Professor Israel Doniach
1911 – 2001
Doniach Lecture 2015
Molecular Pathology – The Future?

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Outline

• What is Molecular Pathology?

• Three Examples
  – HPV and genital tract neoplasia
  – Genomics and the endometrium
  – Where pathology meets imaging

• What next?
What is Molecular Pathology?

The identification of diagnostically and therapeutically relevant molecular abnormalities in clinical samples.

The identification of diagnostically and therapeutically relevant molecular abnormalities in patients.

The molecular investigation of disease processes.
What is Molecular Pathology?

• Molecular abnormalities
  – Global approaches – usually untargeted and discovery driven
  – Targeted approaches – often used to reduce complexity or for validation
  – Specific approaches – defined targets with specific contextual meanings

• -omics usually refers to global approaches
  – Genomics, epigenomics, transcriptomics, proteomics, metabolomics etc
Diagnostic Molecular Pathology

• Diagnostic Histopathology
  – Surrogate markers e.g. p16
  – ‘Genogenic’ immunohistochemistry
    • Identification of specific mutations e.g. *TP53*, *BRAF*
    • Identification of products of translocation e.g. t(2:5)
    • Identification of therapeutic targets e.g. HER2
      Gown AM Diagnostic Histopathology 2002; 8: 193-200
  – In situ hybridisation
    • FISH/CISH e.g. HER2, translocations, viruses

• Ancillary Molecular Testing
  – PCR-based methods – DNA/RNA
  – ‘omics’ technology
Beyond the Microscope

• Ancillary Molecular Testing
  – PCR-based methods – DNA/RNA
  – ‘omics’ technology
• Non/Pauci-cellular Samples
  – ‘The liquid biopsy’
  – Cell-free DNA
• Molecular Imaging
  – Label-free spectroscopy
  – Tomography
  – Probe-based imaging
Discovery

Validation

Implementation

Global approach
Targeted approach
Serendipity

Assay development
Validation sets
Trials etc

Reproducibility
Quality control
etc
Human Papillomavirus Infection

From molecular biology to prevention and treatment
Human Papillomavirus Infection and Anogenital Disease

- HPV infection is present in 99.7% of invasive cervical carcinomas

- Mucosal HPV infection can also cause vulval and vaginal pre-cancerous lesions and genital warts
History

- 1976: Papillomavirus suggested as cause of cervical cancer (zur Hausen)
- 1983-1984: HPV 16/18 genome cloned from cervical cancers
- 1999: HPV DNA present in 99.7% of cervical cancers
- 2006: Vaccine introduced

http://nobelprize.org/nobel_prizes/medicine/laureates/2008/hausenn-lecture.html
Human Papillomavirus is Common

- Transmitted by intimate contact
- An estimated 80% of sexually active women will be exposed to the virus by age 50
- Most infections will regress spontaneously after 6-12 months
- Over time, persistent infection can lead to cancer and other HPV related diseases
In Women, Peak Exposure to Human Papillomavirus Occurs in the Late Teens and Early Twenties

Overall prevalence by age of any (high- and low-risk) human papillomavirus type (%)
HPV and Squamous Neoplasia

HPV Infection

- De-regulation of E6/E7 (p16 positive)

Low Grade SIL

- Telomerase Activation
- Inhibition of Apoptosis
- Genetic Changes
- Immune Response
- Smoking

High Grade SIL

Invasive Cancer

Normal
Human Papillomaviruses

- Non-enveloped, double-stranded DNA viruses
- 8 kb circular genome
- At least 100 genotypes
- Epitheliotropic
- Associated with warts and neoplasia
Molecular Organisation

Doorbar J Clin Sci 2006;110:525-41
The Papillomavirus Life Cycle

Doorbar J Clin Sci 2006;110:525-41
HPV and Neoplastic Progression

<table>
<thead>
<tr>
<th></th>
<th>HPV -ve</th>
<th>Low and high risk HPV</th>
<th>High risk HPV</th>
<th>High risk HPV</th>
<th>High risk HPV</th>
</tr>
</thead>
</table>

Suprabasal
- differentiating cells
- undifferentiated cells

Basal

Normal → CIN1 → CIN2 → CIN3 → Invasive Cancer

LSIL → HSIL

Modified from Doorbar J Clin Sci 2006;110:525-41
What Governs Progression?

- HPV type
  - High-risk HPV types, particularly 16 and 18
- Persistence of HPV infection
- Up-regulation of E6/E7
- Loss of capacity to replicate viral DNA
- HPV integration
  - Chromosome sites random
  - Viral breakpoint consistent (E1/E2)
HPV and Squamous Neoplasia

HPV Infection

De-regulation of E6/E7 (p16 positive)

Normal

Low Grade SIL

High Grade SIL

Invasive Cancer

Telomerase Activation
Inhibition of Apoptosis
Genetic Changes
Immune Response
Smoking
Clinical Applications

• HPV testing
  – Screening
  – Diagnosis

• Vaccination
  – Prophylactic
  – Therapeutic

• Therapeutic possibilities
HPV Testing

• High quality validated HPV testing methodology is now available

• The 3 main areas of application are:
  – Triage of patients with low-grade abnormalities
  – Primary screening
  – Follow-up after treatment

• But remember:
  – The effect of age
  – The role of type-specific persistence
  – The high negative predictive value
HPV Vaccination

• Prophylactic vaccination
  – HPV 16/18 virus-like particles (VLPs)
  – Prevents incident and persistent infection
  – Prevents cytological abnormality

“Vaccines work, but we need more information before widespread immunisation”

Stanley M *Br J Cancer* 2007; 96: 1320-3
Rationale for Designing a Quadrivalent HPV Vaccine (‘Gardasil’)

Anticipated benefits

- **Main impact:**
  - Pre-cancerous lesions & Cervical Cancer
  - Costs

- **Additional impact:**
  - Other HPV-related diseases
  - Costs
  - Abnormal Pap smears

Types 16 and 18
- 75% Cervical cancer

Types 6 and 11
- 90% genital warts
Factors Affecting Outcome

- Vaccine uptake
- Protocol violation
- Other HPV types
- Existing infection with vaccine HPV types
- Vaccine waning
## Cervix - Combined Analysis of 4 RCTs

**HPV16/18-related CIN2/3 or AIS**

<table>
<thead>
<tr>
<th>Population</th>
<th>Vaccine (N=10291)</th>
<th>Placebo (N=10292)</th>
<th>Efficacy</th>
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<tbody>
<tr>
<td></td>
<td>N</td>
<td>Cases</td>
<td>Rate</td>
</tr>
<tr>
<td>Per-protocol susceptible population</td>
<td>8579</td>
<td>1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>9729</td>
<td>3</td>
<td>&lt;0.1</td>
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<tr>
<td>Intention to treat population</td>
<td>10291</td>
<td>142</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Ault KA. Lancet 2007; 369: 1861-8 - ‘Gardasil’
## Vulva / Vagina - Combined Analysis of 3 RCTs

<table>
<thead>
<tr>
<th></th>
<th>Vaccine (N=9087)</th>
<th>Placebo (N=9087)</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Cases</td>
<td>Rate</td>
</tr>
<tr>
<td><strong>HPV16/18-related VIN2/3 or VaIN2/3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per-protocol susceptible population</td>
<td>7811</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Unrestricted susceptible population</td>
<td>8757</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Intention to treat population</td>
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<td>9</td>
<td>0.03</td>
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<tr>
<td><strong>All lesions</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Intention to treat population</td>
<td>9087</td>
<td>27</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Other Issues

• Other HPV types
  – Reduce efficacy of vaccination
  – May cause disease in vaccinated population
  – Nonavalent vaccine

• Cross-protection (e.g. HPV 45, HPV 31)

• Duration of protection
  – Antibody levels maintained for at least 5 years
  – Level required not clear
Cervarix™, GSK’s cervical cancer vaccine, wins tender for UK national immunisation programme

Issued – Wednesday 18 June 2008, London, UK

GlaxoSmithKline’s (GSK) cervical cancer vaccine, Cervarix™, was today confirmed as the UK Department of Health’s vaccine of choice for its national human papillomavirus (HPV) immunisation programme. The programme, which aims to protect against the two types of HPV that are responsible for approximately 70% of cervical cancers¹, will start in September 2008 and will vaccinate girls aged 12 to 13 each year. There will also be a catch up programme for girls aged 14 up to 18 years, which will be implemented over the next 2-3 years.

Cervix wars - score one for Cervarix

The Department of Health has announced that the national contract for human papillomavirus vaccine (HPV) has gone to Cervarix®. This announcement has also been reported in the general media (BBC, The Times).

As previously noted, Gardasil® has a wider range of licensed indications based on the current Summaries of Product Characteristics (Cervarix and Gardasil). In addition to the wider range of indications, Gardasil is also licensed in a wider age group and for both genders.

The official announcement notes that the decision was based on a wide range of criteria such as their scientific qualities and cost effectiveness but that the price remains commercially confidential.

At a time when the NHS has a significant cash surplus it must be hoped that, for reasons of transparency, the rationale for choosing what appears to be an inferior vaccine are made public.
Vaccination for Condyloma / VIN 1

- Vaccination against HPV 6, 11, 16, and 18 provides strong and sustained protection for up to four years against condyloma and low grade vulvovaginal neoplasia related to these four HPV types.

- In generally HPV naive subjects, vaccination reduced the total burden of condyloma by 83%.

Dillner et al BMJ 2010; 341; c3493
HPV Vaccination

• HPV vaccination works, particularly in susceptible populations
• Effects are modified by:
  – Pre-existing infection
  – Non-vaccine HPV types
  – Protocol violation
• Duration of protection not clear
• Effects on invasive disease are assumed, not proven (yet)
Conclusions

- Persistent ‘high-risk’ HPV infection is strongly associated with cervical neoplasia
- HPV testing is established for ‘test of cure’ and may take over from cytology as the primary screening modality
- HPV vaccination is changing things!
- But vaccination is unlikely to be the whole answer
- HPV and cervical cancer is a paradigm for translational research
Endometrial Carcinoma

A Genomics-Driven Classification?
Endometrial Carcinoma

‘Type I’ tumours

- Endometrioid and mucinous phenotypes
- *PTEN, CTNNB1, KRAS, PIK3CA* mutations
- *PTEN* loss and mutation identifiable in morphologically normal proliferative glands
- Microsatellite instability
  - Germline mutation of MMR genes
  - Promoter hypermethylation esp *hMLH1*
Endometrial Carcinoma

- ‘Type II’ tumours
  - Serous and clear cell phenotypes
  - $TP53$ mutation and overexpression
  - Inactivation of $p16$ and $E$-cadherin
  - $PPP2R1A$ mutation in 41% of serous
    McConechy et al J Pathol 2011; 223: 567-573

- Ambiguous and mixed tumours
  - Overlapping morphological and molecular features
  - More frequently MSI-high
  - Dedifferentiation by acquisition of $TP53$ mutation
    Soslow RA. Histopathology 2013; 62: 89-110
Mutation Spectra Across Endometrial Carcinomas

Mutation Spectra Across Endometrial Carcinomas

Diagnostic Algorithm?

- Tumours associated with *POLE* mutation
  - 65% microsatellite stable, 35% p53 mutant
  - Often high grade and morphologically ambiguous
    Hussein et al Mod Pathol 2015; 28: 505-514
  - Excellent outcome
    Meng et al Gynecol Oncol 2014; 134: 15-19

- Microsatellite unstable tumours
  - MMR protein immunohistochemistry

- Serous-like tumours
  - *TP53* mutation

- Endometrioid tumours
  - None of the above
Where Pathology meets Imaging

‘Molecular’ imaging
Applications in Medicine

• Addition to light microscopy
  – Aids microscopic diagnosis
  – Of specific relevance to clinical pathology

• Application to patients
  – Evaluation of skin lesions
  – Use during endoscopy
Raman scattering

The scattered light carries information on the molecular constituents of the cell: chemical composition, molecular structure and molecular interaction in cells and tissues.

- cancer diagnosis
- rapid identification of pathogenic microorganisms
Addition to Light Microscopy

Cells - Cervical Smear

Tissue - Cervical Biopsy

The ability to interrogate individual cells would provide novel information to enhance diagnosis
Application to Patients

• Skin lesions
  – Easily accessible
  – Correlation with gold standard diagnosis (pathology) easy

• Endoscopy
  – Common procedure
  – Correlation with gold standard diagnosis (pathology) easy

• Enhanced diagnosis could remove the need for tissue biopsy
Raman for Medical Applications

**Pros**

- Non invasive
- No labels, stains or chemical agents required
- Sub-micrometric resolution
- Spectra obtained in the "physiological" state
- Chemical ‘fingerprint’ reflecting biological differences

**Cons**

- only 1 in $10^6$ photons are Raman scattered
- **Raman obscured by strong fluorescence background**
- Data interpretation complex
Raman Micro-spectroscopy of Trapped Particles

Jess et al, *Optics Express* 2006; 14: 5779 - 91
Raman Spectra can be Obtained from Trapped Cells

Jess et al, *Optics Express* 2006; 14: 5779 - 91
Raman Spectra can be Obtained from Stacked, Trapped Cells

Jess et al J Raman Spectrosc 2007; 38: 1082-1088
Raman Spectra can be Obtained from Subcellular Compartments of Single Cells

Jess et al, *Optics Express* 2006; 14: 5779 - 91
PCA can Discriminate between Fixed Cell Types

Figure 4

Jess et al, Int J Cancer 2007; 121: 2723-2728
Lung Cancer

- Similar approach
- Normal bronchial epithelial cells
- Normal cells expressing HPV 16 E7 or cdk4
- BEP2D cells (HPV18-immortalised)
- AsbTB2A cells (BEP2D cells transformed with asbestos fibres)
Lung Cells

AsbTB2A
BEP2D
CDK4 cells
E7 cells
Normal cells

Linear Discriminant Analysis
## Confusion Matrix of Predictive Model

<table>
<thead>
<tr>
<th>%LDA</th>
<th>N</th>
<th>CDK4</th>
<th>E7</th>
<th>BEP2D</th>
<th>TB2A</th>
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<tbody>
<tr>
<td>N</td>
<td>75</td>
<td>11</td>
<td>11</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>CDK4</td>
<td>16</td>
<td>58</td>
<td>18</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>E7</td>
<td>16</td>
<td>5</td>
<td>77</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BEP2D</td>
<td>4</td>
<td>10</td>
<td>3</td>
<td>63</td>
<td>20</td>
</tr>
<tr>
<td>TB2A</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>21</td>
<td>70</td>
</tr>
</tbody>
</table>
Modulated Raman Spectroscopy

- Removes fluorescence
- Periodical modulation of laser excitation wavelength

By using the least-squares fit...

- Range of excitation wavelengths $\Delta \lambda \approx 0.1$nm
- Reduced 1/f noise
- Signal at known modulation frequency
- Applied to cell lines (static and flow) and clinical samples

Proof of Principle...

2 mm-polystyrene bead + Sigma BioChemika NIR fluorescent dye

Raman Intensity (arb. un.)

Raman Shift (cm$^{-1}$)

Total Acquisition Time = 10s

Laser Modulation 1 Hz
Number of Acquisitions 100
Integration time 0.1 s

Chinese Hamster Ovary Cells

Standard Raman
- Acquisition time 200s

Modulated Raman
- Laser Modulation 0.2 Hz
- Integration time 200ms
- Number of acquisitions 1000

Improved Spectral Detail from Bladder Cancer Cell Lines

Improved Discrimination Between Bladder Cancer Cell Lines

Sensitivity 97%
Specificity 72%

Sensitivity 98%
Specificity 95%

Improvement of Acquisition Time
Modulated fibre probe Raman spectroscopy
Probe + Modulation

Standard vs modulated Raman mean spectra derived from adipose tissue

Praveen et al., J. Biomed Optics 2012; 17: 077006
Probe + Modulation

Standard versus modulated Raman signal acquired from bovine bone tissue

Praveen et al., J. Biomed Optics 2012; 17: 077006
[a] Acquisition of Raman spectra from an *ex vivo* bovine liver tissue sample using a fibre Raman probe under ambient light conditions [b] standard Raman spectra and [c] modulated Raman spectra acquired in ambient light.
Wide field Raman Imaging: a snapshot. Proof of concept

Standard bright field imaging

Optical eigenmode compressive imaging (20x4 illuminations)

Standard Raman raster scan (676 illuminations)

Kosmeier et al Optica 2014; 1: 257-63
Multimodal Imaging

- Optical coherence tomography
  - Structural information
- Raman spectroscopy
  - Chemical information

Ashok et al Biomed Opt Express 2013; 4: 2179-86
# Multimodal Imaging

<table>
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<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
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</thead>
<tbody>
<tr>
<td>Raman</td>
<td>89%</td>
<td>77%</td>
<td>82%</td>
</tr>
<tr>
<td>OCT</td>
<td>78%</td>
<td>74%</td>
<td>75%</td>
</tr>
<tr>
<td>Combined</td>
<td>94%</td>
<td>94%</td>
<td>94%</td>
</tr>
</tbody>
</table>

Ashok et al Biomed Opt Express 2013; 4: 2179-86
Summary

• Raman spectroscopy can discriminate between normal and tumour cells
• Modulated Raman spectroscopy improves acquisition time and discrimination accuracy
• Fibre-based modulated Raman spectroscopy shows promise for clinical applications
• Wide-field (eigenmode) Raman imaging opens up the possibility of in vivo tissue imaging
• Multimodal Raman/OCT combined the strengths of both modalities
Discovery

Validation

Implementation

Optical Imaging

Endometrial carcinoma genomics

HPV testing and vaccination
Molecular Pathology

Pathology
The Study and Diagnosis of Disease

Integration

Cell and Tissue Analysis
Molecular Analysis
Imaging
Disease Mechanisms
Disease Diagnosis
Disease Management
Acknowledgements

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