Towards Deciphering The Genetics Landscape in Melanoma

Yardena Samuels, Ph.D.
NHGRI/NIH
ESMO Congress, Vienna
September 30th 2012
Disclosure slide

• I have no Conflicts of Interest to declare
Histologic Progression of Melanocyte Transformation


<table>
<thead>
<tr>
<th>Stage</th>
<th>Benign Nevus</th>
<th>Dysplastic Nevus</th>
<th>Radial-Growth Phase</th>
<th>Vertical-Growth Phase</th>
<th>Metastatic Melanoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epidermis</td>
<td>Basement membrane</td>
<td>Dermis</td>
<td></td>
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</tbody>
</table>

Molecular Lesions:
- **BRAF** mutation
- CDKN2A loss
- PTEN loss
- MMP2 expression

Metastasis to lung, liver, or brain
Our Goals

• Mutational analysis of the melanoma genome

• Functional analysis of the most highly mutated genes

• Translation of the genetic and functional data into the clinic
# Tumor Bank Establishment

<table>
<thead>
<tr>
<th></th>
<th>Dr. Rosenberg National Cancer Institute</th>
<th>Dr. Gershenwald MD Anderson</th>
<th>Dr. Robinson Colorado Cancer Center</th>
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<tbody>
<tr>
<td>Metastatic tumor DNA</td>
<td>120</td>
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<td>Matched protein lysate</td>
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</table>
Analysis of the Tyrosine Kinome

Somatic Mutations Found in ERBB4 (19% in total)

Analysis of the tyrosine kinome in melanoma reveals recurrent mutations in *ERBB4*

Todd D Prickett¹, Neena S Agrawal¹, Xiaomu Wei¹, Kristin E Yates¹, Jimmy C Lin², John R Wunderlich³, Julia C Cronin¹, Pedro Cruz⁴, NISC Comparative Sequencing Program⁵, Steven A Rosenberg³ & Yardena Samuels¹

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The diagram shows the regions of the ERBB4 protein where mutations were found, including:
- **Receptor L domain**
- **GFR domain**

The mutations are indicated by arrows and include:
- L39F
- Y111H
- E317K
- S341L
- R393W
- P409L
- R491K
- E542K
- R544W
- E563K
- D609N
- P700S
- E836K
- G936R
- P1033S
- R1174Q
- S1246N

These mutations are located within the indicated domains of the protein sequence.
Somatic Mutations Found in ERBB4

*Samuels Nature Genetics (15/79; 19%)*

*Chin Cell 2012 (18/121; 15%)*

*Hayward Nature Genetics (2/8; 25%)*
ERBB4 ‘Driver’ Statistics

Non-synonymous: Synonymous ratio
24:3 (p<0.01)

Non-synonymous: Synonymous ratio
12:1 (p<0.03)

Mutations above background mutation rate
4.99E-13
ERBB Mutations in Cancer

ERBB Signaling Pathways

3D-Structure of Extracellular Region of ERBB4

Prickett et al., Nature Genetics, 41: 1127-1132 (2009)
ERBB4 Mutants Have Increased Basal Activation

HEK 293T cells
KD-Kinase dead (K751M)

Prickett et al., Nature Genetics, 41: 1127-1132 (2009)
Mutant ERBB4 Displays Increased Basal Activity in Melanoma Cells
Expression of Mutant ERBB4 Provides an Essential Cell Survival Signal in Melanoma

Mutant ERBB4 Sensitizes Melanoma Cells to Lapatinib

1 is 10 nM lapatinib

Clinical Application of the ERBB4 Study

Hypothesis: ~ 20% of current melanoma patients harbor ERBB4 mutations. Exposure of melanoma cells harboring these mutations to lapatinib will result in cell growth inhibition.

CLIA certified ERBB4 sequencing: Paul Meltzer (NCI)
Dose: 500 mg orally twice daily  # of patients: 25
Lapatinib provided by Cancer Therapy Evaluation Program (CTEP)
Multi-site clinical trial involving:
National Cancer Institute
Memorial Sloan Kettering

Clinical trial samples (NCI; MSKCC) (9/35; 25%)
All mutant samples had detectable levels of ErbB4 ectodomain in the serum. Median of 21.2. ng/ml.

In comparison, in 30 healthy individuals the median is 7.8 ng/ml.

T-test-highly significant difference for the two groups (p<4.14E-05)
ERBB4 Interacting Proteins Strategy
The Endogenous Epitope Tagging (EET) Approach

- **a**
  - ERBB4 E452K mutation
  - ERBB4 E872K mutation
  - Targeting cell colonies with an AAV-NEO-Flag construct

- **b**
  - Phase contrast
  - AAV-GFP

- **c**
  - Genomic ERBB4
  - AAV-NEO-Flag vector
  - Targeted ERBB4 (after recombination)
Melanoma Whole Genome Sequencing
Cell Culture

Cell Line
Tumor Genome

Tissue
Tumor Genome

Normal Genome

Melanoma Somatic Variation

Elliott Margulies
Intersection of non-synonymous and nonsense somatic variants in CDS

However, copy number variations were less concordant:
78.9% of tissue CNVs overlap with cell culture CNVs

Comparative Exome Sequencing of Melanoma Lesions Derived from the Same Patient

98% of variants seen in both Metastases

96% of variants seen in both Metastases

However, copy number variations were less concordant: 89% and 65% similarities between the two metastases

Gartner et al, BMC Genomics, Accepted (2012)
Implications

Though just a small pilot of $2\text{ sets}$ these findings indicate the potential to sequence a single metastasis from an individual to identify drivers and determine treatment strategy.
Exome capture sequencing of 14 untreated melanoma samples and their matched normal 180x coverage, 90% bases with high quality genotype calls

Number of potential somatic mutations: 5161

Assemble sequence data (genome build hg18) and filter putative somatic mutations

Number of mutations with a MPG/Coverage ratio \( \geq 0.5 \): 4226

Missense/ nonsense/ splice site mutations: 2813
  Insertions/Deletions: 27
  Synonymous mutations: 1386

Nonsynonymous:synonymous ratio 2:1

Whole Exome Discovery Screen

Nonsynonymous:synonymous ratio 2:1
The Challenge in Cancer Genomics
‘Passengers’ versus ‘Drivers’

• Statistics
  - Recurrently mutated genes: “Hotspots”
  - Highly mutated genes
  - Nonsynonymous: synonymous ratio
  - Mutations above background mutation rate

• Bioinformatics

• Functional studies
Search for recurrent “Hotspot” mutations

9 novel genes with recurring mutations

Validate mutated genes in Additional tumors

Discovery (n=14)
Prevalence (n=70)
Validation set 1 - Colorado Cancer Center (n=39)
Validation set 2 - MD Anderson (n=32)
Commercial cell lines (n=12)
• Functions as part of a histone acetyltransferase complex
• Disruption of TRRAP causes defects in cell cycle progression

The likelihood for the occurrence of 6 identical mutations is approximately $5 \times 10^{-20}$

• A 433 kDa nuclear protein with homology to the ATM/PI-3 kinase family

Validation Screen

Search for recurrent “hotspot” mutations

9 novel genes with recurring mutations

Search for highly mutated genes

16 highly mutated genes (binomial p value < 0.05; mutated in > than 2 discovery samples)

Validate mutated genes in Additional tumors

Discovery (n=14) Prevalence (n=38)
Whole Exome Sequencing in Melanoma Revealed Sixteen Highly Mutated Genes

The table below summarizes the findings:

<table>
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<th>Gene name</th>
<th>P value</th>
<th>% of tumors affected</th>
<th>% of tumors affected</th>
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<td>BRAF</td>
<td>4.80E-05</td>
<td>50%</td>
<td>65%</td>
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<tr>
<td>GRIN2A</td>
<td>6.36E-03</td>
<td>43%</td>
<td>33%</td>
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<td>CCDC63</td>
<td>3.34E-03</td>
<td>29%</td>
<td>11%</td>
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<td>TMEM132B</td>
<td>7.59E-03</td>
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<td>17%</td>
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<td>ZNF831</td>
<td>1.29E-02</td>
<td>29%</td>
<td>17%</td>
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<td>PLCB4</td>
<td>4.39E-02</td>
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<td>15%</td>
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<td>AKR1B10</td>
<td>5.21E-03</td>
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<td>8%</td>
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<td>KHDRBS2</td>
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<td>PTPRO</td>
<td>9.09E-03</td>
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<td>SYT4</td>
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<td>C12orf63</td>
<td>4.46E-02</td>
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<td>9%</td>
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<tr>
<td>PCDHB8</td>
<td>4.80E-02</td>
<td>21%</td>
<td>8%</td>
</tr>
</tbody>
</table>

GRIN2A is Highly Mutated in Melanoma (25%)  

Wei et al., *Nature Genetics*, 43:442-446 (2011)  

COSMIC  
Chin et al, *Cell* (2012) (30/121; 25%)  

Recurrent mutations:
NMDA Receptor Structure and Function

GRIN1
GRIN2A

Ca^{2+}
glycine
LBD
glutamate

p38/JNK activation
MAPK activation
Caspase activation

Apoptosis

LBD – ligand binding domain
Cloned GRIN2A Somatic Mutations

*NMDAR disrupting mutant
(Endele et. al., Nat Genet. 2010 (42) 1021-26)

N615K*

Wei et al, Nature Genetics, 43:442-446 (2011)
Somatic Mutations in GRIN2A Affect Complex Formation with GRIN1

HEK293T transient co-expression

<table>
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<tr>
<th>Vector</th>
<th>Wild type</th>
<th>E371K</th>
<th>W372X</th>
<th>E373K</th>
<th>G889E</th>
<th>W1271X</th>
<th>+ GRIN1 (WT)</th>
</tr>
</thead>
</table>

IP: α-GRIN1
IB: α-GRIN2A

lysates

α-GRIN2A
α-GADPH
Mutant GRIN2A disrupts Ca$^{2+}$ signaling

GRIN1 (WT)/GRIN2A (WT)

GRIN1 (WT)/GRIN2A (Q891X)

GRIN1 (WT)/GRIN2A (R920K)

GRIN1 (WT)/GRIN2A (W1271X)

200µM NMDA added to cells

Fold change (ΔRFU)

Time (sec)
NMDA Receptor Structure and Function

- **Ca^{2+}**
- GRIN1
- GRIN2A
- glycine
- glutamate (NMDA)
- LBD
- Survival

LBD – ligand binding domain
Expression of Wild-Type GRIN2A Inhibits Cell Proliferation

501Mel (GRIN2A-E1175K)

2359 (31T) WT
Pathway Oriented Models in Cancer Genetics
Mutations in the Glutamate Signaling Pathway are Enriched in Melanoma

Binomial test - p-value 0.000237 (Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, and MSigDB)
Photoactivated Localization Microscopy (PALM) Imaging single molecules *in situ*

Photoactivate GFP and mCherry

Photoactivated Localization Microscopy (PALM) Imaging single molecules *in situ*

Erlon Sherman

A375 cells (10% serum)
Exome sequencing identifies GRIN2A as frequently mutated in melanoma

Xiaomu Wei1, Vijay Walia1, Jimmy C Lin2,3, Jamie K Teer3, Todd D Prickett1, Jared Gartner1, Sean Davis4, Lena Samuels1

Melanoma mouse model implicates metabotropic glutamate signaling in melanocytic neoplasia

Pamela M. Pollock1, Karin Hua Zhu2, Christiane Robb, Laura M. Yudt1, Amy Chen Dunn5, Steven M. Crespo-C, Kenneth R. Reuhl4, Michael R. Kuhar6,7

*These authors contributed equally to this work.

Expression of the metabotropic glutamate receptor 5 (mGluR5) induces melanoma in transgenic mice

Kyu Yeong Choi1, Kai Chao1, James M. Birken1, John D. Barder II1, and Katherine W. Roche1

Exon capture analysis of G protein-coupled receptors identifies activating mutations in GRM3 in melanoma

Todd D Prickett1,11, Xiaomu Wei1,11, Isabel Cardenas-Navia1,11, Jamie K Teer2,3, Jimmy C Lin4, Vijay Walia1, Jared Gartner1, Jiji Jiang1, Praveen F Cherukuri2, Alfredo Molinolo5, Michael A Davies6,7, Jeffrey E Gershonwald8,9, Katherine Stemke-Hale7, Steven A Rosenberg10, Elliott H Margulies2 & Yarden Samuels1
Delving Deeper into the Melanoma Genome
Mutation Frequency in Solid Cancers

* Pleasance et al., Nature. 2010 Jan 14;463(7278):191-6
** Greenman et al., Nature. 2007 Mar 8;446(7132):153-8
*** Wei et al, Nature Genetics, 43:442-446 (2011)

Walia V et al, Pigment Cell Melanoma Res. 2012 Jan 19
Integration of Cutaneous Melanoma Somatic Mutation Data from 300 Whole Exomes/Whole Genomes

<table>
<thead>
<tr>
<th>search for</th>
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<tbody>
<tr>
<td>recurrent mutations</td>
</tr>
<tr>
<td>highly mutated genes</td>
</tr>
<tr>
<td>genes mutated in BRAF wild-type melanomas</td>
</tr>
<tr>
<td>significantly mutated pathways</td>
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<tr>
<td>‘genetic instability’ genes</td>
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</table>

“Passengers” versus “Drivers” genes: Statistical and bioinformatics analysis

Expression analysis of significantly mutated genes

Genetically evaluate affected genes in an expanded melanoma panel (n=192)

“Passengers” versus “Drivers” genes: Statistical and bioinformatics analysis such as:

Evaluate affected genes in the TCGA portal

Functionally characterize melanoma ‘driver’ genes
Melanoma Biology
- High-throughput somatic cell knockout in melanoma cells
- Tumorigenesis assays such as growth, migration, invasion in vitro and in vivo under various growth conditions
- Validate novel driver mutations

Melanoma Protein/Protein Interactions
- High-throughput somatic Endogenous Epitope Tagging in melanoma cells
- Ascertain wild-type and mutant binding partners by mass spectroscopy
- Validate and characterize interacting partners

Melanoma Genetics
- Analysis of 300 melanoma exomes
- Identify novel melanoma genes
- Sub-classify melanoma based on mutation signature

Further validation of melanoma driver genes
Further our understanding of melanoma genetics and biology
Integration Across Tumor Types—"Hotspots"

Merge of our data with COSMIC data reveals 16 non-synonymous "hotspot" mutations shared by 1301 samples.

Our data

- UGDH p.F420S (Glioma)
- PLOD2 p.R54Q (Carcinoma, Ovary)
- PSMD6 p.E99K (Glioma)
- PTEN p.F271S (Glioma)

COSMIC data

- PIK3CA
- NRAS
- CDKN2A
- TRP53
- BRAF

Mutations in red indicate found through whole genome screen

Known hotspot mutations removed (PIK3CA, NRAS, CDKN2A, EZH2, TP53, & BRAF) find 7 novel "hotspot" mutations.

All found in one of our samples and one sample in COSMIC

* this number excludes 11389 COSMIC samples containing the BRAF V600E

Mutations in red indicate found through whole genome screen
Genomic Medicine: Cancer Diagnostics

Now

Future

Disease categorized by clinical, pathologic, and somatic mutation signatures

Courtesy Eric Green
### Examples of Targeted Therapies In Cancer

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tumor type</th>
<th>Target gene</th>
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<tbody>
<tr>
<td>Imatinib (Gleevec)</td>
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<td>BCR/ABL</td>
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<tr>
<td>Trastuzumab (Herceptin)</td>
<td>Breast</td>
<td>HER2/NEU</td>
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<td>EGFR inhibitors</td>
<td>Colorectal, Lung</td>
<td>EGFR</td>
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<tr>
<td>Vemurafenib</td>
<td>Melanoma</td>
<td>BRAF</td>
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Acknowledgments

Samuels Lab

Collaborators
Tumor Bank (NCI)
Steven Rosenberg
Paul Robbins
Nicholas Restifo
John Wunderlich
Udo Rudloff
Patti Fetch
Armando Filie

Present
Todd Prickett
Victoria Hill
Brad Zerlanko
Jared Gartner

Past
Xiaomu Wei
Vijay Walia
Lavanya Palavalli
Jiji Jiang

NIH
Elliott Margulies
Nancy Hansen
NISC
Jamie Teer
Steve Parker
Sean Davis

MD Anderson
Jeff Gershenwald
Mike Davies
Victor Prieto
Katherine Stemke-Hale

Colorado Medical Center
Steven Robinson
William Robinson

Nicholas Hayward
(Queensland Institute of Medical Research)

Jimmy Lin (Washington University)

Klaus Elenius
(University of Turku)

Eilon Sherman
(Hebrew University)
The Weizmann Institute of Science

Open positions:
Yardena Samuels, Ph.D.
Email: samuelsy@mail.nih.gov
Glutamate signaling pathways in melanoma

Identification of Melanoma Progression Genes

• Through the help of one of our collaborators we were able to obtain Primary, Metastasis, and Normal sample sets from 5 different patients.
• These samples are fresh DNA extracted from the sample at the time of removal.
• Whole exome sequencing of these samples may provide the opportunity to determine which variants are present in the metastasis and not the primary.
  – We could then evaluate these variants to see if/how they promote metastasis.
ERBB4 Interacting Proteins Strategy
The Endogenous Epitope Tagging (EET) Approach

ERBB4 mutation

WT allele
Tagged

MUT allele
Tagged

Tagged ERBB4
immunoprecipitated

Mass spectrometry

Flag Tag
Preliminary Results

Melanoma cell lines can be infected by AAV

The rAAV construct can integrate into melanoma cell lines

Insertion of NeoR via homologous recombination
Cutaneous Malignant Melanoma
Overview

- 76,250 new cases of melanoma predicted (USA, 2012)
- 9,180 people died of this disease predicted (USA, 2012)

- Median patient survival is **six months** following diagnosis of late stage disease

- Identification of **genetic alterations** may provide new opportunities for **drug development**

What We Know About Cancer

Genetics
Increased AKT Activation in Melanoma Cells Expressing Mutant ERBB4

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<th>wild-type</th>
<th>mutant</th>
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<td>(WT)</td>
<td>7T (E452K)</td>
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<tr>
<td>39T</td>
<td>(WT)</td>
<td>63T (E542K/E872K)</td>
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<tr>
<td></td>
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<td>17T (E317K)</td>
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α-P-AKT (S473)

α-AKT

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<tr>
<td>31T</td>
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α-P-Erk1/2

α-Erk1/2
## Results of Initial Analysis

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<tr>
<th>Sample Set name</th>
<th>Nonsynonymous (NS)</th>
<th>Synonymous (S)</th>
<th>NS + S</th>
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<tr>
<td>130 set</td>
<td>305</td>
<td>173</td>
<td>478</td>
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<tr>
<td>98 set</td>
<td>185</td>
<td>87</td>
<td>272</td>
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<tr>
<td>Totals</td>
<td>490</td>
<td>260</td>
<td>750</td>
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The G Protein Coupled Receptors Screen: Multiplex Capture

Discovery Phase
Location: NISC
No. of samples: 11
No. of genes: 734
Domain: 94% of coding exons

Validation Phase
Location: Samuels lab
No. of samples: 80
No. of genes: 11
Domain: All coding exons

Functional analysis
## Somatic Mutations Identified in GRM3

### Cohort #1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Other names</th>
<th>CCDS accession*</th>
<th>Ref Seq accession*</th>
<th>No. of mutations (% tumors affected)#</th>
<th>Tumor</th>
<th>Exon</th>
<th>Nucleotide†</th>
<th>Amino Acid†</th>
<th>Functional domain‡</th>
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<tbody>
<tr>
<td>GRM3</td>
<td>GLUR3</td>
<td>5600.1</td>
<td>NM_000840</td>
<td>16 (16.3%)</td>
<td>85T</td>
<td>1</td>
<td>G53A</td>
<td>G18E</td>
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<td>21T</td>
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<td>G176A</td>
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<td>Splice Site</td>
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### Cohort #2

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<th>CCDS accession*</th>
<th>Ref Seq accession*</th>
<th>No. of mutations (% tumors affected)#</th>
<th>Tumor</th>
<th>Exon</th>
<th>Nucleotide†</th>
<th>Amino Acid†</th>
<th>Functional domain‡</th>
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</table>
GRM3 Somatic Mutations Activate the MEK Pathway

The likelihood of having 4 identical mutations at the same position in 140 samples is $1.8 \times 10^{-12}$

Adapted from Wheeler C. et al, *Heart* 2005;91:1366-1374

Whole Exome
Exome sequencing of 18 untreated melanoma samples and their matched normals. 180X coverage, 90% bases with high genotype call.

Whole Genome
Genome sequencing of 10 untreated melanoma samples and their matched normals. 41X mean coverage, 95% mean callable genome.

Merge data

Search for highly mutated genes

15 genes found to be highly mutated

4 genes previously identified to be mutated and play a role in melanoma

Search for non-synonymous recurrent mutations

38 recurrent non-synonymous mutations

<table>
<thead>
<tr>
<th>Gene name</th>
<th>P-value</th>
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<td>ADAM29</td>
<td>2.32E-10</td>
<td>18.18</td>
<td>Wei, X. et al. <em>Hum Mutat</em> 32, E2148-75 (2011)</td>
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</table>
Integration of melanoma somatic mutation data from 300 whole exomes/whole genomes

Search for recurrent mutations

Search for highly mutated genes

Search for genes mutated in BRAF wild-type melanomas

Search for significantly mutated pathways

Search for ‘genetic instability’ genes

“Passengers” versus “Drivers” genes: Statistics and Bioinformatic analyses

Expression analysis of significantly mutated genes

Genetically evaluate affected genes in an expanded melanoma panel (n=192)

“Passengers” versus “Drivers” genes: Statistics and Bioinformatic analyses

Evaluate affected genes in the TCGA portal for:
Somatic mutations; expression; copy number variations and methylation

Functionally characterize melanoma 'driver' genes
Criteria for Evaluation

• Criteria was the same as used in our tumor normal whole exome sequencing.
  – MPG score of >10 MPG
  – MPG/Coverage of >0.5
  – For any variant this criteria needed to met in both metastases and the normal.

Gartner et al, *BMC Genomics*, Accepted (2012)
Mutation Specific Immunotherapy for Melanoma

Tumors are infiltrated by Tumor Infiltrating Lymphocytes (TILs) which may control malignant growth

- Whole exome sequencing
- HLA typing
- Predict mutated epitopes
- Predict best HLA binders

Adoptive transfer of autologous TILs stimulated *in vitro*

Blood lymphocytes

Assay for specific TIL responses

Clinical Benefit?

Retrospective study

N=8

Long term survivors

Positive results: PPP1R3B, GAS7

In collaboration

Steven Rosenberg

Paul Robbins
The Kinome Screen

Discovery Phase
Location: NISC
No. of samples: 29
No. of genes: 86
Domain: Kinase

Analysis:
Mutation validation
Somatic mutation validation

Validation Phase
Location: Samuels lab
No. of samples: 80
No. of genes: 19
Domain: All coding exons

Analysis:
Mutation validation
Somatic mutation validation

Functional analysis
Whole Exome Sequencing

**Discovery**
Exome capture (14 tumors/ matched normal)
Agilent SureSelect 37Mb
~20,000 genes and flanking regions
Illumina GAII platform
ELAND followed by cross_match

**Validation**
Sanger

- 2.4% false negative rate
- 81% sensitivity