TS Genes: Overview

• How are TS genes lost from tumor progenitor cells?
• How have TS genes been identified?
• Diverse functions of TS genes; what are the types of data?
Retinoblastoma: “Two-hits”

Familial inherited/multifocal

Sporadic not inherited/unifocal

Chromosome 13

Cells of child

Retinal cells

Retinoblastoma 30,000 fold increased incidence

Overall: $10^{-12}$
Retinoblastoma

thickening of optic nerve
due to extension of tumor

displaced normal retina
retinoblastoma
Pathways for Loss of TS Genes

First ‘hit’: germline or somatic mutation

Second ‘hit’
- Mutation on 2nd allele (rare)
- Loss of whole chromosome
- Mitotic recombination: loss of heterozygosity
Identifying TS Genes

- Early method: search for LOH using polymorphic DNA markers along the chromosome (general evidence for a TS locus)
- Search for homozygous DNA deletions and mutations (more specific evidence)
- Current method – total genome or exome sequencing – look for *homozygous* mutations
Example: the p16 *(CDKN2A)* TS Gene

• LOH mapping: Chr.9p

• Homozygous DNA deletions in cancer cell lines; delimited the general region of the chromosome.

• Somatic mutations in some primary cancers (brain tumors, bladder CA, etc.); directly pinpointed p16 as the key gene.

• Germline mutations in familial melanoma

• p16 encodes a cyclin-cdk inhibitor (regulates the cell cycle).
The p16 Gene in Glioblastomas

LOH

Homozygous Deletions

Point Mutation
Environmental insults; DNA damaging agents; viruses

Inherited mutations in genes affecting DNA repair

Normal cell

DNA damage

Factors affecting CpG methylation

Mutations in the genome of somatic cells

Activation of proto-oncogenes

Alterations in genes that regulate apoptosis

Inactivation of tumor suppressor genes

Tumor progression; genomic instability

Malignant neoplasm
Cancer Epigenetics

The two main components of the epigenetic code

DNA methylation

Histone modifications
DNA Methylation: Heritable Gene Silencing

S-phase: methylation of daughter strands
Epigenetic silencing of tumor suppressor genes by CpG methylation can be quite stable, and is similar in this regard to mutational silencing.

First ‘hit’: mono-allelic DNA methylation

Second ‘hit’: bi-allelic DNA methylation

Methylation on 2nd allele

Loss of whole chromosome

Mitotic recombination: loss of heterozygosity
Epigenetic Silencing of p16: Reactivation by 5aza2‘deoxy-C

Fig. 3  Re-expression of transcriptionally silenced p16 after treatment with 5-deoxyazacytidine in cancer cell lines.  a, Southern blot analysis...
Mapping CpG Methylation: Bisulfite Sequencing

RASSF1A

Normally arrests the cell cycle or promotes apoptosis; when it is silenced by CpG methylation of the gene promoter, the cancer cells proliferate faster.
Newest discoveries: epigenetic “reader” and “writer” genes are frequently *mutated* in human cancers

<table>
<thead>
<tr>
<th>Histone modifications</th>
<th>CpG methylation pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MLL1/2/3</em></td>
<td><em>DNMT3A</em></td>
</tr>
<tr>
<td><em>EZH2</em></td>
<td><em>TET1/2</em></td>
</tr>
<tr>
<td><em>KDM6A</em></td>
<td><em>IDH1/2</em></td>
</tr>
<tr>
<td>(leukemias, carcinomas, medulloblastomas)</td>
<td>(brain tumors, leukemias, etc.)</td>
</tr>
</tbody>
</table>

**Chromatin remodelers**

| **SNF5**               |
| **ARID1A**             |
| (rhabdoid tumors, ovarian CA, etc.) |
## Diverse Functions of TS Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>Rb and p16</strong></td>
<td>Restrain the cell cycle</td>
</tr>
<tr>
<td><strong>p53 and ARF</strong></td>
<td>Checkpoints for DNA damage, hypoxia, oncogenes</td>
</tr>
<tr>
<td><strong>E-cadherin</strong></td>
<td>Membrane protein; promotes cell-cell adhesion</td>
</tr>
<tr>
<td><strong>VHL</strong></td>
<td>Cytoplasmic protein: ubiquitination pathway</td>
</tr>
<tr>
<td><strong>NF-2</strong></td>
<td>Cytoskeletal protein</td>
</tr>
<tr>
<td><strong>TSC-2</strong></td>
<td>Antagonizes the mTOR pathway</td>
</tr>
<tr>
<td><strong>NF-1</strong></td>
<td>GAP homology; antagonizes Ras signaling</td>
</tr>
<tr>
<td><strong>PTEN</strong></td>
<td>Phosphatase; AKT pathway antagonist</td>
</tr>
<tr>
<td><strong>APC</strong></td>
<td>Cytoplasmic; antagonizes Wnt signaling</td>
</tr>
<tr>
<td><strong>PTC</strong></td>
<td>Membrane protein; antagonizes Hedgehog signaling</td>
</tr>
<tr>
<td><strong>WT1</strong></td>
<td>Transcription factor; kidney differentiation</td>
</tr>
<tr>
<td><strong>BRCA1 and 2; MLH1</strong></td>
<td>Roles in DNA recombination and repair</td>
</tr>
</tbody>
</table>
Prototype TS Proteins: Rb and p53

**Rb**

- Gene cloned by a classical positional approach (1986)
- restrains cell proliferation and induces terminal differentiation in normal tissues
- affects transcription as a co-factor, but does not bind directly to DNA

**p53**

- Found as a protein ("tumor antigen") associated with tumor virus infection of human and mouse cells
- activates a “stress checkpoint” that blocks cell proliferation or induces apoptosis in cells that have undergone DNA damage, pathological stimuli for proliferation, or hypoxia
- activates transcription of specific target genes by binding directly to DNA of their promoter regions
Normal cell

Cell with p53 mutation/loss

Stress: DNA damage, hypoxia, oncogene activation

p53 protein is stabilized and activated

p53 activates stress-response/repair genes

Growth arrest

Successful damage repair

Normal cell

Apoptosis (cell death)

Unsuccessful repair

No response

Apoptic pathway is disabled; persistent DNA damage promotes genomic instability

Accumulation of mutations; Malignant cell
Stabilization of p53 protein by DNA damage

- Accumulation of nuclear p53 (seen with anti-p53 antibody) after DNA damage by UV- or X-irradiation.

- The increase is due to stabilization of the p53 protein against ubiquitin-mediated proteolysis; not to transcriptional activation of the p53 gene.
ARF/p16: 2 TS genes at one locus

- p16 regulates Rb function.
- In contrast, the p19ARF protein functions as a tumor suppressor not by inhibiting cyclin-cdk enzymes, but rather by stabilizing p53, via inhibition of the Mdm2 ubiquitin ligase.
Induction of p14ARF by E2F-1

p19ARF is particularly important as a ‘sentinel gene’ that induces p53-dependent apoptosis as a checkpoint response in cells which have been partially transformed by activation of dominant oncogenes, such as mutated Ras genes, over-expressed Myc gene, or other growth stimuli that induce E2F-1. This type of checkpoint is distinct from the checkpoint induced by DNA damage but, remarkably, it still functions through the p53 effector.
Central TS Protein Interactions
Linking p53 and Rb

- p14Arf
- MDM2
- E2F-1
- p53
- CyclinD/Cdk4, 6
- p16

- Transcriptional activation
- Phosphorylation
- Ubiquitin-mediated proteolysis
Genetic Interaction: p53 and pRb

Mouse Retina

Rb⁺ p53⁺

Rb⁻ p53⁺

Rb⁻ p53⁻
Proto-oncogenes and TS Genes in Differentiation Signaling Pathways
Proto-oncogenes and TS Genes in Differentiation Signaling Pathways

“A signaling pathway that normally responds to an extracellular ligand becomes locked into a ligand-independent state of constant activation in cancer precursor cells.

The fully developed cancer therefore grows in a nutrient-limited, rather than signal-limited fashion.”
Adenomatous Polyposis Coli

Biallelic loss of the APC tumor suppressor gene
Mechanism of Action of APC

Identify protein-protein interactions

Beta-catenin

Wnt/Wg signaling pathway

Drosophila genetics  Mammalian cells  Xenopus development
Wnt/APC/β-catenin Pathway

No Wnt signaling, no CTNNB1 mutation; β-catenin degraded

β-TrCP complex

+ Wnt signaling or CTNNB1 mutation; β-catenin stabilized

β-catenin/TCF4

Target Genes → ON

TCF sites

Groucho/HDAC repressive factors displaced from promoter region
APC Gene: TS (inhibitor) in the Wnt signaling pathway

General Principle:

• Signaling pathways that normally respond to extracellular ligands become locked into a ligand-independent state of constant activation in cancer cells

• This concept applies to both proliferation signaling (e.g. RAS; oncogenic pathway) and differentiation signaling (e.g. WNT, HH, TGFβ; tumor suppressor pathways)
APC Mutations in Colon Cancers
Wnt/APC/β-catenin Pathway: key biochemical features

General principles:

- protein-protein interactions
- Phosphorylation events
- ubiquitin-mediated proteolysis
$\beta$-catenin Phosphorylation and Proteolysis

$\beta$-catenin

GSK3b

APC

Axin

Ub

P

26S proteasome

$\beta$-catenin degraded

CK1a

GSK3b

APC

Axin

TRCP (SCF)

ubiquitination

$\beta$-catenin degraded.
Patched (PTC) TS gene: hedgehog (HH) Signaling Pathway in Skin Cancers and Medulloblastomas

Hedgehog-family proteins

• Extracellular ligands essential for normal development in both invertebrates and vertebrates

• Signal transduction from HH ligands occurs through a series of downstream inhibitory components at the cell membrane and in the cytoplasm
Hh mutation in Drosophila

Figure 1. Ventral cuticular pattern of Drosophila larvae. **A:** Wild-type denticle pattern. **B:** Hedgehog mutant denticle pattern. Figure adapted from Nusslein-Volhard C, Wieschaus E. 1980 Nature 287:795–801 with permission of Nature Publishing Group.
Hedgehog signaling in Drosophila

Hedgehog → Smo → PKA

PTC

βTRCP (SCF)

ubiquitination

26S proteasome (partial proteolysis)

Ci155: transcriptional activator (GLI proto-oncogene in humans)

Ci75: repressor of growth genes
Basal Cell Carcinoma
PTC mutations in basal cell CA
Sonic Hedgehog Signaling in Mammals

- Net affects on the levels of the \textit{oncogenic GLI-1} transcription factor (and other GLI family members; Ci homologues)

- \textit{increase in GLI-1 expression} (oncogenic) after exposure of normal cells to Shh and in cancer precursor cells that \textit{lack PTC} or that have \textit{activating mutations in Smoothened}
Skin Tumors in Transgenic Mice Over-expressing $GLI1$

a, c) basal cell carcinoma

e) cylindroma

g) trichoblastoma

mouse  human
Medulloblastoma-like abnormalities in $Ptc$ KO mice
DPC4/SMAD4: TGF-beta Pathway

- $T\beta$RII mutations
- $DPC4$ mutations
  - colon CA
  - pancreatic CA

*Parsons lecture; Tumor Suppressors 2*
VHL: TS Gene Directly Controlling Ubiquitination

Hemangioblastoma (cerebellum)
Von-Hippel Lindau (VHL) Disease

Multiple cysts and tumors in the kidney
VHL Disease

Progressive malignant transformation of the renal cyst epithelium
Classes of Kidney Cancer

Human Renal Epithelial Neoplasms

<table>
<thead>
<tr>
<th>Type</th>
<th>Clear Cell</th>
<th>Papillary Type 1</th>
<th>Papillary Type 2</th>
<th>Chromophobe</th>
<th>Oncocytoma</th>
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</thead>
<tbody>
<tr>
<td>Gene</td>
<td>VHL</td>
<td>Met</td>
<td>FH</td>
<td>BHD</td>
<td>5%</td>
</tr>
<tr>
<td>Percentage</td>
<td>75%</td>
<td>5%</td>
<td>10%</td>
<td>5%</td>
<td>5%</td>
</tr>
</tbody>
</table>

[Linehan et al., 2003]
VEC Complex Homologous to SCF
Targeting of Hif1α by the VEC Complex

Hypoxia-inducible factor 1-alpha: Key factor inducing VEGF
VHL: Summary

Formation of vascular tumors in VHL disease is explained by the deficiency in ubiquitin-dependent degradation leading to increased levels of HIF1α...

• but to explain the dysregulated cell growth in this disorder, there may be additional important substrates of the VHL-containing ubiquitin-conjugating complex.

• There are several other “kidney cancer TS genes” that point to metabolic regulation as the key abnormality in this type of cancer (See article on web site).
5 minute break
Von-Recklinghausen’s Disease: Neurofibromatosis Type-1
Neurofibromatosis Type-1 (NF1)

Most common inherited tumor syndrome (1:3500)

- Neurofibromas (benign nerve tumors)
- Neurofibrosarcomas (malignant peripheral nerve sheath tumors - MPSNT)
- Optic nerve gliomas
- Pheochromocytomas (adrenal tumors)
- Leukemias
- Café-au-lait spots (benign melanocytic lesions)
NF1 Gene: Ras-GAP
Mouse Model of Neurofibromatosis

$p53$ het; $Nf1$ het

$\rightarrow$

LOH in most tumors
**NF2** TS Gene*:
“Central Neurofibromatosis”-Brain Tumors

*Not related to NF1*
Nf2/Merlin: Function

ERM family: ezrin, moesin, radixin
  • link membrane receptors to the cytoskeleton

• Suppression of cell motility
• Suppression of Rac signaling
• Down-regulation of multiple signaling receptors
TS Genes in Familial Cancers

- Wilms tumor: *WT1* (Denys-Drash and WAGR syndromes)
- Breast cancer: *BRCA1, BRCA2* (familial breast and ovarian cancer)
  - Classical 2-hit mechanism; but mutations largely restricted to *familial/syndromic* cases
  - Cancer predisposition based on genetic aberrations during organ development?
Wilms: pediatric kidney tumor arising from metanephric mesenchyme
Histology of the Fetal Kidney
Histology of Wilms Tumor

Blastemal Component

Epithelial Component
Genetic Predisposition to Wilms Tumor

Denys-Drash syndrome
- Genitourinary malformations
- Renal failure (glomerulopathy)
- Wilms tumor

WAGR syndrome
- Wilms tumor
- Aniridia
- Genitourinary malformations
- Mental retardation

WT1 mutations

WT1 deletions
Expression of WT1 in Development
Wt1 Is Essential for Kidney Formation
**WT1 Mutations at Sites of DNA Contact**

A compilation of WT1 point mutations in the zinc finger region in patients with Denys-Drash syndrome.
Cooperative *WT1* and *CTNNB1* mutations in some Wilms Tumors

WT1 loss-of-function  →  nephrogenic rests

WT1 loss-of-function  →  Wnt/beta-catenin activation

Wilms tumor
Beta-catenin (\textit{CTNNB1} gain-of-function) mutations are restricted to \textit{WT1}-mutant Wilms tumors

Columbia U. series of 36 Wilms tumors sequenced for \textit{WT1} and \textit{CTNNB1}:

• 0/18 \textit{CTNNB1} mutations (0\%) in \textit{WT1}-wild type Wilms tumors (all sporadic: “\textbf{Class 1}”)

• 14/18 \textit{CTNNB1} mutations (77\%) in \textit{WT1}-mutant Wilms tumors (17 syndromic; 1 sporadic: “\textbf{Class 2}”)

Li et al., AJP, 2004
Wnt/beta-catenin pathway

No Wnt signaling, no CTNNB1 mutation; beta-catenin degraded

With Wnt signaling or CTNNB1 mutation; beta-catenin stabilized

Target Genes → ON

Groucho/HDAC repressive factors displaced from promoter region
Hypothesis: Loss of WT1 is cell-lethal; activation of the Wnt/beta-catenin pathway rescues WT1-null Wilms tumor precursor cells

- Gene expression profile of WT1-null Wilms tumors (Class 2) should differ from that of WT1-wild-type Wilms tumors (Class 1)

- WT1 and beta-catenin target genes should be enriched among the differentially expressed genes

- Activation (stabilization) of beta-catenin in a mouse model should cause Class 2 Wilms tumors – necessary to also delete Wt1?
Genetics of Class 2 Wilms Tumors

WT1 loss-of-function → nephrogenic rests

WT1 loss-of-function

Wnt/beta-catenin activation; activation of downstream oncogenic target genes...

Wilms tumor
Separating Class 1 from Class 2 Wilms tumors by expression profiling

K-Means Cluster 3

K-Means Cluster 4

P.001  Δ > 3-fold

WT1-positive Class 1

WT1-null Class 2

Li et al., 2004
Northern blot validations

Wilms Tumors

<table>
<thead>
<tr>
<th>FK</th>
<th>Class 1</th>
<th>Class 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>5 12</td>
<td>3 13</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>2 14</td>
</tr>
<tr>
<td>18</td>
<td>19 18</td>
<td>17 15</td>
</tr>
<tr>
<td>16</td>
<td>21 23</td>
<td>24</td>
</tr>
</tbody>
</table>

18S

MEOX2

ID4

FGFR2

β-actin

Wilms Tumors

<table>
<thead>
<tr>
<th>FK</th>
<th>Class 1</th>
<th>Class 2</th>
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<td>17 15</td>
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<tr>
<td>16</td>
<td>21 23</td>
<td>24</td>
</tr>
</tbody>
</table>

28S

PAX2

WIF1

HAS2

β-actin
Activation of beta-catenin in embryonic kidney organ culture

CreER;Ctnnb1 Δex3

cultured kidney

H&E
Strategy to find bona fide Wnt/beta-catenin target genes relevant to human Wilms tumor formation

- Genes activated in beta-catenin* mouse kidney tumors
- Human Class 2 Wilms tumor signature genes
ITF2/E2-2: an interesting target gene for beta-catenin in Wilms tumors
Epigenetics: the *WT2* Wilms Tumor Locus

- frequent (~45%) LOH for 11p15.5 markers
- invariable loss of maternal/duplication of paternal markers
- epigenetic lesions at *H19/IGF2*
- *WT2* overlaps with the BWS locus

Chromosome 11p13; *WT1*

- single classical tumor suppressor gene (*WT1*)
- definite role in a minority of cases of WT
- *WT1* not imprinted in kidney
Reciprocal Expression of *H19* and *IGF2* in Normal Tissues
Silencing of $H19$ and biallelic Expression of $IGF2$ in Wilms Tumor
LOH of Chr11p15 in Wilms Tumor

Pat

Me-CpG

Pat

Me-CpG

Expressed

Silenced

IGF2

H19

Enhancer
**H19 DMR methylation precedes tumor formation**

<table>
<thead>
<tr>
<th>WT-A</th>
<th>Ki-A</th>
<th>Nml Ki</th>
</tr>
</thead>
<tbody>
<tr>
<td>R+C+H</td>
<td></td>
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</tbody>
</table>

100% methylation in Wilms tumor-A (WT-A), but the abnormality is already present as ~50% cellular mosaicism in the surrounding kidney tissue (Ki-A).

![Genomic region diagram](image)

- **H19** probe
- **Control probe**
- **CTCF binding sites**

6 kb
BRCA1 and BRCA2: Hereditary Breast Cancer

• Tumor suppressors mediating DNA repair and chromosome stability

• Mutated mainly in hereditary (not sporadic) cases of breast and ovarian cancer
BRCA2 in DNA Repair

• BRCA2 protein binds to Rad51, a DNA-binding protein essential for DNA repair via homologous recombination

• Brca2 knockout cells accumulate chromosomal aberrations

• Brca2 knockout cells are deficient in homologous recombination
Brca2-null Cells: DNA Damage-sensitive
Chromosome Translocations in *Brca2*-KO Mouse Cells

*Brca2*-hypomorphomic allele (truncated gene)

• Mouse lymphocytes with numerous chromosomal rearrangements
Deficient homologous recombination in Brca1\(^{-/-}\) cells

[Moynahan et al., Mol Cell, 1999]
Deficient Formation of Rad51-foci in *Brca1*-null Cells
what are the interacting proteins?

• BRCA1-BARD1
• Many other candidate interactions, including DNA repair proteins, transcription factors and chromatin-remodeling proteins
**BRCA-1: heterodimer With BARD-1**

Ring domains: *E3 ubiquitin ligase activity*

BRCT domains: common in DNA repair enzymes

Ankyrin repeats

[Baer, Nature Structural Biol, 2001]
Protein-protein interactions involving BRCA1, 2

These interactions are highly dynamic; depending on phase of cell cycle and timing in DNA repair.
Dynamic complexes with BRCA1, 2

Wu, W, et al., Cell Division, 2008
• BRCA2 protein: direct role in modulating homologous DNA recombination (binds RAD51).
• No complete model for BRCA1 function; but this protein is also essential for efficient DNA repair in homologous and non-homologous recombination.
• BRCA1/BRCA2 mutation carriers: *genetic instability in breast/ovarian development*. *Eventual tumor formation (after loss of p53 checkpoint).*