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Unearthing Hidden Genomic Data in Solid Tumor Samples: Are Your FFPE Samples Revealing All? will begin shortly…

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Science Webinar Series

Unearthing Hidden Genomic Data in Solid Tumor Samples: Are Your FFPE Samples Revealing All?

18 September 2013

Brought to you by the Science/AAAS Custom Publishing Office

Participating Experts:

Sarah South, Ph.D.
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Unearthing hidden genomic data in solid tumor samples

Data beyond FISH

Sarah South, PhD, FACMG
ARUP Laboratories
Departments of Pediatrics and Pathology
University of Utah
Fluorescence *in situ* hybridization (FISH)

- First described by Pinkel, Straume, & Gray in 1986
- Label DNA with fluorescent molecule and hybridize to human chromosomes on a slide

**Benefits:**
- Can turn almost any DNA into a probe
- For clinical use, most probes 100-500 kb
- Much higher resolution as compared to G-banding for identifying deletions, insertions, and translocation breakpoints
- Can use cells in any state of the cell cycle as well as archived tissue
- Can analyze results on a cell-by-cell basis
- Shorter TAT since tissue does not need to be cultured for metaphase cells

**Limits:**
- Only going to see the region of the genome complementary to your probe
Molecular Inversion Probe (MIP) technology: how it works


Slide courtesy Josh Schiffman
Advantages of MIP technology

- Ability to choose any non-repetitive sequence of interest
- Ability to include hundreds of thousands of probes across genome in single assay
- Ability to specifically determine genotype of SNPs of interest
- Somatic mutations can be built into SNP analysis
- Reduced DNA requirements compared to aCGH: 80 ng vs. 500 ng
- Due to small footprint for binding, works well on FFPE DNA (150 bps intact segments)
- High copy number dynamic range, amplifications detected up to copy number 60
Evaluation of OncoScan (MIP) at ARUP Laboratories

• Cases from clinical lab signed out as positive by FISH
  – Gliomas – 1p/19q deletion - 9 samples
  – Gliomas – EGFR amplification – 8 samples
  – Adenocarcinomas – EGFR amplification – 2 samples
  – Lipomas – MDM2 amplification – 10 samples
  – Neuroblastoma – MYCN amplification – 1 sample
  – Breast – HER2 amplification – 6 samples

  – % tumor estimated from section cut after sections used for DNA extraction (four 20 micron sections for DNA)
  – No microdissection performed
  – Percentage of tumor ranged from 40-90%
Molecular Diagnostics of Gliomas

Value of single test to determine BRAF-KIAA1549 fusion, IDH1/2 mutation status, p53/17p status, 1p/19q status, 9p status, EGFR status, 10q status

Nikiforova and Hamilton, Arch Pathol Lab Med. 2011;135:558–568
1p/19q deletion positive samples

- All 9 were positive for whole arm deletions of 1p and 19q
  - Size of 1p deletion matters, partial 1p deletions has opposite prognostic significance and is often observed in GBMs
- 8/9 were positive for IDH1 mutations (2/2 confirmed)
- None were positive for EGFR amplifications
- None were positive for 10q loss
- None were positive for 9p loss

Multiple lines of evidence from single test to indicate oligoastrocytoma or oligodendroglioma grade II: Good prognosis and expected response to therapy

![Diagram showing the classification of gliomas based on genetic alterations](Diagram.png)
Note whole arm 1p loss and 19q loss – no other copy number changes or LOH
IDH1 R132H mutation clearly identified in 1p/19q del cases

Confirmed by Sanger

20% positive control
Note 1p, 19q, 9p, chr10 and EGFR normal, but copy-neutral LOH of 17p
Case with CN-LOH of 17p also shows mutation of TP53 – LOH is selecting mutation
EGFR Glioma positive cases

- All 8 showed EGFR amplification
- All 8 showed 9p deletion involving CDKN2 (p16)
- All 8 showed loss 10q (PTEN)
- None had IDH1/2 mutations
- None had 1p/19q loss

Multiple lines of evidence from single test to indicate primary glioblastoma multiforme, grade IV
note 1p loss not whole arm, EGFR amplified, CDKN2 and chr10 loss
Adenocarcinomas – EGFR amplification – 2 samples – concordant to MIP data

Lipomas – MDM2 amplification – 10 samples – concordant to MIP data

Neuroblastoma – MYCN amplification – 1 sample – concordant to MIP data
HER2 amplification positive cases
4/6 confirmed gain of HER2
Very focal gain of HER2, can also determine involvement of nearby TOP2A

Case does not contain gain of TOP2A
Case does contain gain of TOP2A - greater levels than Her2
2/6 HER2 cases were discordant – Example 1:
HER2 normal (as is all 17q)….but region around centromere (and all 17p) lost – skews the FISH ratio

OncoScan identified this as a false positive FISH result
Example 2 for HER2 discordant:

FISH was equivocal, reflexed to qPCR and was called positive for gain
Mosaic monosomy 17 observed by array, may have less loss around HER2?

OncoScan identified this as a likely false positive PCR result
False positive FISH results can be due to relative loss around centromere rather than actual gain of HER2

Gunn et al. BMC Cancer 2010, 10:396
Figure 6: Proposed algorithm for establishment of HER2 status in breast cancer samples by protein expression and genomic analysis.

IHC studies for HER2 protein expression are performed as part of the diagnostic workup and confirmed or resolved by FISH. If FISH and IHC results are concordant, or equivocal IHC results are resolved by FISH, no further testing is done. Equivocal cases by FISH and cases with discordant IHC/FISH results are resolved by array CGH.
Testing for melanoma

- 20% of melanocytic lesions signed out as “atypical”
- 25% discordant rate among pathologists for these challenging lesions
- for every melanoma removed in the U.S., 30 benign nevi are removed
- most highly litigated area of pathology

"ABCD" exam

benign nevus

melanoma

punch biopsy

atypical “rule out melanoma”
Characteristic chromosomal gains/losses associated with 95% of melanomas

- frequent chromosomal gains: 1q, 6p, 7p, 7q, 8q, 17q, 20q
- frequent chromosomal losses: 6q, 8p, 9p, 10p, 10q, 11q
- benign nevi do not display these alterations
- Spitz nevi occasionally show isolated gains of 11p, 7q

Study on V1 of Oncoscan

- Sample set of 64 FFPE melanocytic lesions:
  - 23 benign nevi
  - 11 atypical melanocytic lesions of unknown potential (MLUMP)
  - 27 primary melanomas
  - 3 metastatic melanomas
- 98.5% of samples had passing MIP QC scores
- DNA yield 0.35-31 ng/µL (16-1395 ng total DNA)
- FFPE blocks ranged in age from 1-18 years

MIPs show 100% specificity in benign nevi.

Chromosomal gains/losses consistent with melanoma in 89% of unambiguous samples:

- 24/27 primary melanomas
- Gains: 6p (67%), 7q (48%) and 7p (33%)
- Losses: 9p (59%), 9q (56%), 10q (37%), 10p (33%)

- 3/3 metastatic melanomas showed MIP patterns consistent with melanoma.
4/11 (36%) MLUMP samples contained chromosomal gains/losses associated with melanoma

<table>
<thead>
<tr>
<th>Case #</th>
<th>Clinical follow-up findings</th>
<th>MIP result</th>
</tr>
</thead>
<tbody>
<tr>
<td>2752</td>
<td>No evidence of disease</td>
<td>melanoma</td>
</tr>
<tr>
<td>666</td>
<td>No evidence of disease</td>
<td>melanoma</td>
</tr>
<tr>
<td>5963</td>
<td>Multiple other primaries, no recurrences</td>
<td>melanoma</td>
</tr>
<tr>
<td>1585</td>
<td>Lost to follow-up</td>
<td>melanoma</td>
</tr>
<tr>
<td>3030</td>
<td>No evidence of disease</td>
<td>benign</td>
</tr>
<tr>
<td>3408</td>
<td>No evidence of disease</td>
<td>benign</td>
</tr>
<tr>
<td>105</td>
<td>No evidence of disease</td>
<td>benign</td>
</tr>
<tr>
<td>105</td>
<td>No evidence of disease</td>
<td>benign</td>
</tr>
<tr>
<td>5778</td>
<td>No evidence of disease</td>
<td>benign</td>
</tr>
<tr>
<td>9243</td>
<td>1/7 positive SLN, radical neck resection</td>
<td>benign</td>
</tr>
<tr>
<td>4077</td>
<td>Lost to follow-up</td>
<td>benign</td>
</tr>
<tr>
<td>4236</td>
<td>Lost to follow-up</td>
<td>benign</td>
</tr>
</tbody>
</table>

All MLUMP cases treated by resection
Malignant melanoma FISH pattern: multiple copies of RREB1 (red signals)

Normal melanoma FISH pattern: 2 copies of RREB1 (red), MYB (gold) CCND1 (green) 6 centromere (aqua)

In our series, 6 cases of melanoma would have been missed by this FISH panel

Deletion on 6q proximal to region covered by MelanoSITE FISH probe

15.5 Mb deletion proximal to MYB gene would not be detected using the commercially-available FISH probe set
Ewing Sarcoma genome wide copy number on FFPE Samples – Dr. Schiffman

- Individual Ewing’s Patients (n=45)
  - 40 Primary Tumors (PT)
  - 12 Metastatic Lesions (ML)
  - 5 Post Treatment Tumors (RX)
- Normal paired bone marrow, n=31

FFPE Samples diagnosed from 1994-2010

Jahromi
Cancer Genet.
(2012)
FFPE Clinical data available

- Age
- Gender
- Soft tissue vs. Bone
- Tumor Diameter >10 cm
- Location: Axial vs. Appendicular
- Necrosis >90%
- Event Free Survival (EFS)
- Overall Survival

Jahromi
Cancer Genet.
(2012)
Primary Ewing Tumors (N=40)

**GAINS:** 1q (17.5%), 5p (10%), 8p (35%), 8q (37.5%), 12 (12.5%), 20p (10%), 20q (15%), and 21q (10%)

**DELETIONS:** 16q (10%) and 9p21 [CDKN2A] (5%).

## Multiple CNAs and outcome

<table>
<thead>
<tr>
<th>CNAs</th>
<th>Event-free Survival (P-Values)</th>
<th>Overall Survival (P-Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16q24.1 deletion</td>
<td>0.014</td>
<td>0.030</td>
</tr>
<tr>
<td>16q23.3-24.1 deletion</td>
<td>0.041</td>
<td>0.10</td>
</tr>
<tr>
<td>20q13.2 gain</td>
<td>0.00033</td>
<td>1.9e-07</td>
</tr>
<tr>
<td>20q13.13 [CEBPB] gain</td>
<td>0.012</td>
<td>0.00013</td>
</tr>
<tr>
<td>MYC gain</td>
<td>0.0059</td>
<td>1.74e-05</td>
</tr>
<tr>
<td>Trisomy 5</td>
<td>0.01</td>
<td>0.0027</td>
</tr>
<tr>
<td>Trisomy 8</td>
<td>0.032</td>
<td>0.00038</td>
</tr>
<tr>
<td>Trisomy 20</td>
<td>0.012</td>
<td>0.00013</td>
</tr>
</tbody>
</table>
CEBPB and outcome

**Event-Free Survival (EFS)**

- CEBPB Normal (N=34)
- CEBPB Gain (N=6)

\[ p = 0.031 \]

**Overall Survival (OS)**

- CEBPB Normal (N=34)
- CEBPB Gain (N=6)

\[ p = 0.000125 \]
2 female children with unique malformations (first described in 1994)
- thrombocytopenia
- Robin sequence
- agenesis of corpus callosum
- distinctive facies
- developmental delay

21q22.11-12 Microdeletion Syndrome (recently described in 2010, 2011)
- syndromic developmental delay
- thrombocytopenia
- includes RUNX1 gene
Braddock-Carey Syndrome

FFPE liver biopsy from autopsy, 20 years ago!
Braddock-Carey Syndrome

FFPE – Patient 1

Blood – Patient 2

(Sarah South, John Carey)
Molecular Inversion Probe Array for the Genetic Evaluation of Stillbirth Using Formalin-Fixed, Paraffin-Embedded Tissue

Leslie R. Rowe, Harshwardhan M. Thaker, John M. Opitz, Joshua D. Schiffman, Zaid M. Haddadin, Lance K. Erickson, Sarah T. South

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Copy Number Analysis in Neuropathological Diagnostics – a Tool for Robust Classification of Brain Tumors

Torsten Pietsch, M.D.
Professor and Chairman

Department of Neuropathology
Brain Tumor Reference Center

Science Webinar, September 18, 2013
Genomic analysis of archival formalin-fixed, paraffin-embedded (FFPE) tissues:

- Only small amounts of biopsy material may be available.
- The histological material may contain contaminating normal tissue, reactive changes, bleeding and necrosis so that tissue dissection may be needed before DNA extraction and analysis.
- The tumor tissue may be genetically heterogeneous.
- As a consequence of fixation and embedding, DNA derived from FFPE material is usually highly degraded and consists mainly of short DNA fragments.
- Common genomic methods used to analyse high-quality tumor DNA including WGS, WES and SNP arrays do not show sufficient performance on such samples.
DNA quantification:

260 nm versus dsDNA fluorescent dyes

343 DNA samples from FFPE tissues:
Relation varied between 1 : 1.4 and
1 : 160.7, mean 1 : 8.9
Copy number analysis of FFPE brain tumor tissues:

Methods

- Fluorescence in situ hybridization (FISH)
- Multiple ligation-dependent probe amplification (MLPA)
- Methylation array (450k array)
- Molecular inversion profiling (MIP)
WHO Classification 2007
Copy number alterations in brain tumors

- Typical copy number alterations occur in different brain tumor entities and represent markers of diagnostic and prognostic importance.

- For example, a larger fraction of glioblastomas carry amplifications of wild-type or mutated EGFR.

- A biological distinct group of oligodendroglialomas show typical codeletions of chromosomal arms 1p and 19q.

- The most frequent brain tumor in children, medulloblastomas, consists of at least 4 subentities with different copy number alterations.

- In pediatric ependymomas, gain of chromosomal material of 1q has been associated with worse outcome.
Ependymoma
The prognostic impact of histopathological grading of ependymomas varies between different clinical trials.
• In retrospective series, gain of genetic material of chromosome arm 1q was identified to predict worse outcome.

Carter et al., BJC 2002, 86: 929

Mendrzyk et al., CCR 2006, 12: 2070
• In retrospective series, gain of genetic material of chromosome arm 1q was identified to predict worse outcome.

Carter et al., BJC 2002, 86: 929

Mendrzyk et al., CCR 2006, 12:2070

• This marker was mainly assessed by FISH analysis which typically showed failure rates of 15-20 % in archival material.
• In retrospective series, gain of genetic material of chromosome arm 1q was identified to predict worse outcome.

• This marker was mainly assessed by FISH analysis which typically showed failure rates of 15-20 % in archival material.

• To validate this marker in a homogenously treated patient cohort, we analysed chromosome 1q in 206 consecutive cases enrolled into the German multicenter trial HIT2000 in which formalin-fixed, paraffin embedded material was available for DNA extraction.

Carter et al., BJC 2002, 86: 929

Mendrzyk et al., CCR 2006, 12: 2070
Multiple Ligation-dependent Probe Amplification (MLPA)

1. Denaturation and Hybridization
   - PCR primer sequence X
   - PCR primer sequence Y
   - Hybridization sequence (left)
   - Hybridization sequence (right)

2. Ligation
   - Ligase reaction

3. PCR with universal primers X and Y
   - Exponential amplification of ligated probes only

4. Fragment analysis
   - Electrophoretic gel showing amplified fragments
Multiple Ligation-dependent Probe Amplification (MLPA)

1q gain

No 1q gain
Chromosome 1q gain was associated with shorter OS

17% of tumors had gain of chromosome 1q
Glioblastoma multiforme
WHO Grad IV
Multiple ligation-dependent probe amplification (MLPA)

- **Advantages:**
  - Low amounts of genomic DNA needed (25 – 100 ng)
  - Several loci can be analysed simultaneously
  - Fast & robust method, low failure rate
  - Low costs

- **Disadvantages:**
  - Focal alterations may be missed
  - No detection of novel (unknown) alterations
  - Individual probes have to be controlled for their performance with FFPE DNA
  - Reference probes have to be carefully chosen (these loci should not show alterations in the respective tumour type)
**Molecular Inversion Profiling (MIP)**

Probes for 335,000 copy number markers, median probe spacing 2.4 kb

Probes for 541 frequent somatic cancer mutations

Wang et al.. *Cancer Gen.* 2012:205.341
Detection of chromosome 1q gain in ependymomas by molecular inversion profiling (MIP)
Medulloblastoma

Most frequent malignant brain tumor of childhood
age: 0-40 J, peak at 7 years

Localization: Cerebellum
Anamnesis: short
Symptoms: Brain pressure, cerebellar symptoms
approx. 25% c.s.f. seeding at diagnosis

Therapy: OP, chemotherapy, irradiation
Prognosis: 50-60 % long-term survivors
Progenitor cells

- **lower rhombic lip**
  - WNT activation
  - Monosomy 6

- **ventricular matrix**
  - LOH 17p
  - others

- **classical MB**
  - LOH 9q22
  - Hedgehog activation
  - others

- **Desmoplastic/nodular MB**
- **MB with extensive nodularity**

- **anaplastic MB / large cell MB**

- **midline tumors**

- **EGL**
  - LOH 9q22
  - Hedgehog activation
  - others

- **hemispheric or midline tumors**
Future Medulloblastoma Stratification

Surgery

Molecular Phenotype + Clinical Factors

Low Risk: Reduced Therapy

Standard Risk: Standard Therapy

High Risk: Maximal / novel Therapies

3 weeks
\( \beta\text{-catenin} \) nuclear accumulation

20-1571 (mutated)  
60-221 (wt control)
WNT medulloblastomas frequently show monosomy 6
Progenitor cells

lower rhombic lip

ventricular matrix

EGL

Progenitor cells

WNT activation
Monosomy 6
LOH 17p
others
LOH 9q22
Hedgehog activation
others

classic MB

Desmoplastic/nodular MB
MB with extensive nodularity

midline tumors

myc amplification
others

anaplastic MB / large cell MB

hemispheric or midline tumors
Fluorescence in-situ hybridization (FISH)

• Advantages:
  • Copy number analysis of individual cells (tumor heterogeneity)
  • Internal hybridization controls of chromosomes (e.g. centromere probes)

• Disadvantages:
  • Only single loci / genes are analysed
  • Copy number alterations in other regions may be missed
  • Relatively high drop-out rate in FFPE tissue (10-20%)
  • Many nuclei are cut if sections are analyzed
  • Expensive and time-consuming
Detection of c-myc amplification by MIP
Molecular inversion profiling (MIP)

• Advantages:
  • Genome-wide, high resolution copy number analysis of DNA derived from FFPE
  • high dynamic range in copy number estimation (amplifications)
  • genome-wide LOH detection including copy-neutral LOH detection
  • inclusion of probes of known cancer point mutations possible

• Disadvantages:
  • multiple step assay – turn around time
  • costs
Copy number analysis in the differential diagnosis of medulloblastoma

- **Teratoid/rhabdoid tumors**: frequent loss of chromosome 22

- **Ependymoblastomas**: frequent amplification of chromosome 19q
Copy neutral LOH in medulloblastoma

Allele ratio

Copy number
Copy number analysis by methylation array

Robust molecular subgrouping and copy-number profiling of medulloblastoma from small amounts of archival tumour material using high-density DNA methylation arrays

Volker Hovestadt · Marc Remke · Marcel Kool · Torsten Pietsch · Paul A. Northcott · Roger Fischer · Florence M. G. Cavalli · Vijay Ramaswamy · Marc Zapata · Guido Reifenberger · Stefan Rutkowski · Matthias Schick · Melanie Bewerunge-Hudler · Andrey Korshunov · Peter Lichter · Michael D. Taylor · Stefan M. Pfister · David T. W. Jones

Hovestadt et al., Acta Neuropath. 2013
Summary

• Copy number analysis can significantly contribute in the neuropathological diagnostics of brain tumors.

• While FISH can give copy number informations on the single cell level, DNA from FFPE tissue can be used with suitable methods for genome-wide copy number analysis.

• MLPA is a rapid and robust method to analyze several markers in FFPE derived DNA, but the selection of reference probes is critical.

• Copy number information can also be retrieved from methylation array data.

• MIP is suitable for high resolution genome-wide copy number analysis and detection of copy neutral LOH.
Thank you!

neuropath@uni-bonn.de
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Q&A

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