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 normal retina

retinoblastoma

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
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

Normal cell

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-deoxyazacytidine in cancer cell lines. a, Southern blot analy-
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

VHL  Cytoplasmic protein: ubiquitination pathway
E-cadherin  Membrane protein; promotes cell-cell adhesion
NF-2  Cytoskeletal protein
TSC-2  Antagonizes the mTOR pathway
NF-1  GAP homology; antagonizes Ras signaling
PTEN  Phosphatase; AKT pathway antagonist
APC  Cytoplasmic; antagonizes Wnt signaling
PTC  Membrane protein; antagonizes Hedgehog signaling
WT1  Transcription factor; kidney differentiation
BRCA1 and 2; MLH1  Roles in DNA recombination and repair
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
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
      - Successful damage repair
        - Apoptosis (cell death)
  - 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.
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.
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
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
“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 \textit{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

In the absence of Wnt signaling and no CTNNB1 mutation, β-catenin is degraded.

β-catenin/Tcf4 activates target genes.

Wnt signaling or CTNNB1 mutation stabilizes β-catenin.

Groucho/HDAC repressive factors are displaced from the 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**

- **β-catenin**
  - Phosphorylation
  - Proteolysis

  - Ubiquitination
  - 26S proteasome
  - β-catenin degraded
**Figure 7.24a** The Biology of Cancer (© Garland Science 2007)

- **intestinal lumen**
  - **wild-type crypt**
  - **Apc^-/- crypt**

- **cell cycle arrested, differentiated cells**
  - **β-catenin:Tcf/Lef OFF**
  - **β-catenin:Tcf/Lef remains ON**

- **proliferating, undifferentiated progenitors**
  - **β-catenin:Tcf/Lef target genes induced by Wnts**

- **APC or β-catenin mutation: progenitor-like phenotype; site of future polyp formation**

- **stem cells**

- **stromal cells**

- **bottom of crypt**
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 Nüsslein-Volhard C, Wieschaus E. 1980 Nature 287:795–801 with permission of Nature Publishing Group.
Hedgehog signaling in Drosophila

Hedgehog → Smo → PKA

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

βTRCP (SCF)

ubiquitination

26S proteasome (partial proteolysis)

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 oncogenic GLI-1 transcription factor (and other GLI family members; Ci homologues)

- increase in GLI-1 expression (oncogenic) after exposure of normal cells to Shh and in cancer precursor cells that lack PTC or that have 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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<tr>
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<td>VHL</td>
<td>Met</td>
<td>FH</td>
<td>BHD</td>
<td></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
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

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

*Not related to NF1

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*Figure 1. Pulsed-Field Gel Analysis of Lymphoblast DNA from NF2 Patients*
DNA in agarose blocks was digested with NotI, subjected to electrophoresis, blotted, and hybridized to a radiolabeled 4 kb *NEFH* probe. Lane 1, GUS6274 (affected NF2 unrelated to lanes 3 and 4); lane 2, GUS7870 (normal human); lane 3, GUS5068 (affected daughter); lane 4, GUS5069 (affected mother).
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: \textit{WT1} (Denys-Drash and WAGR syndromes)

- Breast cancer: \textit{BRCA1, BRCA2} (familial breast and ovarian cancer)

  - Classical 2-hit mechanism; but mutations largely restricted to \textit{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

Blastema 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 (*CTNNB1* gain-of-function) mutations are restricted to *WT1*-mutant Wilms tumors

Columbia U. series of 36 Wilms tumors sequenced for *WT1* and *CTNNB1*:

- 0/18 *CTNNB1* mutations (0%) in *WT1*-wild type Wilms tumors (all sporadic: “Class 1”)

- 14/18 *CTNNB1* mutations (77%) in *WT1*-mutant Wilms tumors (17 syndromic; 1 sporadic: “Class 2”)

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

- **sFRP**
- **DKK**
- **sFRP**
- **APC**
- **Axin**
- **GSK3b**
- **Frizzled receptor**
- **LRP**
- **Wnt**
- **WIF**
- **Dsh**
- **PP2A**
- **b-TrCP complex**
- **Ub**
- **P**

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

+Wnt signaling or **CTNNB1** mutation; beta-catenin stabilized

Target Genes → ON

Groucho/HDAC repressive factors displaced from promoter region

β-catenin/TCF

TCF sites

β-catenin/Tcf sites
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 $\rightarrow$ nephrogenic rests

WT1 loss-of-function $\rightarrow$ 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

Non-supervised
Supervised

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

28S

PAX2

WIF1

HAS2

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

A

E2-2/ITF2 (human)

Relative mRNA expression

FKi (n=6)  Class 1 (n=23)  Class 2 (n=16)

B

E2-2/ltf2 (mouse)

Relative mRNA expression

Control (n=13)  Δex3 (n=9)

C

H&E  β-catenin  E2-2 (a.k.a. Itf2, Tcf4)
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

- **Mat**
  - Expressed
  - Silenced

- **Pat**
  - Me-CpG
  - Enhancer

- **IGF2**
- **H19**
Silencing of *H19* and biallelic Expression of *IGF2* in Wilms Tumor
LOH of Chr11p15 in Wilms Tumor

Pat

Me-CpG

Pat

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)

![Image of gel electrophoresis with bands and probe locations labeled H19, WT-A, Ki-A, Nml Ki, and 1.5 kb and .5 kb probes. CTCF binding sites are indicated with dots and lines near the H19 probe position.]
**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
Brca2-hypomorphpic allele (truncated gene)

- Mouse lymphocytes with numerous chromosomal rearrangements
Deficient homologous recombination in \textit{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).*