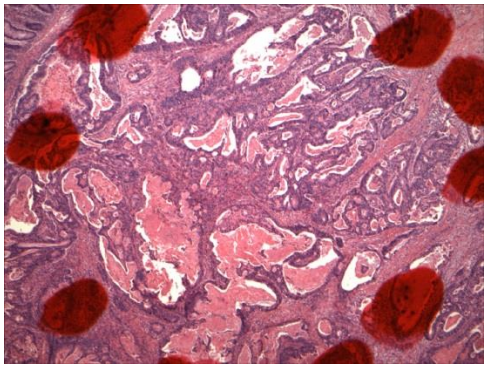
Nontumor controls – flesh out the false positives

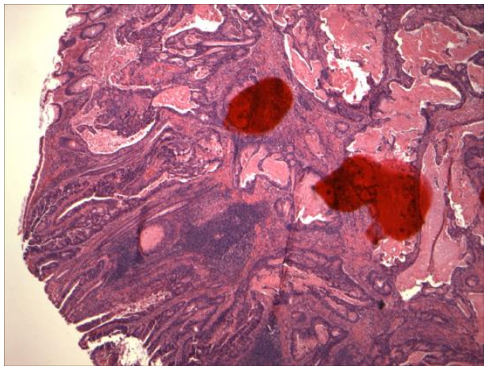
Resources *critical* for Interpretation & Classification

1. Variant Annotator Tools (ideally housed in a LIMS)
    - For each variant lists
        a) allele frequency in 1000 genomes & NHLBI Exomes (6500)
            - Identify germline SNPs
        b) COSMIC link
        c) Internal database allele frequency
            - How classified & interpreted in the past?
        d) Public/private somatic mutation databases
            - The Cancer Genome Atlas, etc.
        e) IGV link – for manual review
2. PUBMED literature review
3. Sequencing analyst
    - Pathologist-only vs. pathologist + M.S. or PhD cancer biologist(s)
4. telephone and email – communication with ordering physician

Tumor Enrichment – Essential Component

Anatomic Pathologist Review & Selection





quality control
increased sensitivity & specificity

Remember – tumors are never pure and are often heterogeneous

Wide range of mutant allele frequencies

images courtesy Wade Samowitz, MD

Important Goals of Development & Validation

- reproducibility of variants *within* runs and *between* runs
- discover software variant-calling errors
- balance sensitivity & specificity
  - adjust software variant caller filter settings to reliably detect X % allele frequency without major sacrifices in specificity
  - establish comfortable reporting threshold. 5% allele frequency?
    - affected by read depth!
- establish procedures for clinical analysis such as
  - manual review of all suspected mutations in IGV
  - manual review of all critical alleles for false negatives
  - multi-director sign-out vs. individual sign out
- feasibility of a ≤ 10 day TAT!!!!

Clinical Reporting

- **What variants will be included in the clinical report?**
  - SNPs
  - intronic
  - UTR
  - Synonymous
  - “mutation”
  - Variant of Uncertain Clinical Significance (VUS)

Options for Variant Classification

- No Classification
  - List all
  - Leave interpretation to ordering physician

- Simple Classification
  - **Mutation**
    - implies significant evidence of "**driver**" mutation status
    - and/or prognostic/therapeutic value (actionable – changes clinical management)
  - VUS
    - Insufficient evidence to determine functional consequences to protein
    - OR to determine whether "passenger" somatic mutation

- Tiered Classification
  - complex stratification schemes based on weighted criteria

Somatic Variant Classification in Cancer

EVIDENCE

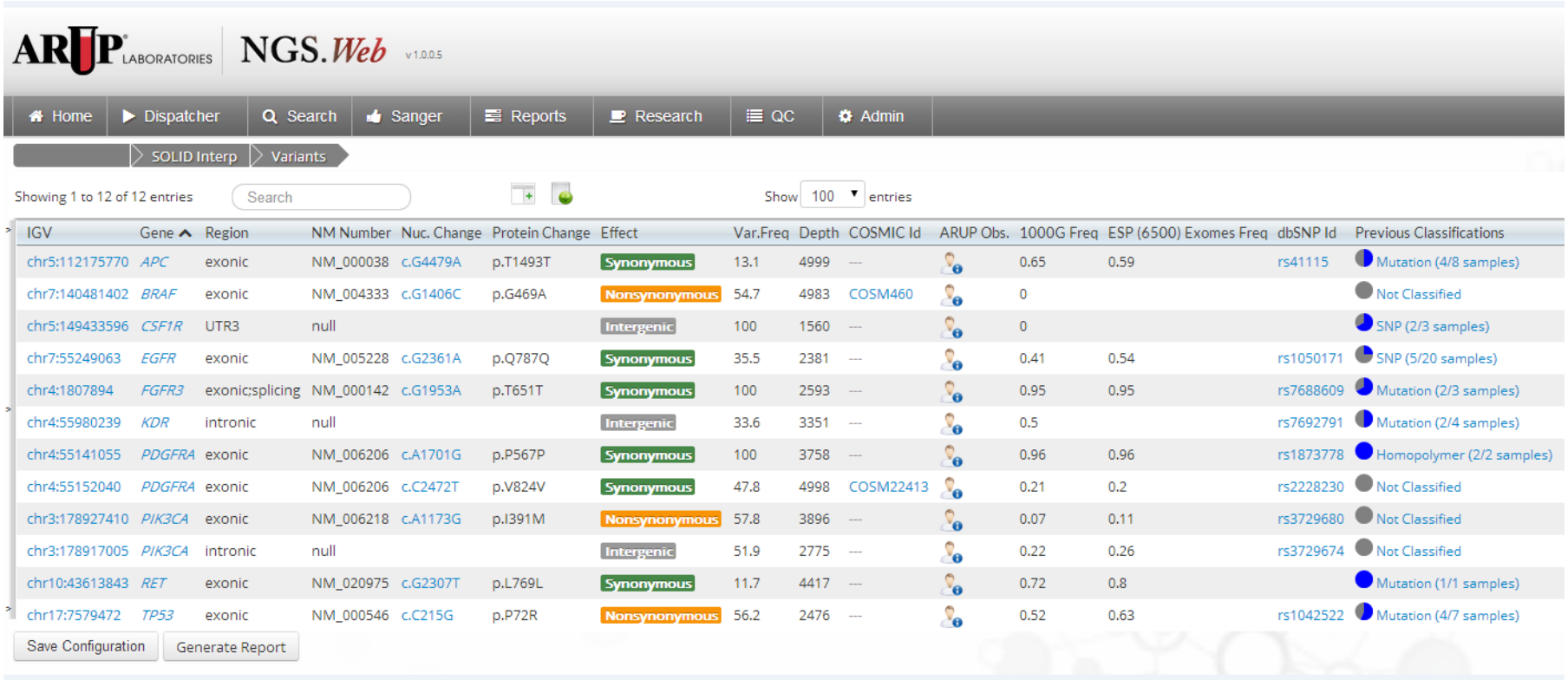
- **Previously reported in any cancer?**
- **Reported in specific tumor in question?**
- **oncogene vs. tumor suppressor?**
  - What protein domain?
  - Oncogene – evidence of activating protein function?
  - Tumor suppressor – evidence of inactivation / deleterious effects?
- **Drug sensitivity?**
- **quality & quantity of published evidence?**
  - Cell lines or animal models vs. patients
  - clinical trial or case series or case reports
  - Incidence in *uncultured* patient samples (ignore tumor cell lines)
  - in vitro proliferation & transformation, in vivo tumor formation

Quality of Interpretive Comments

- Classification with no interpretative comments OR
- If include comments, what content?
  - Has been observed in X cancer types
  - Has/has not been observed in cancer type in question
  - Protein domain?
  - Functional significance to protein / signaling pathway?
  - Predicts survival?
  - Predicts response to X therapy?
  - Provide published data to support a specific therapy?
  - Suggest clinical trials?

Case 1: melanoma

⚠ CAUTION



ARUP LABORATORIES | NGS.Web v1.0.0.5

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SOLID Interp > Variants

Showing 1 to 12 of 12 entries Search Show 100 entries

IGV	Gene	Region	NM Number	Nuc. Change	Protein Change	Effect	Var.Freq	Depth	COSMIC Id	ARUP Obs.	1000G Freq	ESP (6500) Exomes Freq	dbSNP Id	Previous Classifications
chr5:112175770	*APC*	exonic	NM_000038	c.G4479A	p.T1493T	Synonymous	13.1	4999	---		0.65	0.59	rs41115	Mutation (4/8 samples)
chr7:140481402	*BRAF*	exonic	NM_004333	c.G1406C	p.G469A	Nonsynonymous	54.7	4983	COSM460		0			Not Classified
chr5:149433596	*CSF1R*	UTR3	null			Intergenic	100	1560	---		0			SNP (2/3 samples)
chr7:55249063	*EGFR*	exonic	NM_005228	c.G2361A	p.Q787Q	Synonymous	35.5	2381	---		0.41	0.54	rs1050171	SNP (5/20 samples)
chr4:1807894	*FGFR3*	exonic;splicing	NM_000142	c.G1953A	p.T651T	Synonymous	100	2593	---		0.95	0.95	rs7688609	Mutation (2/3 samples)
chr4:55980239	*KDR*	intronic	null			Intergenic	33.6	3351	---		0.5		rs7692791	Mutation (2/4 samples)
chr4:55141055	*PDGFRA*	exonic	NM_006206	c.A1701G	p.P567P	Synonymous	100	3758	---		0.96	0.96	rs1873778	Homopolymer (2/2 samples)
chr4:55152040	*PDGFRA*	exonic	NM_006206	c.C2472T	p.V824V	Synonymous	47.8	4998	COSM22413		0.21	0.2	rs2228230	Not Classified
chr3:178927410	*PIK3CA*	exonic	NM_006218	c.A1173G	p.I391M	Nonsynonymous	57.8	3896	---		0.07	0.11	rs3729680	Not Classified
chr3:178917005	*PIK3CA*	intronic	null			Intergenic	51.9	2775	---		0.22	0.26	rs3729674	Not Classified
chr10:43613843	*RET*	exonic	NM_020975	c.G2307T	p.L769L	Synonymous	11.7	4417	---		0.72	0.8		Mutation (1/1 samples)
chr17:7579472	*TP53*	exonic	NM_000546	c.C215G	p.P72R	Nonsynonymous	56.2	2476	---		0.52	0.63	rs1042522	Mutation (4/7 samples)

Save Configuration | Generate Report

Case 1: melanoma

⚠ CAUTION

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chr4:1807894	*FGFR3*	exonic;splicing	NM_000142	c.G1953A	p.T651T	Synonymous	100	2593	---		0.95	0.95	rs7688609	Mutation (2/3 samples)
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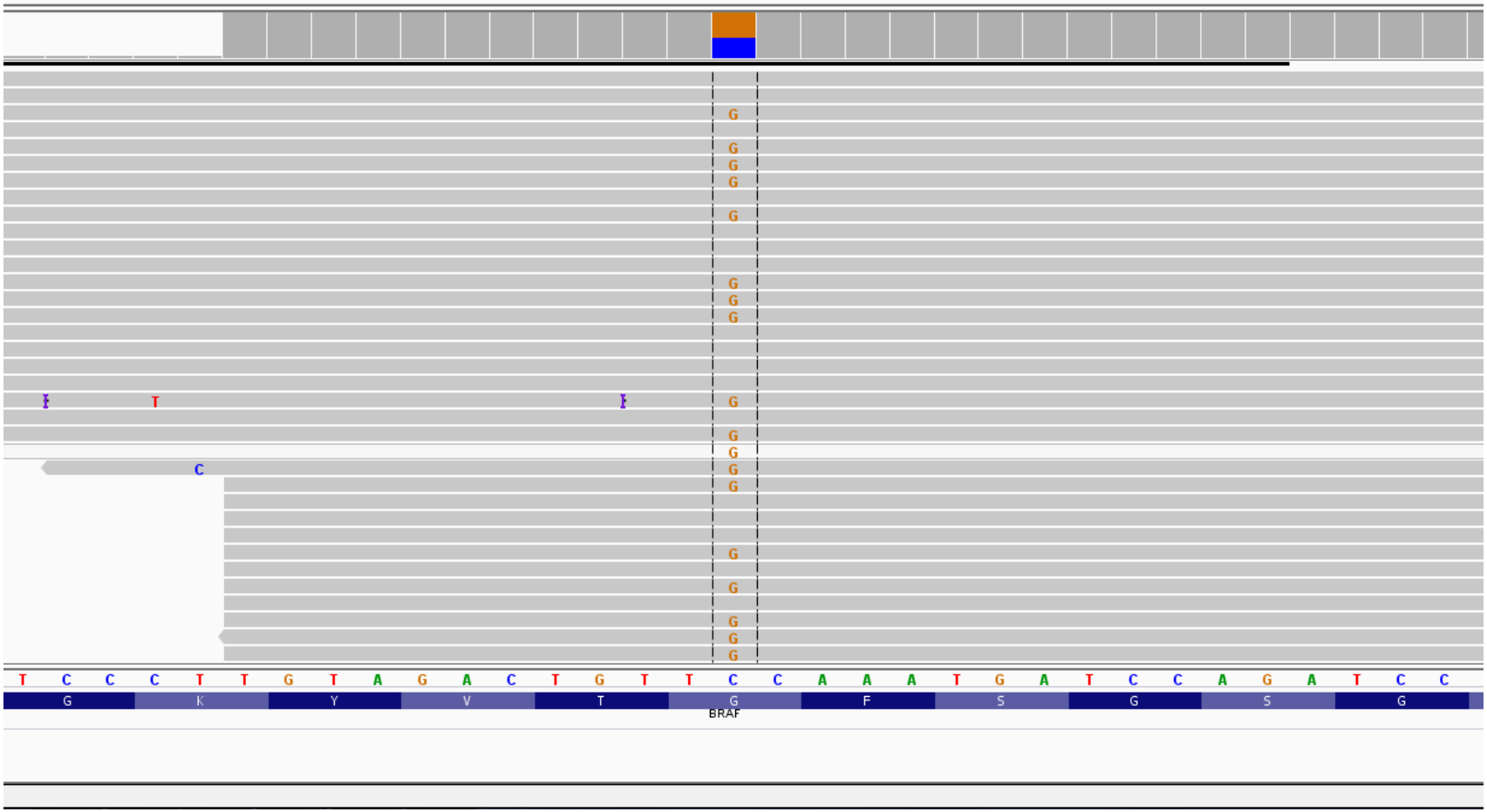
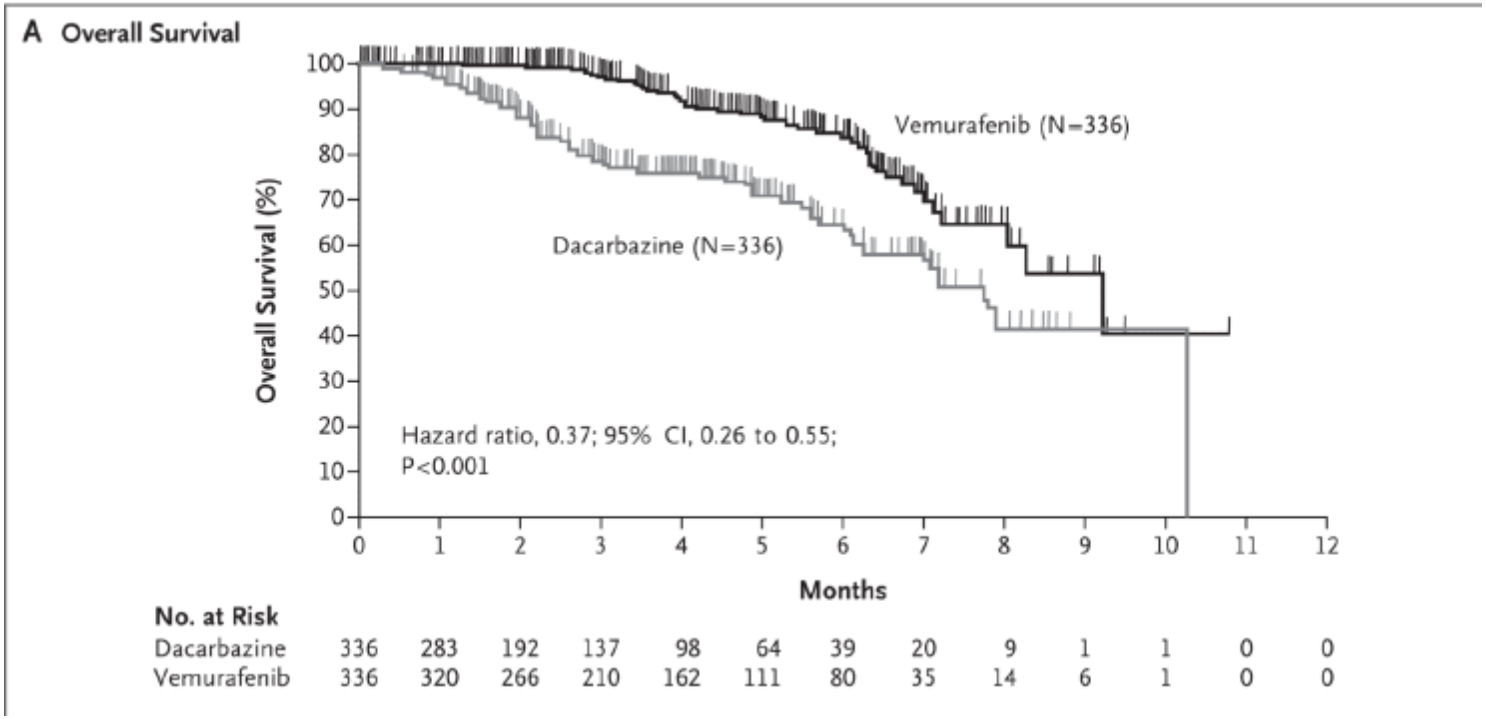
Save Configuration | Generate Report


T C C C T T G T A G A C T G T T C C A A A T G A T C C A G A T C C
G K Y V T G F S G S G
BRAF

BRAF G469A is NOT codon 600!

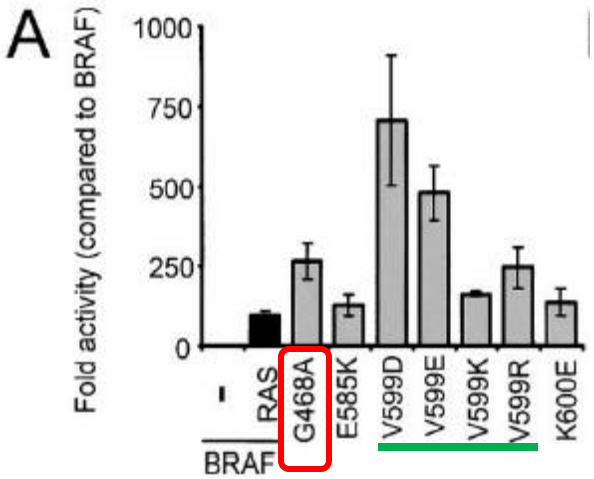
N Engl J Med. 2011 June 30; 364(26): 2507–2516. doi:10.1056/NEJMoa1103782.

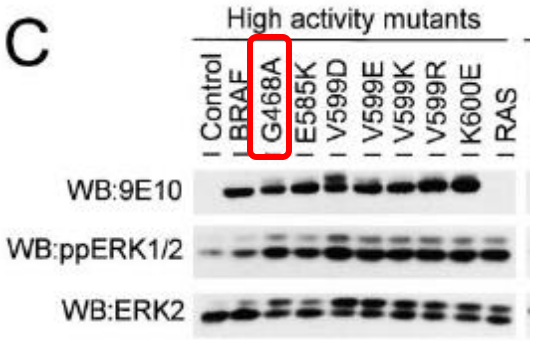
Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation



Cell, Vol. 116, 855–867, March 19, 2004, Copyright ©2004 by Cell Press

Mechanism of Activation of the RAF-ERK Signaling Pathway by Oncogenic Mutations of B-RAF





Mutations of the *BRAF* gene in human cancer

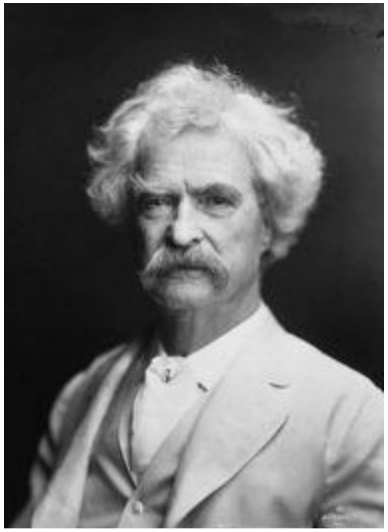
NATURE | VOL 417 | 27 JUNE 2002 |

BRAF G469A transforms fibroblasts in vitro

Table 2 Transforming activity of BRAF mutants

Allele	Transformed foci per μg DNA	Fold increase over wild-type BRAF
^{WT}BRAF	1.3	–
V599E	180	138 ×
DAVE	0	–
L596V	90	70 ×
DALV	0	–
G463V	130	100 ×
G468A	90	69 ×
^{G12V}HRAS	12,000	9,200 ×

NIH3T3 cells were transfected as described in Methods. Transformed foci contained cells like Ras- or Raf1-transformed cells—which are refractile and frequently bipolar—and often contained the giant cells typical of RAS or RAF1 transformation. DAVE and DALV are kinase-inactive versions of V599E and L596V, respectively, in which D593 of the conserved DFG motif is replaced by alanine to generate a kinase-dead variant.

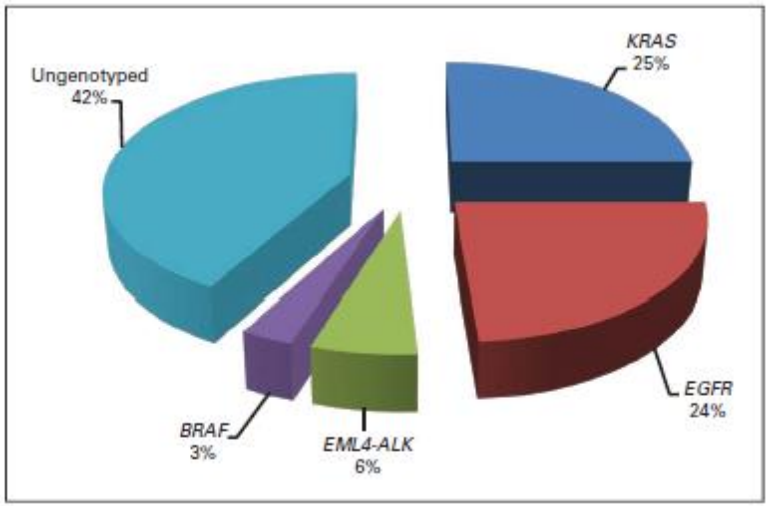


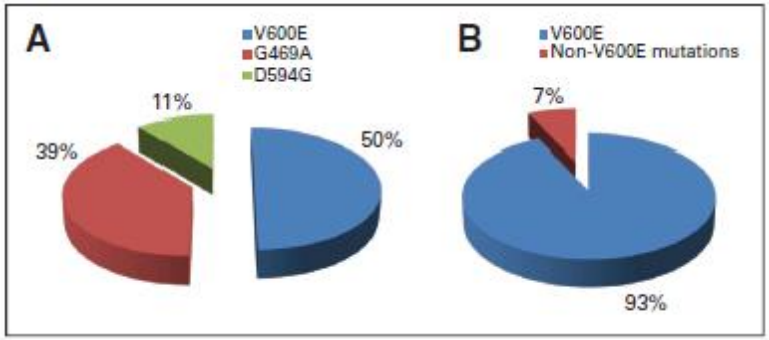
"There is something fascinating about science. One gets such returns of conjecture out of such a trifling investment of fact."

Mark Twain

VOLUME 29 · NUMBER 15 · MAY 20 2011

JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT





Clinical Characteristics of Patients With Lung Adenocarcinomas Harboring *BRAF* Mutations

Paul K. Paik, Maria E. Arcila, Michael Fara, Camelia S. Sima, Vincent A. Miller, Mark G. Kris, Marc Ladanyi, and Gregory J. Riely

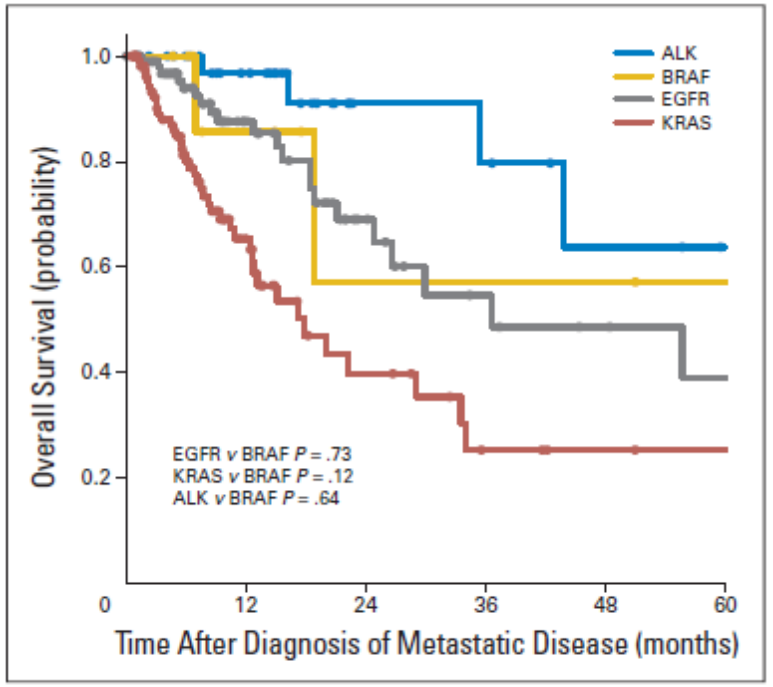


Fig 2. Kaplan-Meier curve for overall survival in patients with advanced stage (IIIB/IV) disease.

BRAF in melanoma

- BRAF targeted therapy is contraindicated in patients with tumors that are WT at V600
  - Paradoxical activation of MAPK
  - Can cause accelerated progression of disease

- Preclinical in vitro data suggests that noncodon 600 – mutated melanoma (G469V) does not respond to BRAF targeted therapy (Yang H et al. 2010 Cancer Res 70: 5518)

Input from ordering physician

“ I will not treat this patient with a BRAF inhibitor without evidence of drug sensitivity demonstrated in a clinical trial. BRAF targeted therapy could harm the patient with wild type codon 600. I will definitely consider alternatives such as MEK inhibitors but only in the clinical trial setting.”

HUNTSMAN CANCER INSTITUTE UNIVERSITY OF UTAH

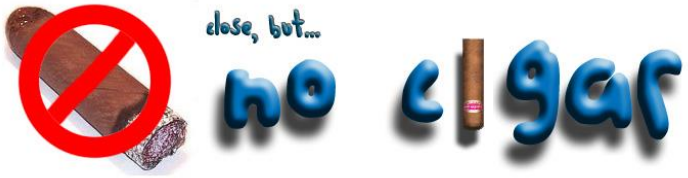
Final classification & Interpretation

Variant of Unknown Clinical Significance
BRAF c.1406G>C, p.G469A

This variant occurs within the highly conserved GXGXXG motif of the kinase domain, and is predicted to activate the MAPK pathway (Davies et al. 2002 Nature 417: 949, Wan 2004 Cell 116: 855). This variant has been reported to be a common *BRAF* mutation in lung cancer (Paik et al. 2011 J Clin Oncol 29:2046). However in melanoma, the clinical significance and effect on drug sensitivity is unknown.

Case 2: Clear cell Renal Cell Carcinoma

c-MET c.2908C>T. p.R970C



Clear Cell RCC with c-MET c.2908C>T. p.R970C

American Journal of Pathology, Vol. 155, No. 2, August 1999

Hereditary and Sporadic Papillary Renal Carcinomas with c-*met* Mutations Share a Distinct Morphological Phenotype

Nature Genetics **16**, 68 - 73 (1997)
doi:10.1038/ng0597-68

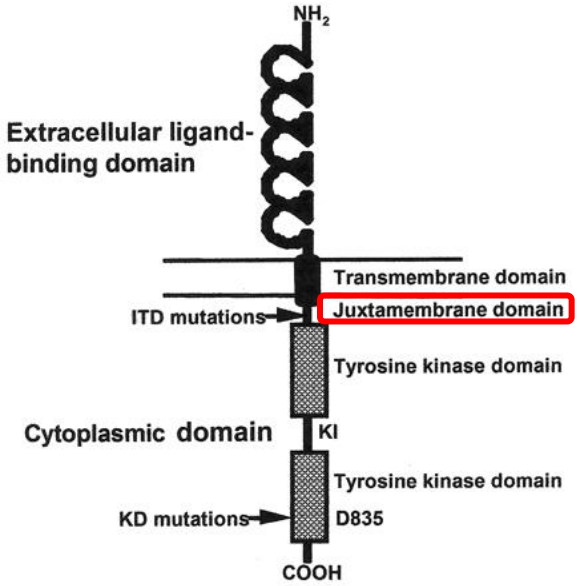
Germline and somatic mutations in the tyrosine kinase domain of the *MET* proto-oncogene in papillary renal carcinomas

Clear Cell RCC with c-MET c.2908C>T. p.R970C

Juxtamembrane domain mutations

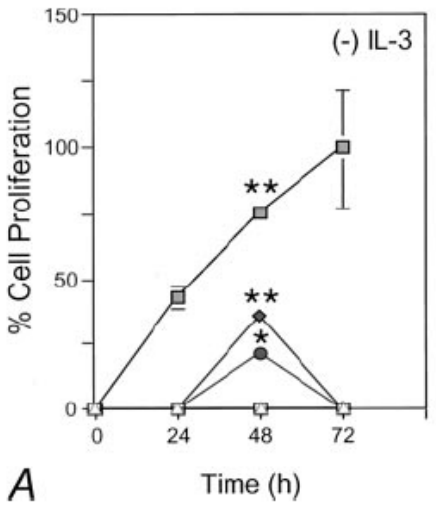
are known to be activating/oncogenic

in Receptor Tyrosine Kinases



Small D. 2006 Hematology 1: 178

c-MET c.2908C>T. p.R970C not reported in Clear Cell RCC

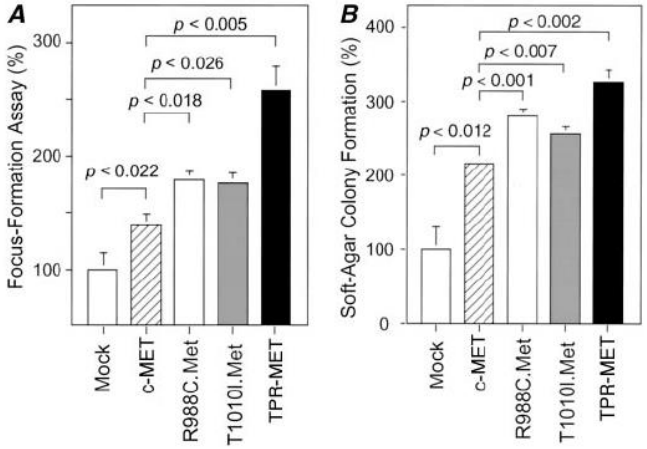


c-MET Mutational Analysis in Small Cell Lung Cancer: Novel Juxtamembrane Domain Mutations Regulating Cytoskeletal Functions

Patrick C. Ma, Takashi Kijima, Gautam Maulik, et al.

Cancer Res 2003;63:6272-6281.

novel JM missense mutation, R988C, was found within exon 14 of both the H69 and H249 cell lines (Fig. 1*B*). Both cell lines were originally derived from patients with extensive-stage SCLC (30). A



Functional Expression and Mutations of c-Met and Its Therapeutic Inhibition with SU11274 and Small Interfering RNA in Non–Small Cell Lung Cancer

Patrick C. Ma, Ramasamy Jagadeeswaran, Simha Jagadeesh, et al.

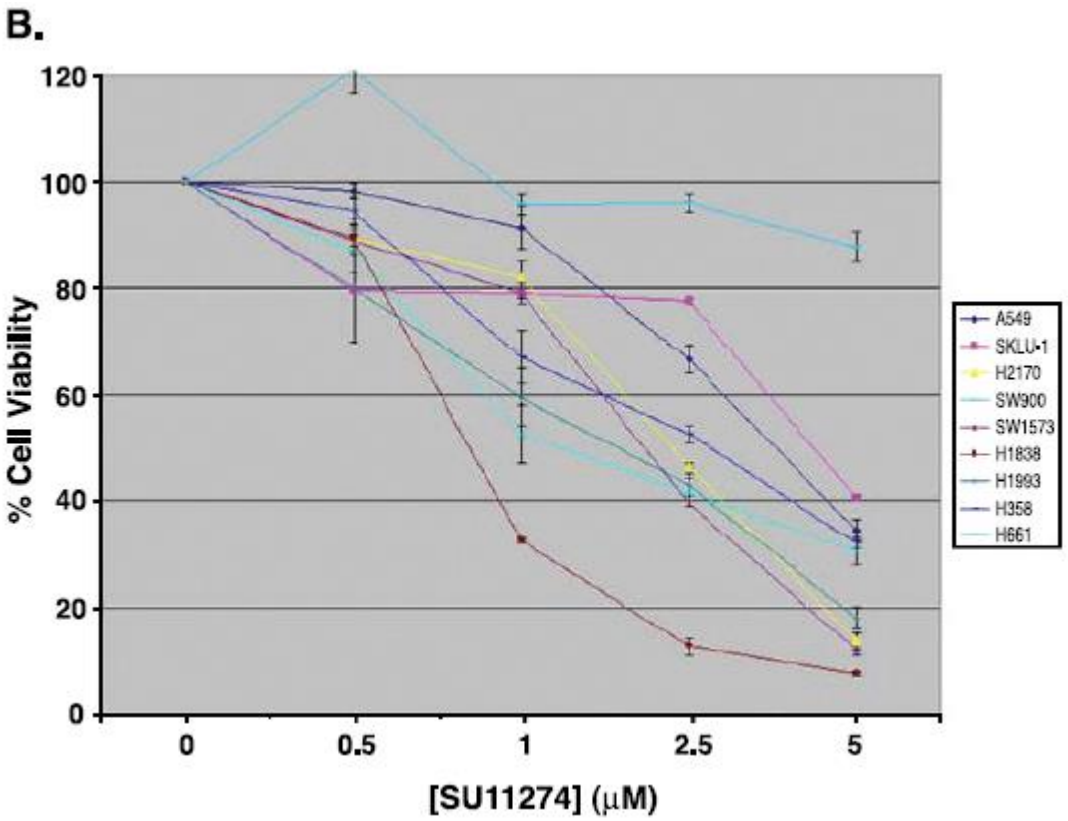
Cancer Res 2005;65:1479-1488.

Table 1. Mutations and sequence variants of *c-Met* identified in NSCLC

Mutations of c-Met in NSCLC

Tumor ID	Nucleotide change	Exon 2 (Sema domain)	Exon 14/15 (juxtamembrane domain)	Mutant genotype	Adjacent "normal" (N)
NSCLC tumor tissues (T1-T127)					
T1	c687G>T (TTG>TTT)	L229F		Heterozygous	–
T9	c2692 C>T (CGC>TGC) + c3029C>T (ACT>ATT)		R988C + T10101	Heterozygous	+ Heterozygous
T63	c1124A>G (AAC>AGC)	N375S		Heterozygous	+ Heterozygous*
T74	c504G>T (GAG>GAT)	E168D		Heterozygous	NA
T80	c1124A>G (AAC>AGC)	N375S		Heterozygous	+ Heterozygous*
T100	c967A>G (AGC>GGC)	S323G		Heterozygous	+ Heterozygous
T103	del 141-bp 2942-3082		Splice variant (exon 14 skipped in-frame)	Homozygous	–
T117	c1124A>G (AAC>AGC)	N375S		Homozygous	NA
T123	c3172T>C (TCT>CCT)		S1058P	Heterozygous	+ Heterozygous
NSCLC cell lines					
A549	Wild-type				
H1395	Wild-type				
H1437	c2692C>T (CGC>TGC)		R988C	Heterozygous	
H2087	Wild-type				

No data on drug sensitivity



Final classification & interpretation

Variant of Unknown Clinical Significance
c-MET c.2908C>T. p.R970C

This variant occurs in the juxtamembrane domain, is recognized in the literature as either R970C or R988C, and shows variable oncogenic capacity. It has been observed infrequently in lung cancer, and colorectal cancer. Some in vitro studies have shown increased cell proliferation and transformation while others show no growth or transformative advantage. This discrepancy may be due to the use of widely different cell lines from unrelated tissue sources. In vivo studies show enhanced tumorigenicity in mice.

Case 3: Anaplastic ganglioglioma

Exceptions to the rules

- PIK3CA c.3140A>G, p.H1047R
- Allele frequency 3.8% (below our threshold for reporting but within the LOD)

Case 3: Anaplastic ganglioglioma

Exceptions to the rules

- PIK3CA c.3140A>G, p.H1047R
- Known activating mutation in oncogene
  - Role in this tumor unknown
- Potentially clinically actionable with targeted therapy
  - Therapeutic efficacy unknown
  - Clinical trials ongoing
- Allele frequency 3.8%
  - below our threshold for reporting, 5%, but within the LOD

Case 3: Anaplastic ganglioglioma

Exceptions to the rules

- PIK3CA c.3140A>G, p.H1047R
- Known activating mutation in oncogene
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- **Variant of Unknown Clinical Significance**

Although seen at low frequency (3.8%) in this case, this mutation has been reported in lung, breast, gastrointestinal and ovarian cancers. This mutation occurs within the highly conserved kinase domain and has been reported to increase p110 catalytic activity, enhancing downstream signaling and oncogenic transformation in vitro.

Case 4: Colorectal Carcinoma

Tumor Specific Classification

- PIK3CA c.3140A>G, p.H1047R
- Known activating mutation in oncogene
  - Role in this tumor KNOWN
  - Predicts resistance to EGFR-targeted therapy
- Potentially clinically actionable with PI3K/AKT targeted therapy
  - Therapeutic efficacy unknown
  - Clinical trials ongoing
- Classified as a MUTATION

Case 5: melanoma

New discoveries?

Obvious Mutations

- *cKIT* c.2464A>T, p.N822Y, This exon 17 mutation has been reported in melanoma (Kong et al. 2011 Clin Cancer Res 17:1684).
- *CTNNB1* c.98C>T, p.S33F. This is an oncogenic mutation that is predicted to activate the WNT/Beta-catenin signaling pathway.

Case 5: melanoma

New discoveries?

Unknown Significance

- *FGFR2* c.755C>T, p.S252L. Although a similar mutation in this codon (S252W) is common in endometrial cancer, this particular missense change has not been reported to our knowledge.
- *PDGFRA* c.2536G>A, p.D846N. Although mutations in this exon 18 codon have been reported, this particular codon change has not been reported and its significance, especially given the KIT mutation, is uncertain.

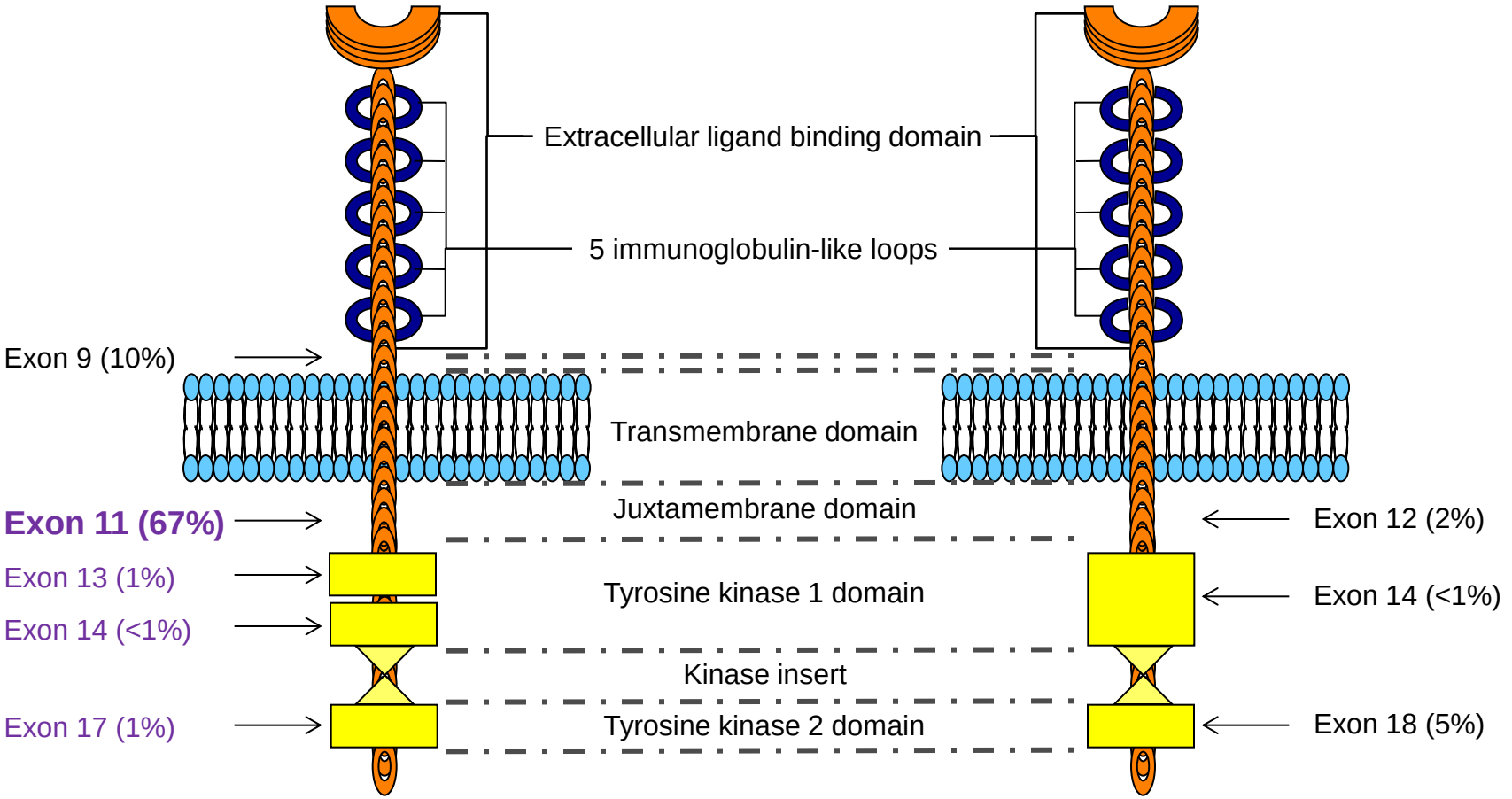
MELANOMA

GIST

KIT

GIST

PDGFRA



Yantiss, Surgical Pathology Clinics, Molecular Oncology, 2012

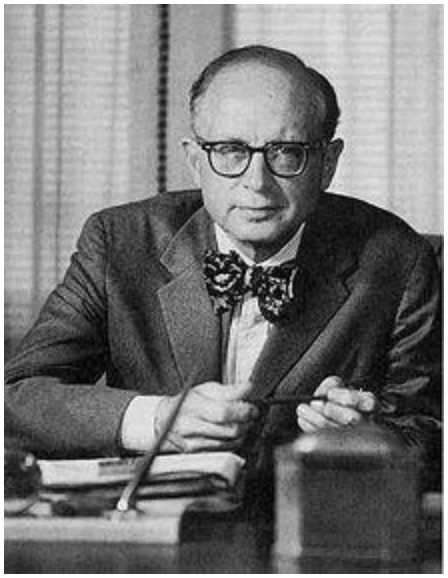
Case 5: melanoma

Obvious Mutations

- *cKIT* c.2464A>T, p.N822Y, This exon 17 mutation has been reported in melanoma (Kong et al. 2011 Clin Cancer Res 17:1684).

Unknown Significance

- *PDGFRA* c.2536G>A, p.D846N. Although mutations in this exon 18 codon have been reported, this particular codon change has not been reported and its significance, especially given the KIT mutation, is uncertain.



"The greatest obstacle to discovery is not ignorance, it is the illusion of knowledge."

Daniel Boorstin

Case 6: urothelial carcinoma

- *c-KIT* c.2458G>A, p.D820N

- Known activating mutation in exon 17 *KIT* oncogene
- Well described in hematopoietic neoplasms and GIST
- Insensitive to imatinib, (other tyrosine kinase inhibitors?)
- Never reported in bladder cancer

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- Driver vs. passenger in this tumor?
- Drug responsive?
- Classified as a VUS

Conclusions

- Interpreting NGS data requires a team approach
- Understanding the clinical context and how NGS report may impact the management of the patient is critical for interpretation
- Each case is unique
- Each variant must be interpreted in the context of the tumor type
- Clinical guidelines for interpretation and classification of somatic variants are needed

Preclinical NGS Research:

take bold RISKs in interpreting variants

http://travel.nationalgeographic.com/travel/united-states/utah-guide/

Clinical NGS interpretations: stay on the groomed trails

http://www.utah.com/ski/ski.htm

Potential Definition of Somatic "Mutation"

- Somatic nucleotide change that is deemed to be pathogenic.
- Pathogenicity implies biologic or clinical significance.
- Clinical significance implies that the somatic DNA alteration is predicted to drive tumor progression, prognosticate survival and/or response to therapy.

Potential Guidelines for Classifying Somatic Variants as Mutations

For <u>oncogenes</u>, any alteration that is well documented and known to:

- activate the protein and drive tumor growth and/or disease progression

or

- predict survival or response to therapy demonstrated in clinical trials

and

- occur as a somatic event in uncultured patient tumors

For <u>tumor suppressors</u>, any alteration that inactivates tumor suppressor, such as:

1. Point mutation leading to a stop codon
2. Small insertion or deletion leading to a frameshift
3. Splice site alteration predicted to affect splicing function, especially positions +1 and +2
4. Large deletions or duplications

Potential Definition of VUS

- A somatic nucleotide change which has an undefined functional effect on the gene product, tumor behavior or patient prognosis.

Potential Definition of VUS

- previously unreported as somatic in uncultured patient samples
- *or*
- previously unreported in the tumor type in question and with little or no evidence for clinical significance
- *or*
- little or no evidence of clinical significance
  - functional data limited to in vitro assays and/or animal models

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