The role of epigenetic modifications in diagnosis and treatment of bladder cancer

Michael Chan, PhD
Department of Biomedical Sciences
Epigenomics and Human Diseases Research Center
National Chung Cheng University
Chia-Yi, Taiwan
National Chung Cheng University
ChiaYi County
250KM from Taipei City (1.5 hrs HSR)
100KM from Kaohsiung (30 mins HSR)
Thanks to Mendel's groundbreaking work in genetics, we now know that peas and men can't breed together, no matter how many times they try.
Epigenetics

Heritable changes that modulate chromatin organization and gene expression without changes in DNA sequence (Riggs et al., 1996)
Prof. Howard Cedar, Hebrew University
The father of DNA methylation

Ancient Bible

Howard Chaim Cedar (Hebrew: חָואָרִיִּים צֶדֶרְרָ; born January 12, 1943) is an Israeli American biochemist who works on DNA methylation, a mechanism that turns genes on and off.

Howard Cedar in 2016

Born
Howard Chaim Cedar
January 12, 1943
(age 79)
New York City, U.S.
• THERAPIST
• THE RAPIST

• KIDS EXCHANGE
• KID SEX CHANGE
DNA Methylation

- Occurs in CG dinucleotide (CpG)
- CpG islands: CG rich region (500bp-2000bp) in the promoter region (of 50% human genes), normally unmethylated
- Methylation of CpG island is associated with transcriptional repression

![Diagram showing the conversion of cytosine to 5-methylcytosine via DNA methyltransferase (DNMT) and demethylase (Tet, 5hmC) reactions involving S-adenosyl methionine (SAM).]
Altered DNA-methylation patterns in tumorigenesis

Normal cell
- Tumour-suppressor gene with promoter CpG island
- ‘Open’ chromatin conformation

Cancer cell
- CpG-island hypermethylation
- ‘Closed’ chromatin conformation

- Entry into cell cycle
- Avoidance of apoptosis
- Defects in DNA repair
- Angiogenesis
- Loss of cell adhesion

- Loss of imprinting and overgrowth
- Inappropriate cell-type expression
- Genome fragility
- Activation of endoparasitic sequences

Unmethylated CpG  Methylated CpG

Tumorigenesis

Nature Reviews | Genetics

Esteller
Nat Rev Genet 2007
Methods for DNA methylation Analysis
<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Analytical step</th>
<th>Locus-specific analysis</th>
<th>Gel-based analysis</th>
<th>Array-based analysis</th>
<th>NGS-based analysis</th>
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<tbody>
<tr>
<td>Enzyme digestion</td>
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<td>• Southern blot</td>
<td>• DMH</td>
<td>• Methyl-seq</td>
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<td>• MCAM</td>
<td>• MCA-seq</td>
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<td>• AIMS</td>
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<td>• mDIP</td>
<td>• mCIP</td>
<td>• mCIP</td>
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<td>• Sanger BS</td>
<td>• BiMP</td>
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<td>• EpiTYPER</td>
<td>• MSP</td>
<td>• GoldenGate</td>
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<td>• Pyrosequencing</td>
<td>• MS-SNuPE</td>
<td>• Infinium</td>
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<td>• COBRA</td>
<td></td>
<td>• WGSBS</td>
<td></td>
</tr>
</tbody>
</table>
Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands

(DNA methylation/tumor suppressor genes/p16/p15)

JAMES G. HERMAN*†, JEREMY R. GRAFF*, SANNA MYÖHÄNEN*, BARRY D. NELKIN*, AND STEPHEN B. BAYLIN*‡

*Oncology Center and ‡Department of Medicine, The Johns Hopkins Medical Institutions, 424 North Bond Street, Baltimore, MD 21231

Communicated by Victor A. McKusick, Johns Hopkins Hospital, Baltimore, MD, June 3, 1996 (received for review April 3, 1996)
Step 1: Denaturation
Incubation at 95°C fragments genomic DNA

Step 2: Conversion
Incubation with sodium bisulfite at 65°C and low pH (5-6) deaminates cytosine residues in fragmented DNA

Step 3: Desulphonation
Incubation at high pH at room temperature for 15 min removes the sulfite moiety, generating uracil

Fragmented Genomic DNA

Cytosine

\[ \text{Cytosine} \rightleftharpoons \text{NaHSO}_3, \text{pH 5.0} \]

\[ \text{Cytosine} \rightarrow \text{SO}_3\text{Na} \] + H$_2$O - NH$_3$

\[ \text{SO}_3\text{Na} \rightarrow \text{OH} + \text{NaHSO}_3 \rightarrow \text{Uracil} \]

5-Methylcytosine (5-mC)

\[ \text{5-mC} \rightarrow \text{NaHSO}_3, \text{pH 5.0} \]

5-mC and 5-hmC (not shown) are not susceptible to bisulfite conversion and remain intact.
Bisulphite-based Methods

Sodium Bisulfite Modification

TUMOR DNA (METHYLATED) vs NORMAL DNA (UNMETHYLATED)

PCR with primers specific for methylated DNA

PCR with primers specific for unmethylated DNA

PAGE, qPCR, microarray, Sequencing/NGS
Urothelial Carcinoma (Bladder Cancer)

- one of the ten most prevalent malignancy in Taiwan (male)

- incidence particularly high in southwestern Taiwan. (Arsenic contamination)

**Diagnosis**:
- imaging (X-ray, CT, IVP)
- urine cytology (sensitivity ~50%)
- cystoscopy

Cooper, Radiographics 2006;
http://www.svuhradiology.ie/case-study/obstructing-bladder-tcc/
Current non-invasive molecular detection for bladder cancer

<table>
<thead>
<tr>
<th>Test</th>
<th>Number of studies analyzed</th>
<th>Median sensitivity [%] (range)</th>
<th>Median specificity [%] (range)</th>
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<td>BTA stat®</td>
<td>36</td>
<td>67 (34–91)</td>
<td>76 (38–96)</td>
</tr>
<tr>
<td>BTA TRAK®</td>
<td>12</td>
<td>63 (17–100)</td>
<td>76 (50–98)</td>
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<tr>
<td>NMP22® Bladder Cancer Test</td>
<td>41</td>
<td>72 (31–100)</td>
<td>80 (43–100)</td>
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<td>NMP22® BladderChek®</td>
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<td>57 (47–85)</td>
<td>86 (40–90)</td>
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<tr>
<td>UBC™-Rapid</td>
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<td>67 (21–84)</td>
<td>80 (49–98)</td>
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<td>HA–HAase</td>
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<td>88 (83–91)</td>
<td>81 (61–93)</td>
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<tr>
<td>UroVysion®</td>
<td>19</td>
<td>73 (13–87)</td>
<td>90 (40–100)</td>
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<tr>
<td>ImmunoCyt®</td>
<td>16</td>
<td>81 (39–100)</td>
<td>75 (62–95)</td>
</tr>
</tbody>
</table>

Mitra and Cote, Nat Rev Urol 2010
Screening of urological cancer by DNA methylation

Figure 1 | Screening for genitourinary cancer. Tumour cells and free tumour DNA from dead cancer cells can access urine through secretion from the prostate into the urethra, and the proximity of urine to the transitional cell lining of the bladder and the renal system. Tumour cells that lack the capacity to metastasize and free tumour DNA can also access the circulatory system. Direct, but invasive, access by needle biopsy is performed for prostate cancer and, less commonly, renal cancer. DNA is isolated from the clinical specimen urine, blood or biopsy, and analysed for the presence of gene methylation by quantitative real-time methylation-specific PCR (qMSP).

Meo et al., Mol Cancer 2017
Hypermethylation of Multiple Genes in Tumor Tissues and Voided Urine in Urinary Bladder Cancer Patients

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FREQUENT HYPERMETHYLATION OF PROMOTER REGION OF RASSF1A IN TUMOR TISSUES AND VOIDED URINE OF URINARY BLADDER CANCER PATIENTS

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²Department of Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

RESEARCH ARTICLE

Distinct DNA methylation epigenotypes in bladder cancer from different Chinese sub-populations and its implication in cancer detection using voided urine

Pi-Che Chen¹, Ming-Hua Wu¹, Wei Huang¹, Chung-Yu Lo¹, Chun-Jen Chen¹, and De-Ching Chang²
¹Department of Urology, Division of Medical Oncology, Cancer Research Institute, National Taiwan University Hospital, Taipei, Taiwan, R.O.C.
²Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan, R.O.C.

Methylomics analysis identifies ZNF671 as an epigenetically repressed novel tumor suppressor and a potential non-invasive biomarker for the detection of urothelial carcinoma

Chia-Ming Yeh¹,²,*, Pi-Che Chen³,*, Hsiao-Yen Hsieh²,⁵, Yeong-Chin Jou³, Chang-Te Lin³, Ming-Hsuan Tsai¹, Wen-Yu Huang¹,², Yi-Ting Wang¹,², Ru-Inn Lin¹,⁶, SZu-Shan Chen¹,², Chun-Liang Tung³, Shu-Fen Wu¹,², De-Ching Chang¹,², Cheng-Huang Shen³, Cheng-Da Hsu⁵ and Michael W.Y. Chan¹,²,*
Methylation analysis in bladder cancer tissues and urines by MSP
Table 8  Comparison of sensitivity and specificity between methylation markers and cytology

<table>
<thead>
<tr>
<th></th>
<th>Methylation markers(^a)</th>
<th>Cytology</th>
<th>DAPK</th>
<th>RARβ</th>
<th>E-cadherin</th>
<th>p16</th>
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<tbody>
<tr>
<td><strong>Sensitivity (%)</strong></td>
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<tr>
<td>All cases</td>
<td>90.9</td>
<td>45.5</td>
<td>45.5</td>
<td>68.2</td>
<td>59.1</td>
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<td>Grade 1</td>
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<td>11.1</td>
<td>55.5</td>
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<td>69.2</td>
<td>38.4</td>
<td>69.2</td>
<td>53.8</td>
<td>7.6</td>
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<tr>
<td><strong>Specificity (%)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>All cases</td>
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<td>Positive predictive value (%)</td>
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<td>All cases</td>
<td>83.3</td>
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<td>100</td>
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<td>100</td>
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<tr>
<td>Grade 1</td>
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<td>100</td>
<td>100</td>
<td>60.0</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Grade 2–3</td>
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<td>100</td>
<td>100</td>
<td>69.2</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Negative predictive value (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>All cases</td>
<td>86.6</td>
<td>58</td>
<td>58.6</td>
<td>65.0</td>
<td>65.3</td>
<td>47.2</td>
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<tr>
<td>Grade 1</td>
<td>100</td>
<td>68</td>
<td>80.9</td>
<td>81.3</td>
<td>85</td>
<td>70.8</td>
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<tr>
<td>Grade 2–3</td>
<td>86.6</td>
<td>80.9</td>
<td>68.0</td>
<td>76.4</td>
<td>73.9</td>
<td>58.6</td>
</tr>
</tbody>
</table>

\(^a\) Any one of the genes showed methylation in urine samples.

\(^b\) Cases where either DAPK showed methylation or cytology diagnosed as cancer or suspicious.
Methylomics analysis in bladder cancer tissue by Illumina 450K methylation microarray

Yeh et al, Oncotarget 2015
ZNF671 is epigenetically silenced by DNA methylation in bladder cancer cell lines

Yeh et al, Oncotarget 2015
Ectopic expression of ZNF671 suppress tumor growth *in vitro* and *in vivo*

Colony formation assay

Invasion assay

Yeh et al, Oncotarget 2015
ZNF671 is epigenetically silenced in urothelial carcinoma patient samples

Yeh et al, Oncotarget 2015
Patients with higher ZNF671 methylation is associated with recurrence

Yeh et al, Oncotarget 2015
Table 5: Sensitivity and specificity of cancer detection using voided urine samples

<table>
<thead>
<tr>
<th></th>
<th>IRF8</th>
<th>SFRP1</th>
<th>ZNF671</th>
<th>IRF8 or SFRP1</th>
<th>ZNF671 or IRF8</th>
<th>ZNF671 or SFRP1</th>
<th>aMarker panel</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity(%)</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>All cases (n=26)</td>
<td>61.5%</td>
<td>50.0%</td>
<td>57.7%</td>
<td>88.4%</td>
<td>80.8%</td>
<td>84.6%</td>
<td>96.2%</td>
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<td>Low grade (n=10)</td>
<td>50.0%</td>
<td>60.0%</td>
<td>40.0%</td>
<td>90.0%</td>
<td>60.0%</td>
<td>80.0%</td>
<td>90.0%</td>
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<td>High grade (n=16)</td>
<td>68.8%</td>
<td>43.8%</td>
<td>68.8%</td>
<td>87.5%</td>
<td>94.1%</td>
<td>87.5%</td>
<td>100%</td>
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<td>Primary (n=22)</td>
<td>68.2%</td>
<td>45.4%</td>
<td>54.5%</td>
<td>86.3%</td>
<td>81.8%</td>
<td>81.8%</td>
<td>95.4%</td>
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<td>Recurrent (n=4)</td>
<td>25.0%</td>
<td>75.0%</td>
<td>75.0%</td>
<td>100%</td>
<td>75.0%</td>
<td>100%</td>
<td>100%</td>
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<tr>
<td><strong>Specificity(%)</strong></td>
<td>94.7%</td>
<td>94.7%</td>
<td>89.5%</td>
<td>89.5%</td>
<td>84.2%</td>
<td>89.5%</td>
<td>84.2%</td>
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<tr>
<td><strong>Positive predictive value (%)</strong></td>
<td>94.1%</td>
<td>92.8%</td>
<td>88.2%</td>
<td>92.0%</td>
<td>87.5%</td>
<td>91.6%</td>
<td>92.6%</td>
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<tr>
<td><strong>Negative predictive value(%)</strong></td>
<td>64.2%</td>
<td>58.0%</td>
<td>60.0%</td>
<td>85.0%</td>
<td>76.2%</td>
<td>80.9%</td>
<td>94.4%</td>
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</tbody>
</table>

*a* Any one of these genes (IRF8, SFRP1 or ZNF671) showed methylation in urine samples.
Summary I

• Combination of DNA methylation biomarkers, including ZNF671, can be a sensitive non-invasive tool for bladder cancer detection and recurrent monitoring.
Treatment of Urothelial Carcinoma (Bladder Cancer)

- **Intravesical chemotherapy**
  - BCG
  - Mitomycin-C
  - Cisplatin

- **Radiotherapy**

- **Immune checkpoint inhibitors**
  (only for advanced cancer with PD-L1 expression ≥ 5%)

Cooper, Radiographics 2006;
http://www.svuhradiology.ie/case-study/obstructing-bladder-tcc/
Cyproheptadine (CPH, Periactin)

I. The first-generation anti-histamine serotonin antagonist

II. Treatment of allergic reactions
   - Hay Fever (1950s)
   - atopic dermatitis
   - appetite stimulant
   - anorexia
   - dyspeptic symptoms
   - migraine prophylaxis
   - antidepressant
   - melatoninergic properties
   - anti-cancer effect

III. Common side effects
   - drowsiness
Case 1: Advanced HCC patient with lung metastasis, prescribed with Thalidomide (50mg BID) and Cyproheptadine (for skin itching, 4mg TID) for 6 months: complete remission of tumors in lung and liver.
Cyproheptadine significantly improves the overall and progression-free survival of sorafenib-treated advanced HCC patients

Yu-Min Feng¹, Chin-Wen Feng², Chin-Li Lu³, Ming-Yang Lee⁴, Chi-Yi Chen¹, and Solomon Chih-Cheng Chen³,⁵,*

Overall Survival

Progression-free survival

P=0.017

P=0.004
Selective anti-cancer effect of CPH in bladder cancer 

*in vitro* and *in vivo*

Hsieh et al., Cancer Lett 2016
CPH induced G1 arrest in bladder cancer

Hsieh et al., Cancer Lett 2016
IRF6 is overexpressed in bladder cancer cells treated with CPH or epi-drugs.

**BFTC905 RNA Seq data**

- Control
- CPH 24hr

**IRF6 level in UC cell lines**

- SV-HUC1
- UMUC3
- J82
- TCCSUP
- TSGH8301
- BFTC905
- HT1376

**UC cell lines treated with CPH**

- DMSO
- CPH 24hr
- CPH 48hr
- CPH 72hr

**IRF6 level in UC cell lines**

- DMSO
- TSA
- 5aza
- 5aza+TSA

* P<0.05
** P<0.01
*** P<0.001
Treatment of CPH or epi-drugs induced DNA hypomethylation in IRF6 promoter

IRF6-Pyro in UC cell lines

IRF6 Methylation level %

UMUC3-DMSO
UMUC3-CPH 24hr
UMUC3-CPH 48hr

IRF6 Methylation level %

UMUC3-DMSO
UMUC3-TSA
UMUC3-5AZA
UMUC3-5AZA+TSA
Overexpression of IRF6 inhibited tumor growth in vivo

** p<0.01
** **

Tumor volume (mm$^3$)

*** p<0.001

Tumor Weight (g)

*** p<0.001
IRF6 is epigenetically silenced in high-staged bladder cancer patients

IRF6 promoter CpG island

TCGA bladder cancer (n=382)

Correlation between IRF6 methylation and expression

\[ r = -0.3 \]
\[ p < 0.0001 \]

CG16030177 methylation (β-value)
Summary II

• IRF6 is a potential tumor suppressor that is epigenetically silenced in bladder.
• CPH induced IRF6 promoter hypomethylation in bladder cancer cells
Treatment of Urothelial Carcinoma (Bladder Cancer)

- **Intravesical chemotherapy**
  - BCG
  - Mitomycin-C
  - Cisplatin

- **Radiotherapy**

- **Immune checkpoint inhibitors**
  (only for advanced cancer with PD-L1 expression $\geq 5\%$)

Cooper, Radiographics 2006;
http://www.svuhradiology.ie/case-study/obstructing-bladder-tcc/
The Nobel Prize in Physiology or Medicine 2018 was awarded jointly to James P. Allison and Tasuku Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation."
Figure 2 Immune checkpoint blockade

Table 1. FDA approvals of anti-PD-1/PD-L1 immunotherapeutic drugs in bladder and other cancers

<table>
<thead>
<tr>
<th></th>
<th>Atezolizumab</th>
<th>Durvalumab</th>
<th>Avelumab</th>
<th>Nivolumab</th>
<th>Pembrolizumab</th>
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</thead>
<tbody>
<tr>
<td>May 2016, pre-treated AMUC (bladder cancer)</td>
<td><strong>May 2017, pre-treated advanced/metastatic (bladder cancer)</strong></td>
<td><strong>March 2017, metastatic Merkel cell carcinoma</strong></td>
<td><strong>December 2014, advanced melanoma</strong></td>
<td>September 2014, advanced melanoma</td>
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<td></td>
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<td>May 2016, Hodgkin lymphoma</td>
<td>October 2016, first line treatment of metastatic NSCLC</td>
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<td>November 2016, head and neck cancer</td>
<td>March 2017, classical Hodgkin lymphoma</td>
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<td><strong>February 2017, pre-treated AMUC (bladder cancer)</strong></td>
<td><strong>May 2017, AMUC (bladder cancer)</strong></td>
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<td>August 2017, metastatic colorectal cancer with MSI or MMR deficiency</td>
<td>May 2017, any solid cancer with MSI or MMR deficiency</td>
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<tr>
<td></td>
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<td>September 2017, pre-treated hepatocellular carcinoma</td>
<td>September 2017, pre-treated advanced/metastatic gastric, gastroesophageal cancer</td>
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<tr>
<td></td>
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<td>August 2018, pre-treated SCLC</td>
<td>June 2018, pre-treated advanced/metastatic cervical cancer</td>
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<tr>
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<td>June 2018, pre-treated PMBCL</td>
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</tr>
</tbody>
</table>

AMUC = Advanced/Metastatic UC
Avelumab Maintenance Therapy for Advanced or Metastatic Urothelial Carcinoma


NEJM 383:13  NEJM.ORG  SEPTEMBER 24, 2020

JAVELIN Bladder 100 trial (NCT02603432)
A Overall Population

Median Overall Survival (95% CI)

Avelumab: 21.4 (18.9–26.1) mo
Control: 14.3 (12.9–17.9) mo

Stratified hazard ratio for death, 0.69 (95% CI, 0.56–0.86)
P = 0.001

No. at Risk
Avelumab: 350 342 318 294 259 226 196 167 145 122 87 65 51 39 26 15 11 5 3 0
Control: 350 335 304 270 228 186 153 125 105 83 68 55 41 33 18 12 9 2 1 0
Editorial

Firing up Cold Tumors - Targeting the Epigenetic Machinery to Enhance Cancer Immunotherapy

Guan-Ling Lin¹,², Leah H.J. Tsai¹,³, Peter J.K. Kuppen³,⁴, and Michael W.Y. Chan¹,²,*

2021 May, Vol 5 Issue 2
Combinatorial Epigenetic and Immunotherapy in Breast Cancer Management: A Literature Review

Yu-Ting Lee, Yu-Ming Chuang, and Michael W. Y. Chan

2020 December, Vol 4 Issue 4
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