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

Cells of child

Retinal cells

Chromosome 13

30,000 fold increased incidence

Overall: $10^{-12}$
Retinoblastoma

thickening of optic nerve due to extension of tumor

displaced retinoblastoma
normal retina

Figure 7.4b  The Biology of Cancer (© Garland Science 2007)
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

First ‘hit’ - mutation

Second hit - LOH
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

DNA damage

Mutations in the genome of somatic cells

Factors affecting CpG methylation

- 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-deoxycytidine 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

**Histone modifications**
- **MLL1/2/3**
- **EZH2**
- **KDM6A**
  (leukemias, carcinomas, medulloblastomas)

**CpG methylation pathways**
- **DNMT3A**
- **TET1/2**
- **IDH1/2**
  (brain tumors, leukemias, etc.)

**Chromatin remodelers**
- **SNF5**
- **ARID1A**
  (rhabdoid tumors, ovarian CA, etc.)
Diverse Functions of TS Genes

- **Rb and p16**: Restrain the cell cycle
- **p53 and ARF**: Checkpoints for DNA damage, hypoxia, oncogenes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL</td>
<td>Cytoplasmic protein; ubiquitination pathway</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Membrane protein; promotes cell-cell adhesion</td>
</tr>
<tr>
<td>NF-2</td>
<td>Cytoskeletal protein</td>
</tr>
<tr>
<td>TSC-2</td>
<td>Antagonizes the mTOR pathway</td>
</tr>
<tr>
<td>NF-1</td>
<td>GAP homology; antagonizes Ras signaling</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase; AKT pathway antagonist</td>
</tr>
<tr>
<td>APC</td>
<td>Cytoplasmic; antagonizes Wnt signaling</td>
</tr>
<tr>
<td>PTC</td>
<td>Membrane protein; antagonizes Hedgehog signaling</td>
</tr>
<tr>
<td>WT1</td>
<td>Transcription factor; kidney differentiation</td>
</tr>
<tr>
<td>BRCA1 and 2; MLH1</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

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

Cell with p53 mutation/loss

- No response
- Apoptotic 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 p14<sup>ARF</sup> 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**
- **Rb**
- **CyclinD/Cdk4, 6**
- **p16**

**Transcriptional activation**

- **Rb**

**Phosphorylation**

- **ubiquitin-mediated proteolysis**

- **transcriptional activation**

- **ubiquitin-mediated proteolysis**
Genetic Interaction: p53 and pRb

Mouse Retina

<table>
<thead>
<tr>
<th>Rb</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
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<td>-</td>
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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/\(\beta\)-catenin Pathway

- sFRP
- DKK
- CK1/2
- GSK3b
- APC
- Axin
- Frizzled receptor
- Wnt
- LRP
- Dsh
- PP2A

\(\beta\)-TrCP complex

- No Wnt signaling, no \(CTNNB1\) mutation; \(\beta\)-catenin degraded
- \(\beta\)-catenin/TCP4
- Target Genes \(\rightarrow\) ON

- 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
β-catenin Phosphorylation and Proteolysis

UB

Ub

Ub

Ub

GSK3b

APC

Axin

β-catenin

ubiquitination

βTRCP (SCF)

GSK3b

APC

Axin

β-catenin degraded

26S proteasome

β-catenin

GSK3b

APC

Axin

CK1a
Figure 7.24a The Biology of Cancer (© Garland Science 2007)
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

Ci155

Cos2

Fu

Su(fu)

Ci75:

repressor of growth genes

βTRCP (SCF)

ubiquitination

26S proteasome (partial proteolysis)

Ci155:

transcriptional activator (GLI proto-oncoprotein in humans)

Ci75: repressor of growth genes

26S proteasome (paral proteolysis)
Basal Cell Carcinoma
PTC mutations in basal cell CA
Sonic Hedgehog Signaling in Mammals

- Net affects on the levels of the \textbf{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

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

[Linehan et al., 2003]
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**

A diagram illustrates the role of neurofibromin in regulating the GTPase activity of Ras. Neurofibromin (GAP domain) stabilizes GDP-bound Ras, preventing it from converting to GTP-bound active form, thus inhibiting cell growth. The absence of neurofibromin leads to increased cell growth due to the constitutive activation of Ras.
Mouse Model of Neurofibromatosis

\[ p53 \text{ het;} \ Nf1 \text{ het} \]

↓

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

- Metanephric mesenchyme
- Ureteric bud
- Proximal nephron
- Distal nephron
- Wilms tumor
Histology of the Fetal Kidney

Mesenchyme

CM

NM

UB

Epithelium
Histology of Wilms Tumor

- Blastemal Component
- Epithelial Component
Genetic Predisposition to Wilms Tumor

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

WT1 mutations

WAGR syndrome
- Wilms tumor
- aniridia
- genitourinary malformations
- mental retardation

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: \textit{Class 1})

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

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

- sFRP
- DKK
- LRP
- sFRP
- WIF
- Wnt
- Frizzled receptor
- Dsh
- PP2A
- CK1/2
- GSK3b
- Axin
- b-TrCP complex
- APC
- β-catenin
- Target Genes → ON

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

β-catenin/TCF

TCF sites

Groucho/HDAC repressive factors displaced from promoter region

+ Wnt signaling or CTNNB1 mutation; β-catenin stabilized

β-catenin degraded

CTNNB1 mutation; β-catenin stabilized
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

Non-supervised

K-Means Cluster 4

Supervised

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

18S

MEOX2

ID4

FGFR2

β-actin

Wilms Tumors

<table>
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<td>3 13 10 6 2 14 19 18 17 15 16 21 23 24</td>
</tr>
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</table>

18S

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

Chromosome 11p15; WT2
- 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

![Diagram showing reciprocal expression of H19 and IGF2 in normal tissues.](image)
Silencing of *H19* and biallelic Expression of *IGF2* in Wilms Tumor
LOH of Chr11p15 in Wilms Tumor

IGF2  H19

Expressed
Silenced

Enhancer
**H19 DMR methylation precedes tumor formation**

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)
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-hypomorphc allele (truncated gene)

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

[Moynahan et al., Mol Cell, 1999]
Figure 12.30  The Biology of Cancer (© Garland Science 2007)
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

Figure 12.34a  *The Biology of Cancer* (© Garland Science 2007)
Dynamic complexes with BRCA1, 2

Wu, W, et al., Cell Division, 2008
BRCA1, BRCA2: Summary

• 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/BRCAC2 mutation carriers: *genetic instability in breast/ovarian development*. 
  
  Eventual tumor formation (after loss of p53 checkpoint).