Ministry of Higher Education and Scientific Research
University of Baghdad
College of Science
Department of Biology

Molecular Basis of Cancer Biology

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المرحلة ـ الدكتوراه

الفصل الدراسي الثاني

تدريسي المادة :

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Lecture1: An introduction to the molecular basis of cancer

What is Cancer?

- A heterogeneous group of diseases in which single cells acquire the ability to proliferate abnormally, resulting in an accumulation of progeny.
- Cancers are those tumours that have acquired the ability to invade through surrounding normal tissues.
- The most advanced form of this invasive process is metastasis, a state in which cancer cells escape from their original location, travel through the blood or lymphatic systems and take up residence at distant sites.

Cancer classification

Classification important for cancer diagnosis and management.

- **Tissue of origin:**
  - Carcinomas – epithelial cells (i.e. endoderm or ectoderm).
  - Sarcomas – mesoderm.
  - Leukaemias and lymphomas – blood cell precursors.
- **Benign** (localised, non-invasive) vs. malignant (capable of invasion and metastasis).
- **Histology** (e.g. tissue architecture and cellular morphology).
- **Genetic** or expression markers.

General Concepts

- Key processes: development, invasion, metastasis, angiogenesis.
- Cancer is a genetic disease.
- Most cancers are derived from a single cell
- At the level of the individual, cancer is a common occurrence, whereas at the level of the cell it is a rare event
- Cancer development is a multi-step phenomenon
- Some benign tumors can progress to malignancy- this is known as progression.

Cancer as a genetic disease

Mutational theory of cancer

- **Mutation: A stably inherited change in the genetic material.**

Cancer can result from:

- **Germ-line mutations.**
- **Somatic mutations.**

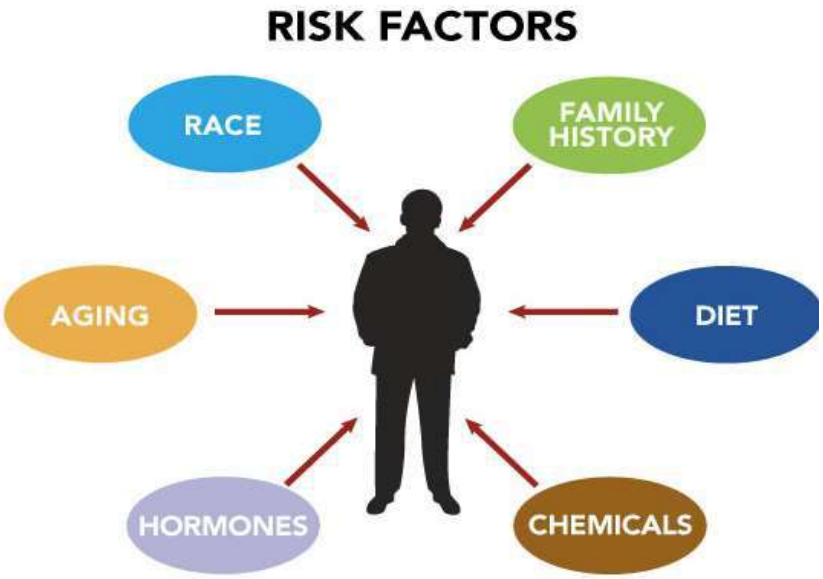
Evidence for a genetic basis of cancer

- Susceptibility to some cancers inherited in families.

- Mutations found in **sporadic cancers** and subsequently found to be transforming.
- Chromosomal changes are common in cancers
- Some cancers associated with particular chromosomal abnormalities.
- Agents that damage DNA are thought to increase the susceptibility to cancer development.
- Some diseases are thought to arise from defects in DNA repair mechanisms that result in an elevated cancer risk.
- Infection with certain viruses associated with development of specific cancer.

What causes cancer?

Risk factors and carcinogenesis



Types of mutations found in cancers

- Chromosome translocations.
- Amplifications.
- Interstitial gains and losses (10's – 1,000,000's bps).
- Small deletions, insertions and single base pair mutations.
- Exogenous sequences e.g. viruses in cervical cancer (HPV), Burkitt's lymphoma (EBV), Hepatocellular carcinomas (hepatitis virus) and Kaposi's sarcoma (HIV virus).
- Epigenetic mechanisms – promoter hypermethylation.

✓ Why is cancer such a rare event at the cellular level?

- DNA repair mechanisms.
- Apoptotic (cell death) mechanisms.
- Mutations in many genes do not affect cell growth.
- Most DNA does not code for protein.
- Rapidly dividing cells (eg intestinal epithelium and skin) are lost from the body so it does not matter if they contain mutations (unless they occur in the stem cell population).
- Many cells do not divide all the time- they differentiate and lose their potential to divide (eg muscle cells).
- No single mutation can circumvent these defenses.

Cancer is a multi-step disease

- The development of cancer is a multi-step phenomenon.
- Multiple successive errors/cell (~6-7) are required to circumvent this protection and give rise to abnormal growth and a malignant cancer.
- Chances of this are rare.

However, two general mechanisms allow this progression to happen:

Accumulation and selection of multiple mutations in cancer

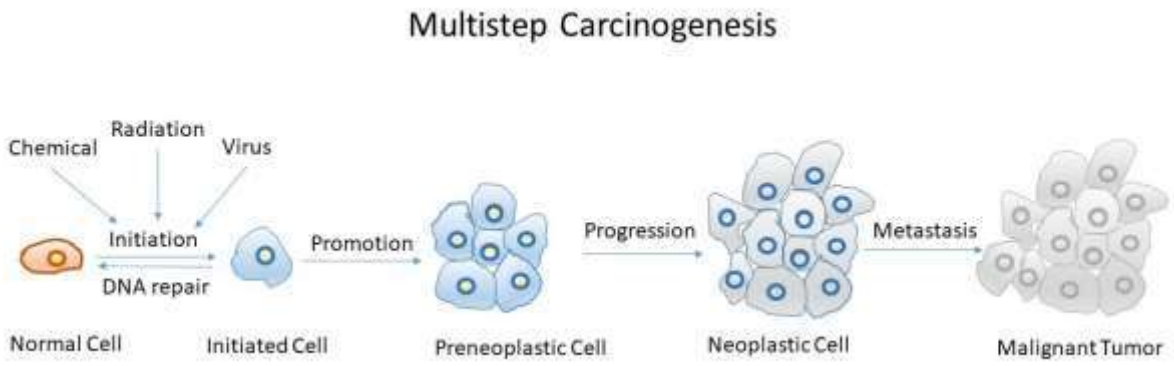
Mutations have two general mechanistic effects:

1-Increase cell growth, division, survival:

Expand target population for subsequent mutations.

2-Alter genomic stability:

Increase mutation rate and susceptibility to further mutations.



What types of genes are targets of mutations in cancer?

Two general categories

1- Oncogenes:

Normal activity promotes proliferation, growth, invasion etc.

Gain-of-function mutations in cancer (1 allele):

Excessive or inappropriate activity.

Normal cellular versions termed proto-oncogenes.

2. Tumor suppressor genes:

Normal role: inhibit events leading to cancer

(eg. negative regulation of cell cycle, pro-apoptotic, genomic stability and repair)

Loss of function mutations (affecting 2 alleles) required for inactivation and cancer development.

Functional classification of tumor suppressor genes

- Gatekeepers: Directly control proliferation.
- Caretakers: Control rate of mutation.

Lecture 2

Oncogenes

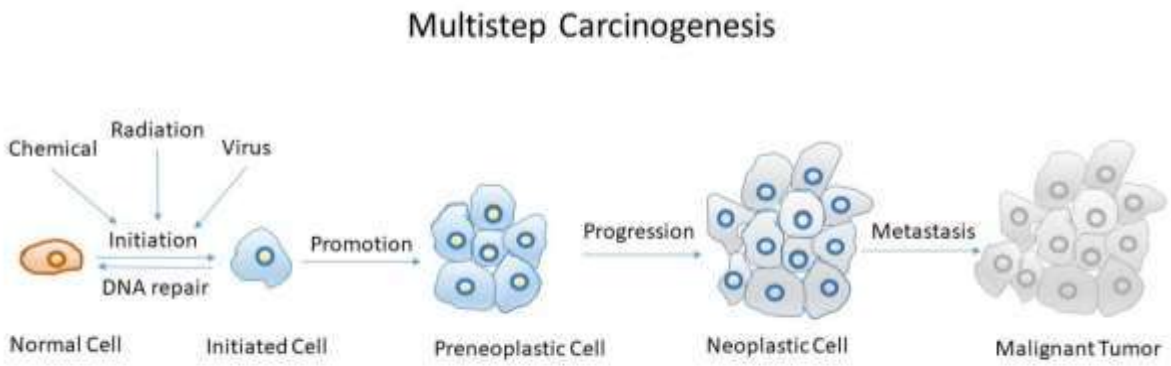
Revision 1: Accumulation and selection of multiple mutations in cancer

- **Mutations have two general mechanistic effects:**

Increase cell growth, division, survival: Expand target population for subsequent mutations.

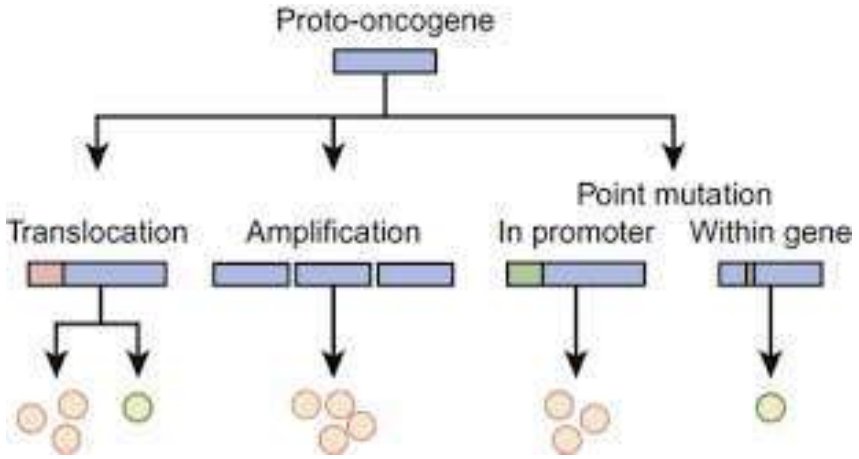
- **Alter genomic stability:**

Increase mutation rate and susceptibility to further mutations



Malignancy	Re-arrangement	Chimeric gene	Chimeric product function
Chronic myelogenous leukaemia (CML)	t(9;22)(q34;q11)	*BCR-ABL*	Tyrosine kinase
Ewing sarcoma	t(11;22)(q24;q12)	*EWS-FLI1*	Transcription factor
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX3-FKHR*	Transcription factor
AML	t(16;21)(p11;q22)	*FUS-ERG*	Transcription factor
ALL	t(11;19)(q23;p13)	*MLL-ENL*	Transcription factor

Mechanisms of oncogene activation



❖ A history of oncogene discovery

- **1960s – animal cancers caused by viruses**
- **DNA or RNA (retro-) viruses (Retroviruses: are any of a group of RNA viruses which insert a DNA copy of their genome into the host cell in order to replicate, e.g. HIV)**
- **Retroviruses have transforming properties – contain one extra gene – the *oncogene***
- **Viral oncogenes (v-*onc*) represent copies of normal cellular genes (c-*onc*) incorporated into viral particles.**
- **Viral versions are activated and cause transformation.**
- **Most human cancers do not depend on viruses, but their related proto-oncogenes become activated.**

❖ Discovery of cellular oncogenes

- **Analysis of transduced cellular gene sequences in acutely transforming retroviruses (eg *neu*).**
- **Identification of the preferred integration sites of retroviruses (eg *wnt1* in mice).**
- **Characterisation of chromosomal translocations (eg *BCR-ABL*).**
- **Characterisation of amplified DNA sequences (eg *NMYC*).**

- **Direct gene transfers experiment to assay for the biological activity of cellular transforming genes (eg *NRAS*).**
- **Roles in control of cellular functions predicted to be disturbed in cancer.**
- **Over 100 oncogenes identified to date (ie. contain dominant activating mutations in human cancer).**

VIRAL DISEASE	v-onc	c-onc (proto-oncogene)	LOCATION	FUNCTION
Simian sarcoma	v-sis	*PDGFRB*	22q13.1	Platelet-derived growth factor receptor B subunit
Chicken erythroleukaemia	v-erbb	*EGFR*	7p12	Epidermal growth factor receptor
Harvey rat sarcoma	v-ras	*HRAS*	11p15	G-protein signal transduction
Abelson mouse leukaemia	v-abl	*ABL*	9q34.1	Protein tyrosine kinase
Avian sarcoma 17	v-jun	*JUN*	1p31-p32	AP1 transcription factor
Avian myelocytomatosis	v-myc	*MYC*	8q24.1	DNA-binding transcription factor
Mouse osteosarcoma	v-fos	*FOS*	14q24.3-q31	DNA-binding transcription factor

❖ Activation of proto-oncogenes in tumor development

- **Activation involves inappropriate and fixed gain of function.**
- **Quantitative – increase in production of unaltered product.**
- **Qualitative – production of modified or novel product.**
- **Dominant effect.**
- **Activation mechanisms affect only one allele.**

Lecture 3:

Tumour Suppressor Genes

Identification

❖ What types of genes are targets of mutations in cancer?

2. Tumor suppressor genes:

- Normal role: inhibit events leading to cancer
- (eg. negative regulation of cell cycle, pro-apoptotic, genomic stability and repair)
- Loss of function mutations (affecting 2 alleles) required for inactivation and cancer development.

❖ Evidence for tumor suppressor genes.

• Cell fusion experiments: transformed phenotype can often be corrected *in vitro* by fusion of the transformed cell with a normal cell.

ie. normal cells express repressor properties that inhibit cancerous properties

• Tumorigenesis involves not only dominant activated oncogenes, but also recessive, loss-of-function mutations in other genes.

• These other genes are the tumour suppressor (TS) genes.

• Sometimes termed anti-oncogenes *or* repressor genes.

• TS genes can have a variety of functions.

• The rare eye tumour, retinoblastoma, has been used to define the concepts and methods of TS gene research.

❖ Retinoblastoma Knudson's "two-hit" hypothesis

- Retinoblastoma is a rare, aggressive childhood tumor of the retina .
- 60% of cases are sporadic and unilateral . (Unilateral Retinoblastoma. About 60% of children have cancer in only one eye, and this is called unilateral retinoblastoma. Most children have only one tumour, but some develop more than one tumour in one eye (multifocal unilateral retinoblastoma). ... This can be inherited and poses increased cancer risks throughout life).
- 40% are inherited as an autosomal dominant trait, which was mapped to 13q14.
- Familial cases are often bilateral
- In 1971 AG Knudson noted that the earlier age of onset of bilateral cases was consistent with a single "hit" or mutation, while later-onset sporadic were consistent with two "hits".

• Proposed that two successive mutations ('hits') were required to turn a normal cell into a tumor cell, and that in familial forms one of the hits was inherited.

❖ Two hit inactivation of Rb on 13q14

• Cavenee *et al.* (1983) proved Knudson's hypothesis, and established the model for laboratory investigations of TS genes.

• Sought evidence of somatic mutations at the *RB1* locus in sporadic retinoblastoma by typing surgically removed tumour material with a series of markers from chromosome 13.

• When they compared the results on blood and tumour samples from the same patients, they noted several cases where the healthy (blood) DNA was heterozygous for one or more chromosome 13 markers, but the tumour cells were apparently homozygous. They reasoned that what they were seeing was one of Knudson's 'hits': loss of one functional copy of a tumor suppressor gene.

• Combining cytogenetic analysis with studies of markers from different regions of 13q, Cavenee *et al*. were able to suggest a number of mechanisms for the loss. Later studies confirmed this interpretation by showing that in inherited cases, it was always the wild-type allele that was lost in this way.

❖ What types of mutation affect TS genes?

• Mutations detected in TS genes are typically consistent with gene inactivation.

• Deletions.

➢ Single bases to entire gene or surrounding region.

• Truncating (insertions / deletions / nonsense (point)).

➢ eg. introduction of premature stop codon.

• Missense (point)

• So a missense mutation is when just one base has been changed, resulting in a codon for a different amino acid.

➢ Affect critical protein regions or protein structure.
➢ Can be dominant negative (eg. P53).

❖ Functions of Tumor Suppressor Genes

• Negatively regulate multiple aspects of the cancer phenotype.

• Transcriptional regulators of the cell cycle (RB1).

• Transcriptional regulators of cell cycle arrest or apoptosis (P53).

• Other transcriptional regulators (WT1).

• Cyclin dependent kinase inhibitors (eg. INK4a).

• Bind and regulate b catenin transcriptional activity (APC).

• Transcriptional regulation and DNA repair (BRCA1 and 2).

• Transduction of TGFb signals (SMAD4).

• Cell adhesion regulators (E cadherin).

• Mismatch repair (MSH2, MLH1, PMS1, MSH6).

• Angiogenesis regulators (VHL).

• Identification of tumor suppressor genes

• TS genes have been discovered genetically by three main routes (each looking for evidence of 'hits'):

– Positional cloning of the genes causing rare familial cancers.

– Defining chromosomal locations commonly deleted in tumour cells-scanning techniques.

– Testing tumours for mutations in known candidate genes (eg. cell cycle regulators, proteins in cancer pathways).

• Studies of transforming DNA viruses have also led to TS gene identification.

❖ Defining chromosomal locations of TS genes in tumor cells using scanning techniques.

• Identification of loss of heterozygosity (LOH) using polymorphic markers

• Polymorphic markers differentiate maternal and paternal alleles on basis of size or sequence difference.

• Commonly used markers:

• Single nucleotide polymorphisms

• Microsatellite length polymorphisms (di-, tri-, tetra-nucleotide repeats – variable numbers of repeats)

• Analyse allele status in tumour vs. healthy DNA by PCR etc.

Epigenetic inactivation of TS genes by promoter hypermethylation

• Epigenetic TS gene inactivation

• TS genes inactivated by promoter hypermethylation (leading to transcriptional silencing) is a very common mechanism.

• Tumor cell DNA is hypomethylated compared to normal cells.

• Tumor-specific de novo methylation of specific CpG dinucleotides in promoter region is associated with transcriptional silencing of TS genes.

• Associated with transcriptionally inactive closed chromatin.

• Some TSGs–methylation occurs in conjunction with genetic events. Others – methylation is only mechanism identified for loss of function (eg. RASSF1A, HIC-1).

❖ Problems in the identification of epigenetically inactivated TS genes.

- Not detected by standard genetic methods (mutation, LOH screening).
- Complicate Knudson's hypothesis – alleles may appear genetically normal, or only one genetic 'hit' may be detectable, if methylation is involved.
- Assessment of methylation status must be performed in addition to genetic detection methods for complete understanding.
- Role and importance may be underestimated requires development of specific methods for the detection of methylation events.

❖ DNA viruses and TS genes

- DNA viruses act by v-onc protein binding to and inactivating host TS proteins.
- Host proteins are negative regulators, encoded by TS genes.
- P53 TS gene first identified by interaction with Large-T antigen of SV40 virus.
- HPV binds and inactivates the P53 and RB TS proteins.
- Both cause loss of growth control and altered proliferation.
- P53/RB binding is a common pathway for cancer-causing DNA virus action.
- Unlike retroviruses, DNA viruses play an important role in certain human cancers.

❖ Functional classification of tumor suppressor genes:

- Gatekeepers: Directly control proliferation.
- Caretakers: Control rate of mutation or protect genomic integrity.

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❖ TS genes in cell cycle control and apoptosis:

Oncogenes and tumour suppressor genes play key roles in interpreting these signals.

❖ Cell cycle control and checkpoints:

Cells that decide to divide – 3 major checkpoints:

1-G1/S checkpoint: DNA replication blocked if unrepaired DNA damage exists. Irreparable damage – apoptosis.

2-G2/M checkpoint: mitosis blocked unless replication/repair complete.

3-The spindle (metaphase) checkpoint.

TS genes in cell cycle control 1.

The RB TS gene (The retinoblastoma tumor suppressor gene)

- Retinoblastoma is a rare, aggressive childhood tumour of the retina.
- 60% of cases are sporadic and unilateral 40% are inherited as an autosomal dominant trait, which was mapped to 13q14.
- Familial cases are often bilateral (Children born with a mutation in the RB1 gene usually develop retinoblastoma in both eyes (known as bilateral retinoblastoma).
- Caused by mutation of the *RB1* TS gene at 13q14.
- One mutation in familial cases, two in sporadic.
- Patients with germ line mutations of *RB1* are also at elevated risk for development of a rather limited number of tumour types (osteosarcomas, soft-tissue sarcomas, and melanomas later in life).
- *S*omatic *RB1* mutations have been observed in a wide variety of other cancer types, including breast, small cell lung, bladder, pancreas, and prostate cancers.
- The protein product of the RB1 gene is a nuclear phosphoprotein with a molecular weight of about 105,000 Daltons known as p105-Rb or, more commonly, as pRb.
- Restoration of RB1 function suppress aspects of retinoblastoma tumorigenesis.
- Mutations inactivating the ability of oncoproteins (a protein that is coded for by a viral oncogene which has been integrated into the genome of a eukaryotic cell and that is involved in the regulation or synthesis of proteins linked to tumorigenic cell growth) to bind to pRb also inactivate their transforming function.

- DNA tumor viruses might transform cells by inactivating tumor-suppressor gene products.
- DNA tumor viruses harness the cell's machinery for replication of the viral genome.

pRb inactivation positively regulates the cell cycle

NORMAL: pRb inactivation by phosphorylation leads to cell cycle entry.

CANCER: pRb inactivation by mutation or viral oncoprotein binding results in uncontrolled cell proliferation.

Lecture 4

TS genes in cell cycle control:

The P53 TS gene.

P53 and tumorigenesis:

- 1970s: a cellular 53KDa phosphoprotein binds to SV40 T antigen and other viral oncogene products – p53 (at 17p13).
- Chromosome 17p LOH (Loss of heterozygosity (LOH) is a common genetic event in cancer development, and is known to be involved in the somatic loss of wild-type alleles in many inherited cancer syndromes. ... It is the loss of an allele in tumor DNA compared to matched normal DNA from the same individual) common in colorectal, bladder, breast, and lung cancer etc.
- Detailed mapping showed that minimal region of 17p included the p53 gene.
- Remaining p53 allele was mutated in many 17p LOH cancers.
- Based on the types of tumors involved and the prevalence of p53 mutations, p53 is believed to be the most frequently mutated gene in human cancer.
- The vast majority of the somatic mutations in p53 are missense mutations leading to amino acid substitutions in the central portion of the protein.

P53 function

- Controls cellular responses to DNA damage and other forms of genotoxic stress in order to maintain genomic stability.
- Has been called “cell cycle checkpoint gene” or “guardian of the genome”, protecting the genome from excess mutations.
- DNA damage – cell cycling stalls for repair. If not repairable, apoptosis is triggered. P53 is central to these processes
- Cellular P53 levels normally low – rapidly degraded
- Signals from cell stress / damage sensors lead to phosphorylation and stabilisation of P53
- Expression of high levels of wild-type p53 can cause cell cycle arrest or apoptosis

• Transcription factor - enhances the rate of transcription of genes involved in either cell cycle arrest or apoptosis

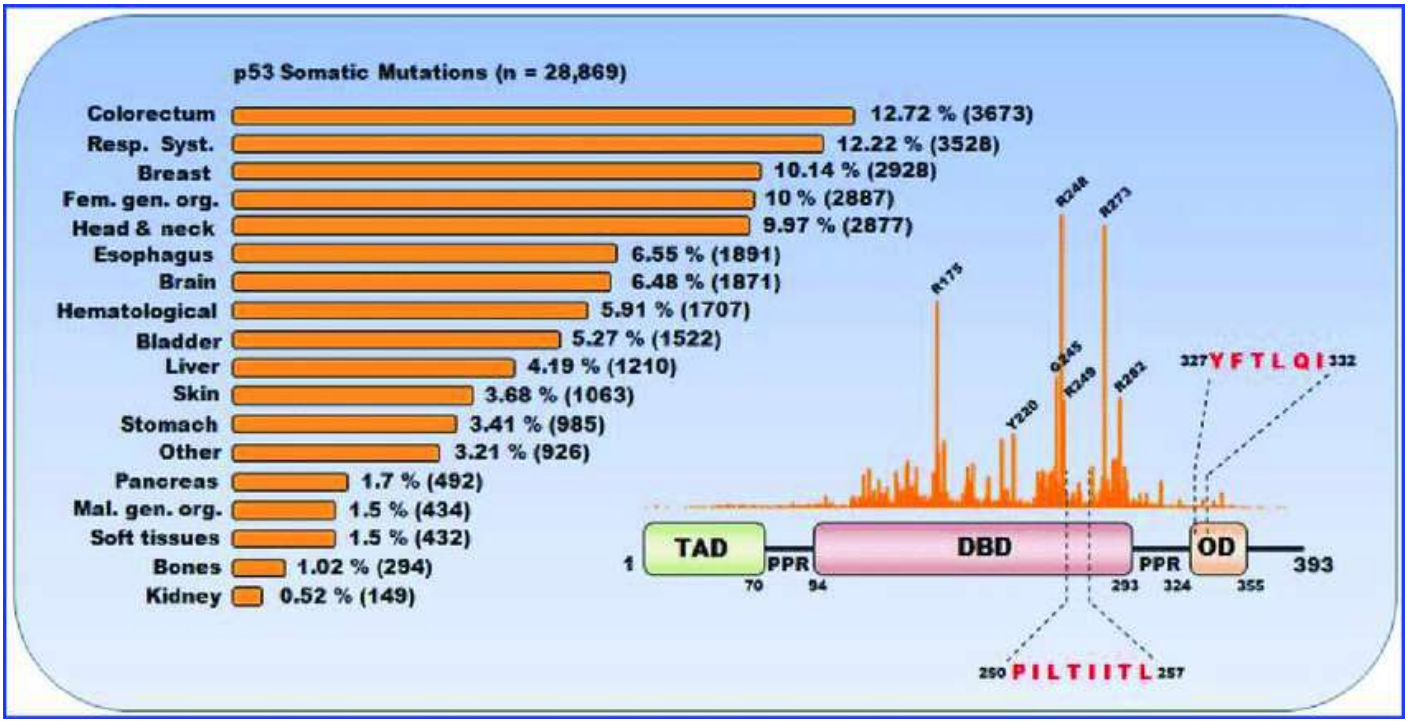
P53 mutations: functional effects

• The vast majority of *p53* mutations are missense mutations Scattered through the central domain of the *p53* coding region (exons 5 to 9).

• All appear to have marked effects on p53 protein's capability to bind to its cognate DNA recognition sequence through either of two mechanisms:

A. Some mutations (eg. codons 248 or 273) alter p53 sequences that are directly responsible for sequence-specific DNA binding.

B. Others (eg. codon 175) affect the folding of p53 and thus indirectly affect its DNA binding ability.



TS genes in cell cycle control: The INK4/ARF locus

❖ LOH of 9p frequently found in tumor types, including melanomas, gliomas, and nonsmall cell lung, bladder, head and neck cancers, leukemia.

❖ Subset of such tumors had homozygous (complete) deletions affecting the 9p21 region – strongly suggests TS locus.

❖ Families with inherited melanoma – linkage studies mapped locus to the same region of 9p.

- Positional cloning at 9p21 identifies MTS1/P16 gene.
- Inhibitor of cdks 4 and 6 – named INK4.
- Another highly related gene, mapping immediately next to the *p16/MTS1*, was found to encode a second INK4 protein, known as p15.
- p16 protein termed *INK4a (CDKN2A).*
- p15 is termed *INK4b (CDKN2B).*
- Heterozygous mutations in *INK4a* are present in some patients with inherited melanoma.
- Somatic mutations in *INK4a* identified in many different cancer types (not just 9p LOH tumours).
- Deletion / homozygous deletion is a fairly common mechanism of *INK4a* inactivation – may also include INK4b.

NORMAL: The ARF protein responds to proliferative signals. When these signals exceed a critical threshold, ARF triggers a p53-dependent response that induces growth arrest and/or apoptosis.

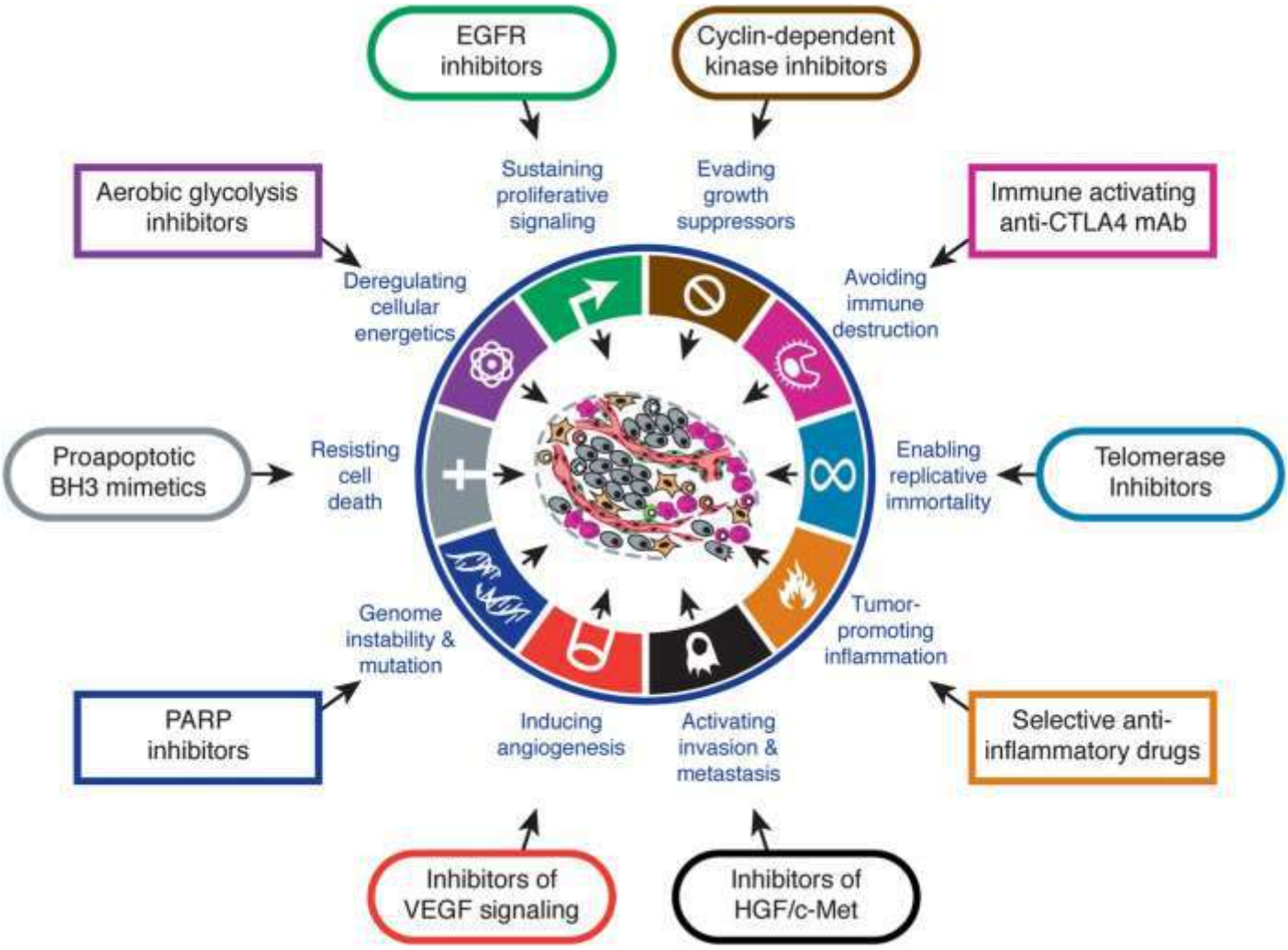
ARF binds and destabilises the MDM2 oncogene product, leading to a P53 response (accumulation).

CANCER: Loss of ARF – excess MDM2 – destruction of P53 Loss of P53-dependent control of cell cycle /apoptosis.

Hanahan and Weinberg's Principles

• The transition of a normal cell to a cancer cell requires acquisition of six specific capabilities:

1. Independence of external growth signals
2. Insensitivity to external anti-growth signals
3. Avoidance of apoptosis
4. Indefinite replication
5. Angiogenesis
6. Invasion and metastasis


EGFR inhibitors
Cyclin-dependent kinase inhibitors
Sustaining proliferative signaling
Evading growth suppressors
Aerobic glycolysis inhibitors
Immune activating anti-CTLA4 mAb
Deregulating cellular energetics
Avoiding immune destruction
Proapoptotic BH3 mimetics
Resisting cell death
Enabling replicative immortality
Telomerase Inhibitors
Genome instability & mutation
Tumor-promoting inflammation
PARP inhibitors
Inducing angiogenesis
Activating invasion & metastasis
Selective anti-inflammatory drugs
Inhibitors of VEGF signaling
Inhibitors of HGF/c-Met

Lecture 5: **Metastatic Cascade**

Metastasis

• The spread of a tumour **from primary organ** to **distant sites** in the body

• Responsible for death of majority of patients suffering from the major forms of cancer

• Difficult to treat metastatic disease.

• Need to develop more specific therapies.

Routes of metastatic spread

• Blood vessels – Capillaries: single layer endothelial cells (form lumen). External basement membrane (glycoproteins)

– Liver: HPV (Human *Papillomavirus*)breaks down to sinosoids (direct contact with hepatocytes)

• Lymphatics – Lymph nodes & capillaries (no basement membrane) – LV drain into 2 veins (form inferior vena cava), drain into heart

• Movement within body cavities – Peritoneal and pleural – Difficult to treat?.

Hypotheses to explain the site of metastatic spread

Metastatic sites only predicted for ~ 50% cancers –additional factors cause cancers to go to preferred site(s):

- Seed and Soil – Paget (1889): 735 BC patient autopsies
- Mechanical – Ewing (1928): pattern of blood flow dictates unequal distribution.

Metastatic inefficiency

• Cells in the primary tumour are **extremely heterogeneous**.

• Most metastatic cells are destroyed in transit.

• To successfully metastasise, a malignant tumour cell has to complete every step of the metastatic cascade.

• Metastases can be **dormant** for long periods

The metastatic cascade

1. **Growth and angiogenesis of primary tumour**
2. **Local invasion**
3. **Penetration of blood vessels or lymphatics (intravasation)**
4. **Survival in the circulation**
5. **Arrest and escape from blood vessels or lymphatics (extravasation)**
6. **Colonisation and angiogenesis at the secondary site.**

Metastasis is poorly understood at the molecular level

- Advanced tumours are cytogenetically very complex.
- Difficult to distinguish **metastasis-associated genes** from **metastasis-determining genes**
- Difficulty in creating relevant animal models
- Metastasis is a multi-step process in which quantitative and qualitative changes in the expression of several genes is necessary.

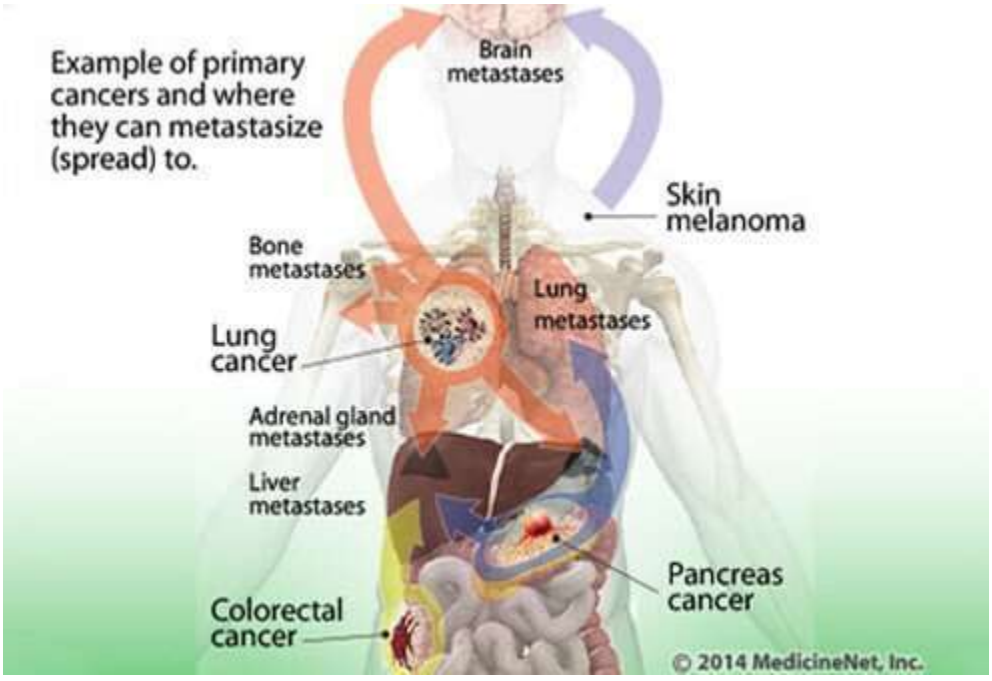
Metastasis-associated processes

1. Invasion
2. Adhesion (cell-cell and cell-ECM)
3. Motility
4. Immune Evasion
5. Angiogenesis

Secondary sites of metastases

Blood-borne cancer: liver first pass organ (common metastatic site)

Lymphatics: drain into lymph nodes (BC metastases frequently armpit nodes)



Adhesion CAMs (**C**ell ***adhesion*** **m**olecules)**have Important roles in metastatic process**

• Four classes of membrane receptors

– Integrins

– Cadherins

– Selectins

– Immunoglobulin family

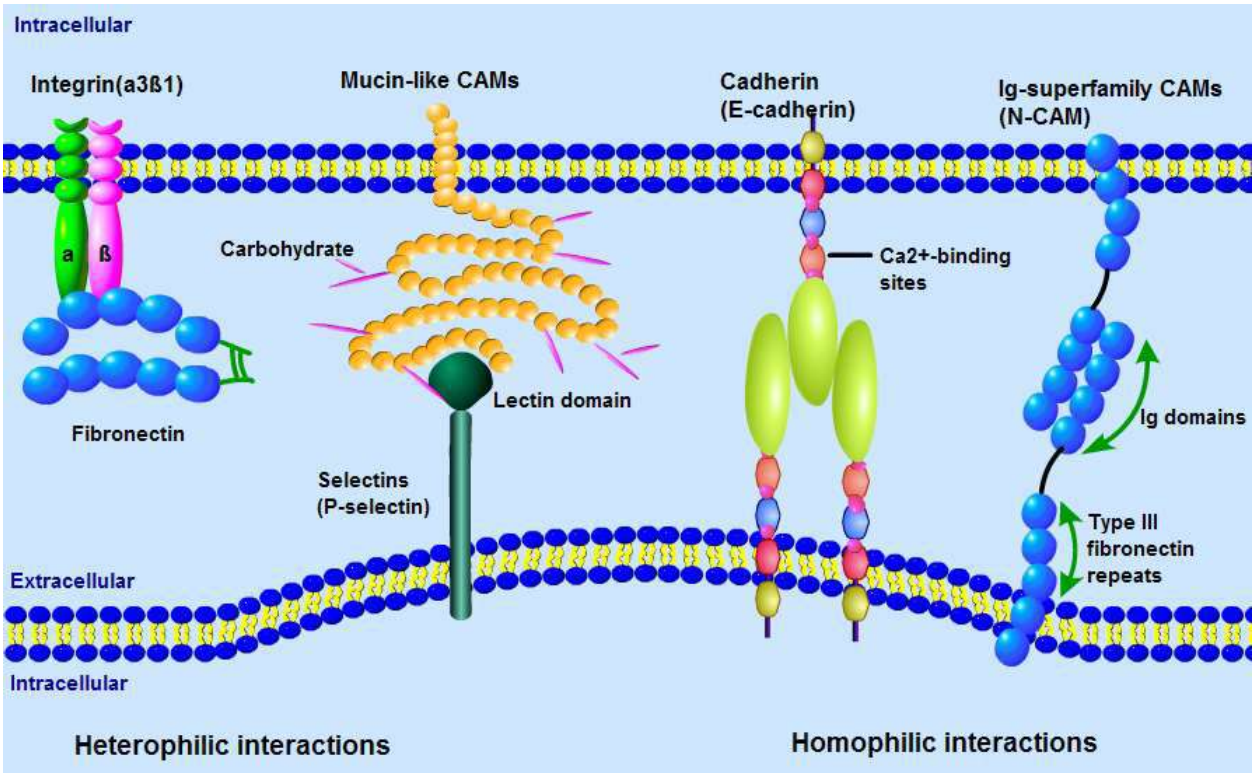
• Types of interaction (cell-cell; cell-ECM);**ECM** means = extracellular matrix

– Homotypic (eg. E cadherin, N-CAM)

– Heterotypic (eg. Selectins)

– Cell-ECM (eg. Integrins, CD44)

Adhesion Molecules



Changes in adhesion in metastasis

• Loss of homotypic adhesion in primary tumour (eg loss of E cadherin, N-CAM)

• Loss of integrin expression e.g. loss of a2b1 in anchorage independent cells, lost in breast cancer)

• Increase of integrin expression e.g. avb3broad ligand specificity, increased in metastatic melanoma. Laminin receptors a3bl and a6b1 elevated in endometrial and breast cancers.

Arrest

• Platelets adhere to proteoglycans (P-selectin binds to fibrinogen on endothelial cell)

• Endothelial cells possess organ-specific cell membrane determinants (eg L-CAM on lung endothelial cells)

• Integrin a4b1 on tumour cells and lymphocytes attach these cells to both fibronectin and V-CAM on endothelial cells

• V-CAM is up-regulated in activated endothelium by cytokines produced by lymphocytes and tumour cells

• Endothelial and Platelet selectins - arrest movement of tumour cells by binding to carbohydrate side chains (E selectin) or membrane glycoproteins (P-selectin) on cancer cell

Escape from blood vessels

• Retraction of endothelial cells exposes glycoproteins of the basement membrane – Laminin, collagen IV, Fibronectin (integrin ligands)

• Cancer cells attach via integrins and digest using proteases and glycosidases

• Different cancers display different patterns of integrin expression; this contributes to "soil" specificity – Osteosarcoma (increased A1B1, recognising collagen and laminins) – Colon cancer (increased A6B4, recogniselaminin).

Invasion

• Metastatic cells have an imbalance of proteolysis favouring invasion

• Increased expression of proteases

• Decreased expression of inhibitors of proteases

Proteases

• **Metalloproteinases:** Require zinc or calcium ions: (e.g. collagenase types I and IV, gelatinases, stromelysin)

• Serine proteases: serine at active site, do not require metal ions (e.g. plasminogen)

• Secreted as inactive precursors

Properties of matrix metalloproteinases (MMPs)

• Large protease family (22 in human), 8 structural classes (3, membrane bound)

• Zn dependent endopeptidases, synthesised as inactive zymogens

• Pro-peptide cysteine, Active site Zn ion

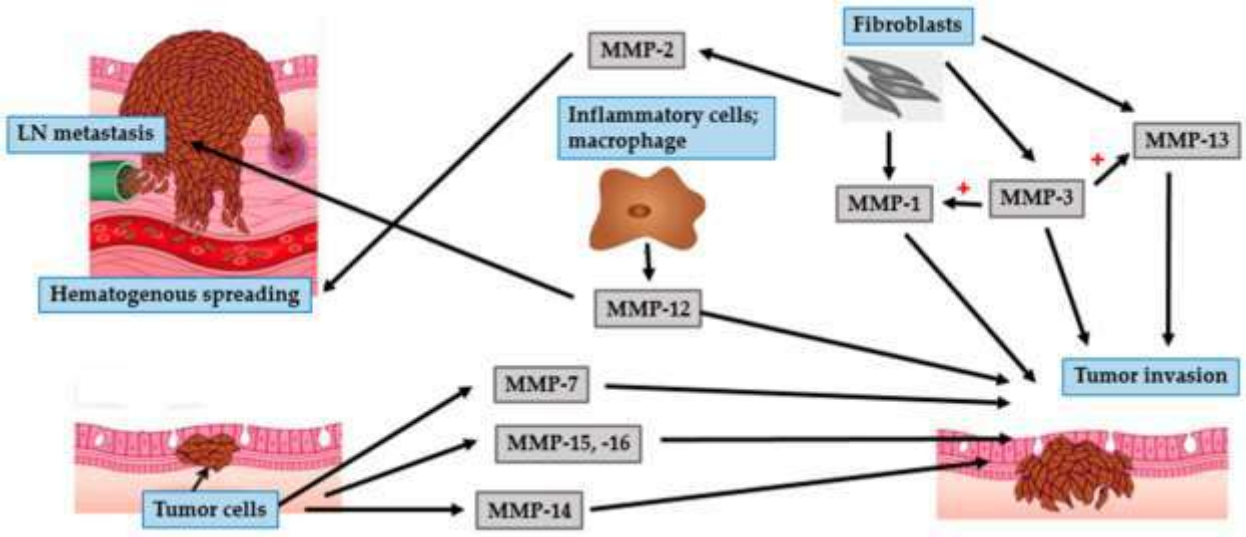
• Cleave structural components of ECM

• Activity regulated by TIMPs

• Upregulated in cancer

• Increase proliferation, migration, invasion, metastasis and angiogenesis.

The function of MMPs in metastasis



Migration

• Achieved by alternate attachment of the leading edge of the cell to matrix proteins and detachment of the rear edge.

• Mediated by **integrins**: transduce external signals from the ECM to the cytoskeleton via GTP binding proteins such as Rho and Rac

• Peptides released during ECM degradation act as chemoattractants

• Motility factors eg HGF (scatter factor)

Identification of Metastasis-associated Genes:

- Somatic cell hybridization
- Cytogenetics
- Transfection of candidate genes into suitable recipient cell lines
- Differential expression of mRNA in cell lines derived from tumour material/animal models
- Transfection of high molecular weight DNA from non-metastatic into metastatic cell lines.

Identification of metastasis related proteins from a heterogeneous cell population

• Laser capture microdissection of individual tumour gland

• Two-dimensional gel analysis of cancer cell proteome (transcriptome)

• Identification of proteins by Mass spectometry

• Confirm protein alteration in cancer patient biopsies.

Study of metastasis-associated genes

• In vitro

– Tumour-derived material

– Cell lines

– Assays –examples include proliferation, adhesion, migration

• In vivo

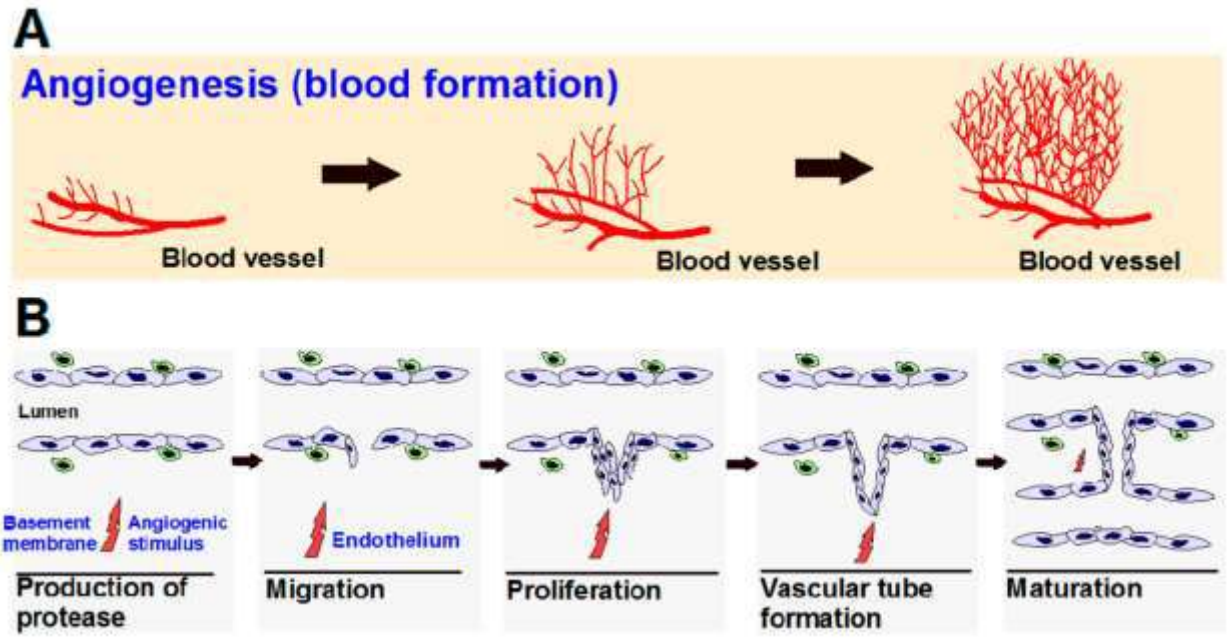
– Animal models

– Imaging

Lecture 6: Tumour Angiogenesis

Angiogenesis: Growth of new blood vessels from the host vasculature

Formation of Blood Vessels



Angiogenesis in Health and Disease

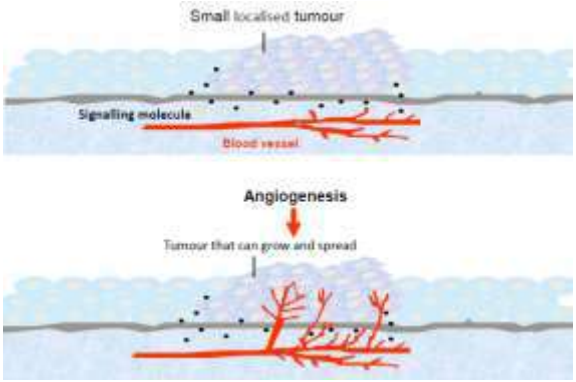
Health

1- Development and growth
2- Reproductive system
3- Repair

Disease

1- Vascular malformations (Haemangiomas)
2- Chronic inflammatory diseases and syndromes
3- Malignant tumours

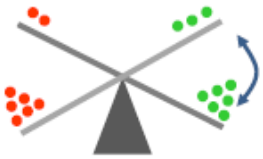
Tumour Angiogenesis



- Tumours cannot grow beyond 1-2 mm3 without a blood supply
- The lack of nutrients and oxygen causes release of factors that stimulate angiogenesis
- Unlike the normal vasculature up to 10% of tumour-associated endothelial cells are dividing at any one time
- Tumours may become vascularised by vessel incorporation

Angiogenesis Regulators

- ✓ Balance of promoters and inhibitors
- ✓ Inhibitors normally predominate



Positive regulators (•)

. Fibroblast growth factors
Placental growth factor
Vascular endothelial growth factor
Transforming growth factors (a + b)
Angiogenin
Interleukin-8
Hepatocyte growth factor
Granulocyte colony-stimulating factor
Platelet-derived growth factor b
Angiopoietin 1

Negative regulators (•)

. Thrombospondin-1
. Angiostatin
. Interferon a
. Prolactin fragments
. Metalloproteinase inhibitors (TIMPs)
. Platelet factor 4
. Interleukins
. Placental proliferin-related protein
. Transforming growth factor beta
. Endostatin (type XVIII collagen fragment)
. Tumstatin (type IV collagen fragment)

Factors Regulating Angiogenesis

1- **Proteolytic Enzymes**: Break cell-cell and cell-matrix contacts for endothelial cell motility.
2- **Soluble Factors (e.g. Cytokines and growth factors)**: Stimulate migration, proliferation and differentiation.

- ✓ VEGF (5 members): Vascular Endothelial cell growth Factor (potent, EC specific mitogen)
- ✓ FGF (20+ members): Fibroblast growth factor (including acidic and basic FGF)
- ✓ TGFb(3 members) : Transforming growth factor beta (1-3, dose dependent effects)

3- **Stress Factors (e.g. hypoxia, glucose deprivation etc):** Regulate gene expression of many angiogenicfactors.

4- **Cell adhesion molecules**: Cell positioning / differentiation

Proteolytic Enzymes

- ✓ Matrix Metalloproteinases(MMPs 1-22)- up-regulated in most of tumours
- ✓ Tissue Inhibitors of MetalloProteases(TIMPs)- block the angiogenesis response.

However:

A balance is required between **MMPs** and **TIMPs** for angiogenesis. When the level of proteolysis is too high, angiogenesis is inhibited.

Under normal circumstances matrix remodelling is a tightly controlled process balanced by signals promoting and inhibiting proteolysis'

In Repair: PA (plasminogen activator)and MMPs are secreted with their inhibitors ensuring a definite start and stop to the process.
In disease: Regulation is lost during tumour growth and metastasis leading to excessive MMP and PA activity in solid tumours and enhancing angiogenesis.

Growth Factors:

VEGF-A: Clinical Importance

1- Produced by the majority of tumours

2- Autocrine signalling by tumour

3- Correlation of VEGF levels with: Tumour burden and survival

4- VEGF-A –VEGFR-2 major growth factor axis in tumour angiogenesis

Other Soluble Stimulating Factors

- **Proteins**
- Angiogenin
- Interleukin-8
- Tumour Necrosis Factor alpha
- Thymidine Phosphorylase
- Fibrin
- Fibrin E-fragment
- Thrombin

- **Small Molecules**
- Adenosine
- Nicotinamide
- Prostaglandins
- nitric oxide

Stress Factors

The angiogenic switch

1- As tumours grow they alter their microenvironment:

✓ decrease pH
✓ decrease concentration of nutrients
✓ increase in interstitial pressure
✓ decrease in oxygen tension (hypoxia)
✓

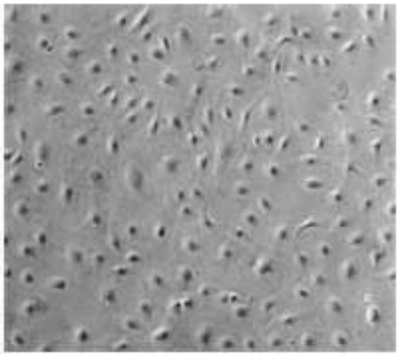
2- Cells sense these changes and respond by generating angiogenicmolecules e.g. VEGF.

3- An extended blood vasculature resulting from angiogenesis relieves the pressures from these stress factors

4- Cancer cells that have acquired angiogenic phenotype are capable of inducing phenotypic changes in ECs.

Assays to Study Angiogenesis

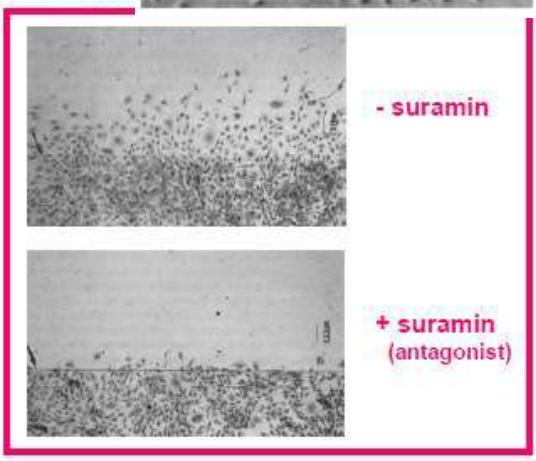
Endothelial cells: The cell type that is involved most in angiogenesis;
Can be isolated and studied in culture

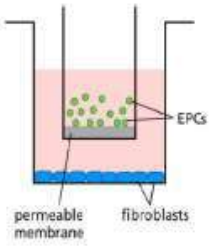


- **Intracellular molecular signalling events**
- **Interaction with ECM(*extracellular matrix)* proteins**
- **Migration**
- **Proliferation**
- **Tubule Formation**

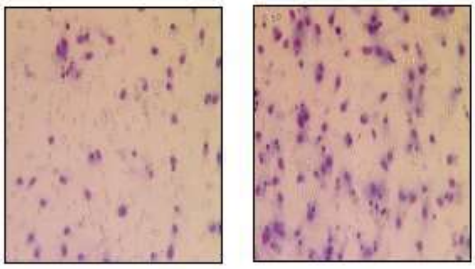
In vitro Assays of Angi

- **Endothelial cell Migration:**
- Boyden chamber
- 'Wound healing'
- Phagokinetic track assay





Endothelial precursor cell migration across a Boyden chamber



Migration of EPC across a filter in response to nothing or VEGF

31

Endothelial cell Proliferation:

Net cell number :

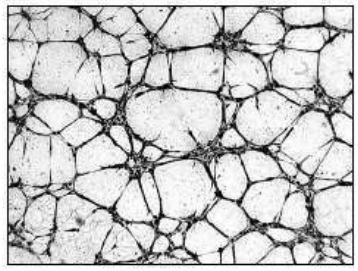
- Haemocytometer
- Coulter Counter
- MTT (assays mitochondrial activity)

Cell cycle kinetics

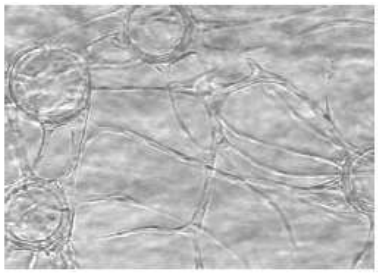
- Thymidine incorporation (DNA synthesis)

Tubule formation:

- Matrigel
- Collagen
- Fibri
- 3D-cultures in gels or on beads



Tubules on Matrigel



Tubules in 3D culture on beads

Targeting Tumour Angiogenesis

'Importance of angiogenesis to tumour growth and metastasis suggested that the tumour vasculature could be a potentially effective target for therapy'

Problems with conventional therapies:

- Delivery of blood borne agents to tumour cell targets in solid tumours has proved difficult due to

1. Abnormal blood flow
2. Lack of lymph drainage
3. High interstitial pressure
4. Resulting in poor access to the majority of tumour cell mass

- Tumour cells are inherently unstable and quickly become resistant to therapy

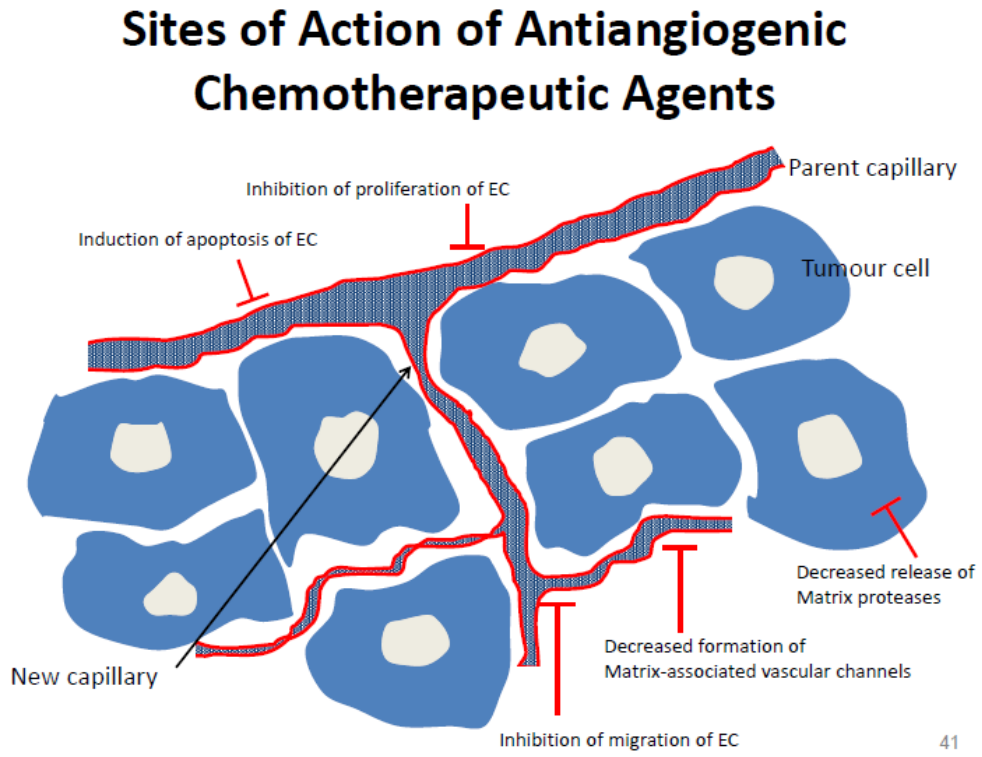
Targeting tumour-associated endothelium

- The only proliferating endothelium within the body of an otherwise healthy adult

- Tumour vasculature targeting would also simultaneously affect any metastases

Therefore <u>drugs are being developed which target different parts of the angiogenic pathway</u>?

Destruction of the tumour vasculature would lead to the **demise of tumour by depriving it of oxygen and nutrients** essential to its growth'



Summary Angiogenesis

- Essential in health
- Up regulated in a number of disease states, including cancer
- Regulated by a balance between pro-and anti-angiogenicfactors
- Controlled by proteolyticenzymes, growth factors, stress and celladhesion molecules
- Studied *in vitro* using functional Endothelial cell assays and *in vivo*
- Anti-angiogenicagents have been designed for use against cancer

These new agents are beginning to show efficacy in combination with conventional

Lecture 7: Carcinogenesis: why some people develop cancer

- Many people develop cancer over their lifetime.
- **Lifetime cancer risk is 1 in 2 to 1 in 3 in the developed world**.
- **Why does cancer affect some people and not others?**

The good news - Cancer survival rates are improving: Success in improving survival rates is down to improved detection and treatment.

But what factors influence an individual's chances of developing cancer?

A. **Age**
B. **Heredity (Genetic factors)**
C. **Environment/ life style**

A- Cancer and age - The incidence of most cancers increases with age (age-related diseases)

B- Heredity (Genetic factors) the effects of the genes inherited from Mum and Dad

1. **Hereditary cancer syndromes:**
   - **Individual inherits a defective version of a tumour suppressor gene e.g. p53, Rb, BRCA1, BRCA2**
   - **Rare in the population (<0.1%).**
   - **Confer high risk of early cancer development**

2. **Common polymorphisms**

Individual inherits one or more weaker acting genetic polymorphism (variants) that alter the risk of developing one or more types of cancer

- Can be common in the population (~10%), frequency and significance may vary between racial groups
- Modest increase (or decrease) in probability of developing cancer
- May depend on other genetic factors or on environmental factors
- May include DNA repair genes or genes encoding metabolic enzymes that activate or deactivate carcinogens (more later)
- Example – several loci are associated with elevated risk of lung cancer

❖ **-Hereditary factors can affect cancer risk –**

For some types of cancer there can be a strong genetic family link....and genetic testing can lead to possible preventative strategies…Along with genetic testing, counseling is essential

Angelina Jolie-Pitt:

Her mother diagnosed with ovarian cancer in her 40s

Carries a faulty BRCA1 allele which confers very high risk of breast and ovarian cancer

Chose to have preventive bilateral mastectomy with reconstructive surgery, and later surgery to remove ovaries and fallopian tubes

Note *BRCA1/2*- associated familial breast cancer cases make up ~2% of all BC cases

Note, numerically, most cancer cases **are not associated with** inherited defects of a tumour suppressor gene such as *P53* or *BRCA1*.

That is, they arise **sporadically** via **acquired defects in oncogenes and tumour suppressor genes.**

However, some of the same genes are involved, for example inherited *p53* defects are the cause of most Li Fraumeni syndrome cases, and *p53* is very frequently altered in sporadic cancers where it has acquired a mutation during the development of the tumour.

Impact of inherited *BRCA1* and *BRCA2* (mutations)risk alleles on cancer risk – much higher incidence and earlier onset

TYPE OF CANCER	RISK IN GENERAL POPULATION	BRCA1 CARRIER	BRCA2 CARRIER
Breast cancer - women	12%	40-87%	18-88%
Ovarian cancer	1-2%	22-65%	10-35%
Breast cancer - men	0.3%	small increase	up to 6%
Prostate cancer	12%	small increase	up to 35%
Other cancers		slight increase	slight increase

BRCA1 and *BRCA2* are not the only genes associated with hereditary cancer syndromes

- **Examples of hereditary cancer syndromes and the associated genes:**

***P53* – Li Fraumeni syndrome - multiple early-onset cancer diagnoses within families, onset often in children or adolescents**

***BRCA1 & BRCA2* – Early onset breast and ovarian cancer**

***Rb* – Retinoblastoma**

***XP* genes (DNA repair of UV DNA damage) – Xeroderma pigmentosum**

***FPC* - Familial adenomatous polyposis**

***MLM1, MSH2, MSH6* (mismatch repair genes) - Hereditary non-polyposis colon cancer**

All these genes have a role in maintaining genome stability – later

❖ Genetic factors in lung cancer

an example of genetic polymorphism and cancer risk

- **>80% of the population risk of lung cancer** can be ascribed to tobacco smoking, but several lines of evidence indicate that **inherited genetic factors influence the development and progression of lung cancer in smokers**.
- Epidemiological studies have consistently shown an elevated risk of **lung cancer in relatives of lung cancer cases after adjustment for smoking**.
- **Genome-wide association studies** have identified common variants **(SNPs)** at **15q25,** 5p15.13, and 6p21.33 that **are associated with lung cancer risk.**
- **Mechanisms?**
  - **15q25** contains the nicotinic acetylcholine receptors *(CHRNA3 & CHRNA5)* polymorphisms of which may be associated with nicotine dependence .
  - **5p15.13 locus** *TERT* - *CLPTM1L* – roles in genome stability
  - **6p21.33 locus** *BAT3* - *MSH5* – DNA repair

Genetics can interact with lifestyle and/or environmental factors to affect cancer risk

…although *BRCA1* mutation carriers have a greatly elevated lifetime risk of BC, mutation carriers born in the earlier part of the 20th century appear to have a lower risk of cancer than those born later.

…the reasons for this are unclear, but may include diet, earlier menarche and later first pregnancy….

C – Environment

- Exposure to carcinogenic substances
- Radiation
- Oncogenic viruses and bacteria

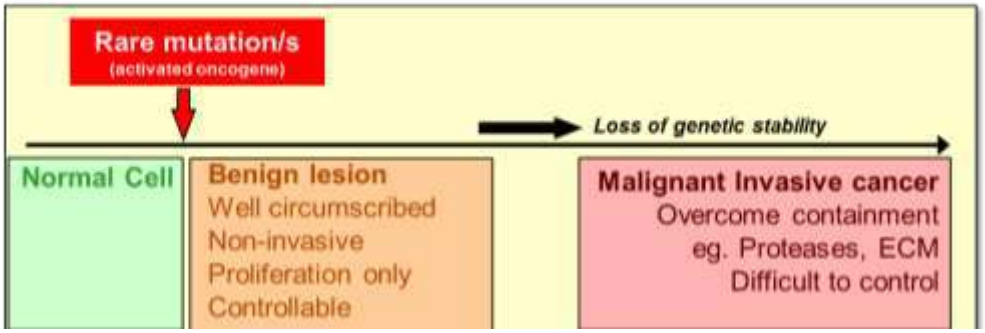
Lifestyle

- Diet
- Smoking
- Weight
- Exercise

What are carcinogens and how do they lead to cancer?

- **Carcinogens interact with DNA and cause DNA damage (INITIATION)**
- **Increase conversion of DNA damage to permanent mutation (PROMOTION)**
  - Increasing cell division
  - Increasing error prone DNA-repair / decreasing accurate repair?
  - Increasing survival of damaged cells / reducing apoptosis

- **Tumours are initiated by somatic mutation leading to activation of oncogenes and loss of tumour suppressor gene function.**
- **Carcinogens cause point mutations and/or chromosome rearrangements that have the potential to activate oncogenes and inactivate tumour suppressors.**



❖ **DNA double strand breaks generated by exogenous DNA damage**

Exogenous DNA Damage	Dose Exposure (mSv)	DNA Lesions Generated	Estimated Number Lesions/Cell
Chest X-rays	0.02	DSBs	0.0008
Dental X-rays	0.005	DSBs	0.0002
Mammography	0.4	DSBs	0.016
Body CT	7	DSBs	0.28
Head CT	2	DSBs	0.08
Coronary angioplasty	22	DSBs	0.88
Tumor PET scan (^{18}F)	10	DSBs	0.4
^{131}I treatment	70–150	DSBs	2.8–6
External beam therapy	1800–2000	DSBs	72–80
Airline travel	0.005/hr	DSBs	0.0002/hr
Space mission (60 days)	50	DSBs	2
Chernobyl accident	300	DSBs	12
Hiroshima and Nagasaki atomic bombs	5–4000	DSBs	0.2–160

Although efficient repair mechanisms exist, DSBs can lead to deletions, duplications and gross chromosome translocations – see later lectures. These are mechanisms that can inactivate a tumour suppressor gene (deletion) or activate an oncogene (duplication, chromosome translocation)

DNA damaging agents including certain alkylating agents and ionising radiation are also used to treat cancer (chemotherapy and radiotherapy.

Not all DNA damage is caused by exogenous agents such as chemical carcinogens or radiation. DNA damage also occurs as a result of endogenous cellular processes.

❖ Endogenous DNA damage and potential for mutation

Estimated rates of endogenous DNA damage

Endogenous DNA Damage	DNA Lesions Generated	Number Lesions/Cell/Day
Depurination	AP site	10000
Cytosine deamination	Base transition	100–500
SAM-induced methylation	3meA	600
	7meG	4000
	O^6meG	10–30
Oxidation	8oxoG	400–1500

Depurination – Loss of purine base, leaving an apurinic site (AP site) repaired by base excision repair (BER).

Cytosine deamination – cytosine converted to uracil* through deamination. Most repaired by uracil glycolase and BER. Since uracil base pairs with adenine, replication yields a permanent C-G to T-A transition mutation.

Methylation – See earlier slide, O^6-MeG is associated with permanent G-C to T-A transition.

Oxidation – Most common DNA lesion resulting from oxidative damage is 8-oxo-gunanine. 8oxoG is repaired by BER employing the gycolase OGG1. 8oxoG is associated with permanent G-C to T-A transition (mitochondria have their own OGG1 enzyme).

The accumulation over time of somatic cell mutations resulting from exogenous and endogenous DNA damage is a major factor in the overall age profile of cancer

Strength of evidence for an increased risk of cancer due to tobacco consumption

Cigarette smoking	**Sufficient:**	Lung, oesophagus, larynx, pharynx, oral cavity, pancreas, bladder, nasal cavity and sinuses, stomach, liver, kidney, cervix, myeloid leukaemia, bowel and ovary (mucinous)
Environmental tobacco smoke	**Sufficient:**	Lung
	Possible:	Bladder, larynx
Pipe and cigar smoking	**Sufficient:**	Lung, oesophagus, larynx, pharynx, oral cavity, liver, bladder, bowel, stomach and pancreas
Smokeless tobacco	**Sufficient:**	Oral cavity, pancreas, oesophagus

❖ BMI*, Diet and cancer risk

BMI (obesity) “Major studies confirm that being overweight or obese increases your risk of various cancers. The WHO says that overweight and obesity are the most important known avoidable causes of cancer after tobacco”.

Strength of evidence for an increased risk of cancer in relation to dietary factors and alcohol

Factor	Sufficient	Probable
Alcohol	Bowel, breast, larynx, liver, oesophagus, oral cavity, pharynx	
Fat		Breast
Fibre		Bowel
Fruit and vegetables		Larynx, lung, oesophagus, oral cavity, pharynx, stomach
Milk		Bowel
Processed and red meat	Bowel	
Salt		Stomach

Red denotes increased risk and blue denotes reduced risk

Probable mechanisms – promoting / suppressing production of carcinogens in the gut (bowel cancer), production and storage of oestrogen (breast cancer), irritant (alcohol).

Processed meat products can contain chemicals that form during meat processing or cooking including N-nitroso compounds and polycyclic aromatic hydrocarbons that are known carcinogens.

❖ Hormones and cancer risk

Reproductive hormones summary box

Evidence for an association between exogenous hormones and cancer

	Sufficient
Combined oral contraceptives	Endometrium Ovary Breast Cervix
HRT (oestrogen-only)	Breast Endometrium Ovary
HRT (oestrogen-progestagen)	Breast Endometrium Ovary

Red denotes positive association and blue denotes negative association

Effects quite small: For breast cancer there are likely to be 5-20 extra breast cancers diagnosed in every 1,000 women taking combined oestrogen and progesterone HRT for 10 years. In the case of oral contraceptive the increased risk of breast cancer is slight and transient.

Probable mechanism – oestrogen promotes growth of early tumour cells in oestrogen-responsive tissues – greater number of cells in which further changes can occur.

❖ Viruses, bacteria and cancer

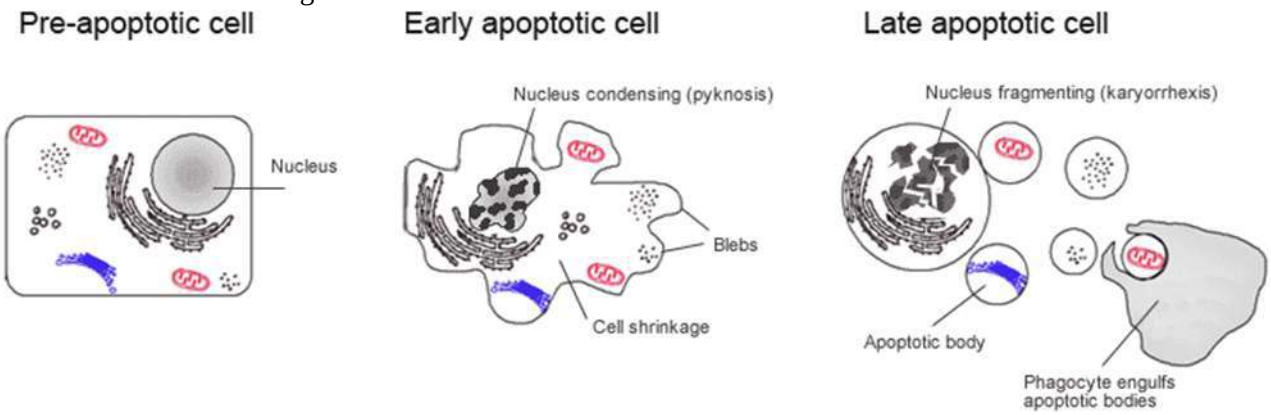
Infection with some microbes increases the risk of certain cancers

Virus	Cancer
EBV – Epstein Barr Virus	Hodgkin's lymphoma, Burkitt's lymphoma, nasopharangeal carcinoma
HPV – Human Papilloma Virus	Cervical carcinoma *(vaccine now in use)*
Helicobacter pylori (a bacterium)	Stomach cancer Estimated that ~30% of stomach cancers are linked to *H .pylori* infection
HTLV – Human T-cell Leukaemia Virus	Leukaemia
KSHV - Kaposi's sarcoma-associated herpesvirus	Kaposi's sarcoma
Hepatitis viruses	Liver cancer (Hepatocellular carcinoma) Chronic Hep B or C infection estimated to account for 75-80% of liver cancers worldwide, but probably <20% in UK

Lecture 8: Apoptosis

What is apoptosis?

- Programmed cell death.
- Series of biochemical events that lead to characteristic cell changes and cell death / "recycling".
- These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.



- Apoptosis is regulated by carefully controlled cell signalling pathways.
- In the context of carcineogenesis, apoptosis also provides a robust self-destruct mechanism for damaged cells and is a crucial anti-cancer mechanism.

How is apoptosis relevant to cancer biology?

1. **Unrepaired or excessive DNA damage leads to apoptosis**. This prevents damaged DNA (esp **DNA DSBs** and unrecoverable replication fork damage) converting to mutations in progeny cells, i.e. prevents the propagation of deleterious mutations.

2. **Inappropriate growth signals (such as those resulting from oncogene activation) can lead to apoptosis**. So activation of a growth-promoting oncogene can be pro-apoptotic (archetype c-MYC).

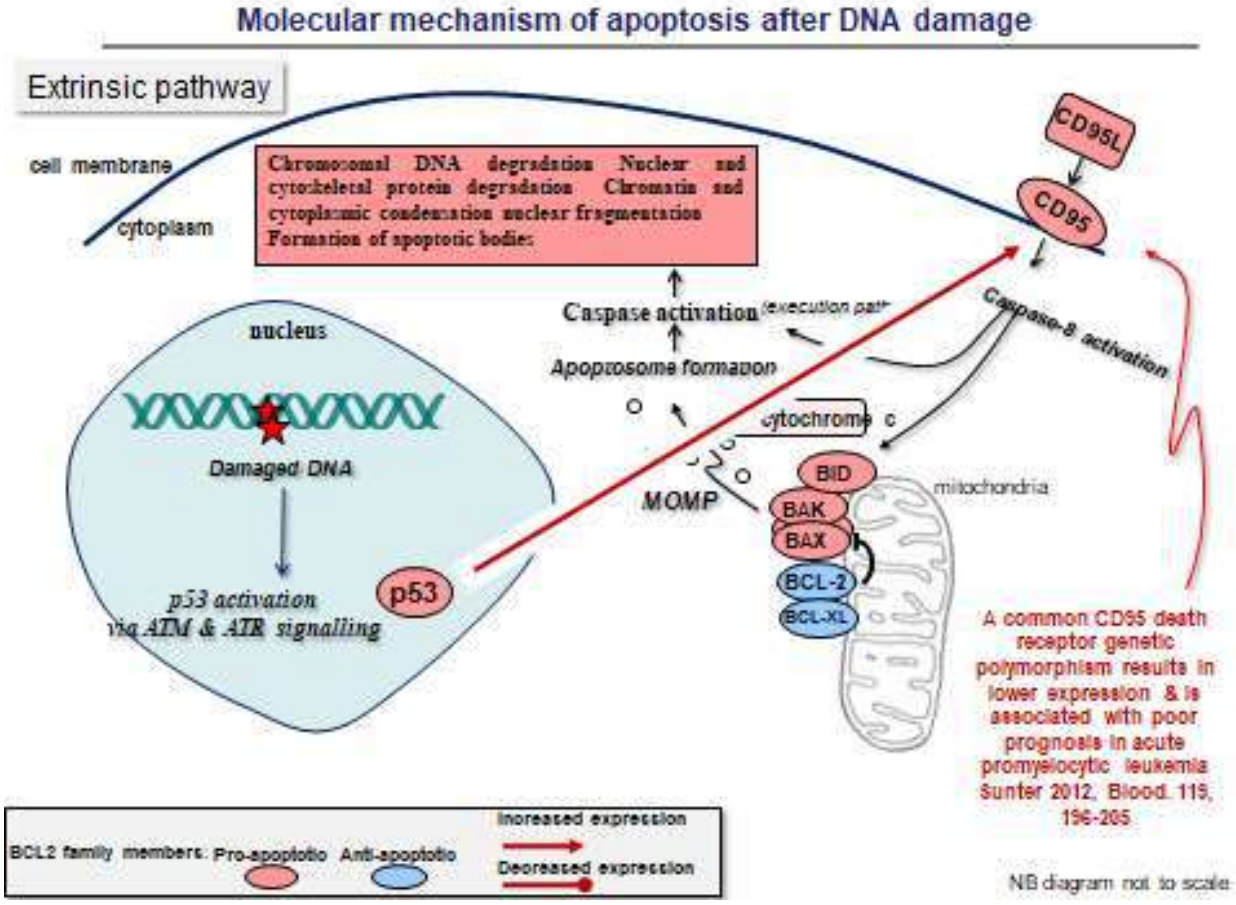
3. **As a result, apoptosis is a strong tumour suppressor mechanism and evasion of apoptosis is a key feature of malignant tumours.**

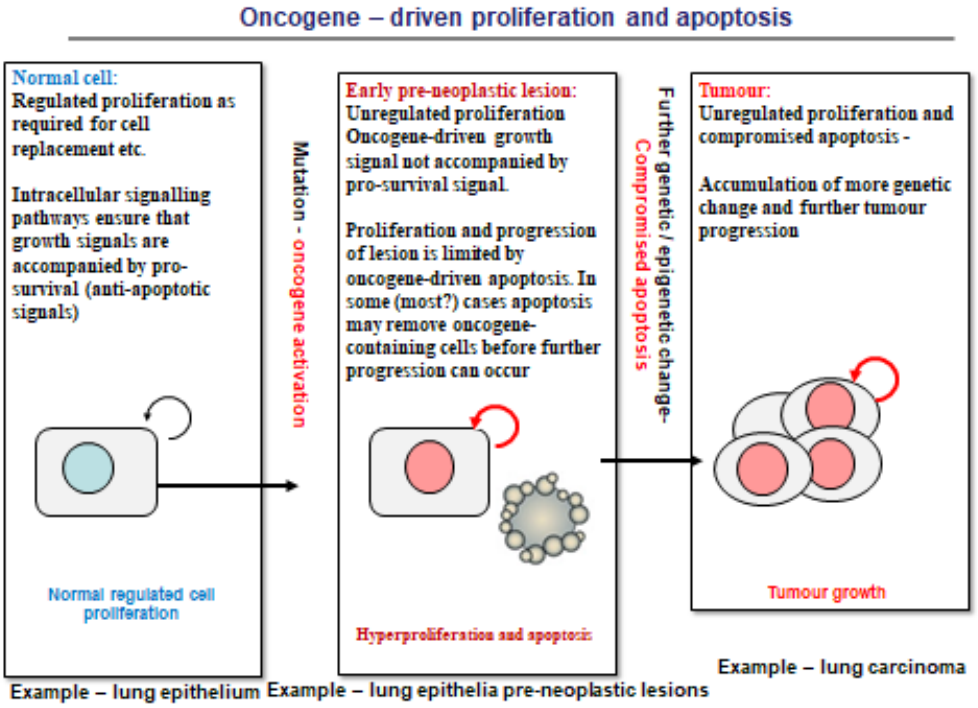
BCL-2 and the regulation of apoptosis

- The BCL-2 family of proteins comprises both pro-apoptotic and anti-apoptotic (pro-survival) members. They are key regulators of apoptosis and control mitochondrial outer membrane permeabilisation (MOMP, see next slide).

- The balance of **pro-apoptotic** versus **anti-apoptotic** BCL-2 family members determines a cell's sensitivity to apoptotic signals.

Pro-apoptotic	Anti-apoptotic
BAX, BAK, BOK	**BCL-2, BCL-$_{XL}$**, BCL-$_{w}$, A1
BID	MCL1
BIM, BIK, BAD, BMF,HRK	
NOXA, PUMA	
Induce mitochondrial outer membrane permeability (MOMP). NOXA and PUMA transcriptionally induced by p53	Preserve mitochondrial integrity and prevent release of cytochrome C (Inhibit MOMP)





Apoptosis & Senescence notes

Apoptosis and Cancer

In normal growth (i.e. during development **wound healing**, or in tissues with a high **cellular turnover**) growth signals are accompanied by finely balanced **pro-survival / pro-apoptotic signalling**. Activation of a growth (proliferation)-promoting oncogene provides a growth signal "**out of context**" and results in apoptosis. In addition, **most normal cells do not express telomerase** and have a **limited proliferative life time**. Activation of an oncogene that stimulates uncontrolled growth soon reaches this limit and telomere shortening results in telomere dysfunction. This results in **damage-signalling promoting apoptosis or cellular senescence**, where the cell **permanently exits the cell cycle**.

The **Ras oncogene promotes cell survival** (i.e. reduces sensitivity to pro-apoptotic signals). Thus, Ras (pro-survival) and Myc (uncontrolled growth) oncogenes for example can **act together (synergistically) in oncogenesis**.

Control of apoptosis, the intrinsic pathway

- The key regulatory step in apoptosis is mitochondrial outer-membrane permeabilisation (**MOMP**) which is accompanied by release of **cytochrome C** from the mitochondria into the cytoplasm. Cytochrome C interacts with a protein called **APAF-1**, causing a conformational change in APAF-1 and its oligomerization to form the **apoptosome** or "**wheel of death**" as it is sometimes known.

- The **apoptosome** activates **caspase 9**, which in turn activates **caspases 3 and 7** that carry out many of the apoptotic process including **chromosomal fragmentation**, **cytoskeletal protein degradation**, **condensation of chromatin and cytoplasm**, **nuclear fragmentation** and **formation of apoptotic bodies**.

- **MOMP** is controlled by **BCL-2 family members**. Anti-apoptotic BCL family members including **BCL-2 and BCL-XL** are potent inhibitors of apoptosis and work by **guarding mitochondrial membrane integrity** and thus **preventing the release of cytochrome C.** Upregulation of BCL-2 is common in cancer. Pro-apoptotic proteins BAX and BAK induce permeabilization of the mitochondrial outer membrane and efflux of **cytochrome C** into the cytoplasm. BAK and BAX are preset in inactive forms in non-stressed cells. Other **pro-apoptotic BCL** family proteins including **NOXA** and **PUMA** are regulated at the transcriptional level by **p53**, they **antagonise the effect BCL-2** and **BCL-XL**. Apoptosis induced by DNA damage is largely dependent on p53 activation (see later for details of upstream events activating p53), but precise mechanism probably depends on cell type. The pro-apoptotic factors BAX, NOXA and PUMA are direct transcriptional targets of **p53**, thus **p53** activation raises their cellular levels. **BCL-2** and **BCL-XL** are antagonised by p53.

- A p53-independent pathway involves the p53-related factor p73, which upregulates the expression of **PUMA**.

- The pathway as described above is known as the ***Intrinsic Pathway***.

Control of apoptosis the extrinsic pathway

- In the ***extrinsic pathway*** apoptosis is initiated by the cell-death receptor, CD95, located at the cell membrane. CD95 activation leads to activation of the upstream caspase, **caspase-8** which can lead to activation of the execution pathway independently of MOMP, but can also lead to MOMP via activation of the proapoptotoc BCL-2 family member BID.

- **CD95** is activated by binding of CD95L/FAS ligand, a process which is important in immune development.

- CD95 and CD95L expression are enhanced by **DNA damaging agents** and CD95 expression is induced by p53. In some cell types at least this death-receptor pathway is important for the apoptotic response to DNA damaging agents.

Oncogene activated apoptosis

- In normal cells mitogenic signals (growth factors) lead to upregulation of MYC which promotes cell cycle progression.
- Thus, deregulation of MYC in an otherwise normal cell might be expected to lead to uncontrolled proliferation.
- But in fact it can sensitise cells to apoptotic signals and lead to a much higher rate of apoptosis.
- This works through **$p14^{ARF}$** which responds to proliferative signals through MYC. **When these exceed a threshold** (such as in the case of overexpressed MYC) $p14^{ARF}$ triggers a p53-dependent response that includes growth arrest and apoptosis. This is achieved by **$p14^{ARF}$** interference with **HDM2**, **leading to increased p53 activity**.
- In normal cells **mitogenic signals**(growth factors) not only up-regulate MYC, but also activate pro-survival (**anti-apoptotic**) pathways which include the **RAS / PI3** kinase signalling pathways and **up-regulation of AKT** which increases the activity of HDM2, maintaining p53 at low levels. Normal cell proliferation depends on the fine balance between **proliferation** and **survival/apoptotic factors**.
- Oncogenic activation of RAS provides a **pro-survival signal** that antagonises the pro-apoptotic effect of **MYC overexpression**.
- Thus MYC and RAS are said to **cooperate in transforming cells**.
- Note – **$p14^{ARF}$** is a tumour suppressor, and loss of function of $p14^{ARF}$ is common in cancer.
- Up-regulation of ARF is not the only way that **oncogenic signalling** can lead to apoptosis.
- The unscheduled and uncontrolled growth signal from an activated oncogene (in this example, MYC) leads to oxidative stress (through increased metabolism) and replication stress. The latter includes more **frequent replication fork errors**, at least partly due to falling nucleotide pools. Replication stress and oxidative stress result in the accumulation of **DNA DSBs** and activation of the DNA damage response (see lecture 13), which leads to increased levels of **p53**. Signs of oncogene-driven DNA damage can be seen in early neoplastic lesions.

Cellular senescence

- Cellular senescence, like apoptosis, provides a **potent anti-tumour mechanism**. The respective signalling pathways utilises distinct but overlapping signalling molecules. In experimental systems MYC-driven p53 induction leads primarily to apoptosis, while **oncogenic RAS provokes cellular**

senescence. In vivo however, the mechanisms that determine whether the apoptotic or a senescent response is activated in response to oncogene signalling are poorly understood.

- Tumour development relies on loss of activity of these "**failsafe**" **mechanisms**.

Lecture 9 : Epigenetic Modifications and Cancer

Epigenetics is one of the most rapidly growing fields of biology and can be defined as heritable alterations in the patterns of gene expression that occur without any change in the DNA sequence. **DNA methylation** and **histone modifications** are the major mechanisms of epigenetics regulation. These mechanisms play a fundamental role in the **control of chromatin structure** and the **transcriptional activity of genes**. However, even though they are **heritable alterations**, in contrast to genetic aberrations epigenetic changes are **reversible phenomena** in response to environmental exposure or **pharmacological intervention**.

DNA methylation has been the best studied epigenetic change. It plays (1) an essential role in normal mammalian **development**, (2) **embryogenesis**, (3) cellular differentiation, (4) **chromosome integrity**, (5) **control** of **DNA repair** and **replication**. (6)**Regulation of gene expression** through altered DNA methylation is critical for several developmental processes, such as **X-chromosome inactivation** and **genomic imprinting**.

The DNA methylation of promoter CpG islands is associated with the **transcriptional inactivation** (gene silencing) of the associated gene. This gene repression can occur via **two potential mechanisms**:

Firstly, some transcription factors are unable to bind to their recognition sequence if it contains a methylated cytosine.

Secondly, the recruitment of methyl-CpG binding domain (MBD) proteins to methylated cytosine sites results in the blocking of transcription factor binding sites and an increase in chromatin compaction. Therefore, in contrast to generally silent heterochromatic regions in terms of gene expression, transcribed genes may be present in methylation-free regions that are highly accessible to transcription factors, or in **hyper-acetylated** chromatin regions through the action of histone acetyltransferases (***HATs***)

DNA methylation

DNA methylation involves the **addition of a methyl group** at position C5 of the cytosine ring. The genomic methylation status **is maintained by** three methyltransferases (*DNMT1, DNMT3a* and *DNMT3b*) that use S-adenosyl-methionine as the methyl donor source (Figure 1). The vast majority of DNA methylations occur in the context **of a CpG dinucleotide** (i.e. a cytosine immediately followed by a guanine, which is called a **CpG site**).

In general, CpG sites comprise approximately **1%** of the mammalian genome. However, these CpGs are underrepresented in the genome, probably because 5'methylcytosine (5mC) has a comparatively high mutation rate due to its spontaneous deamination to thymine. It is interesting that the CpG sites across the genome are **not distributed randomly**; in fact, up to **85%** of these CpGs are methylated and based mainly in the genome repetitive regions. The remaining **15%** of the CpGs are largely methylation-free and are preferentially found in clusters of short DNA sequence called **CpG islands**.

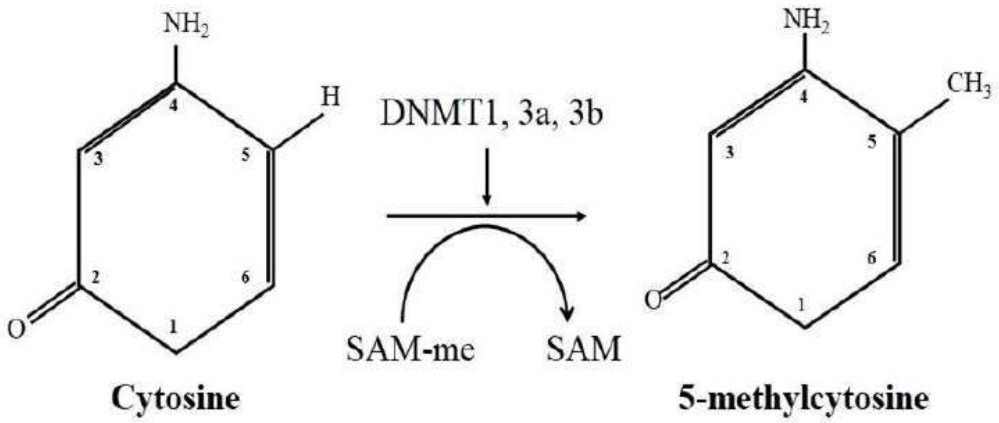


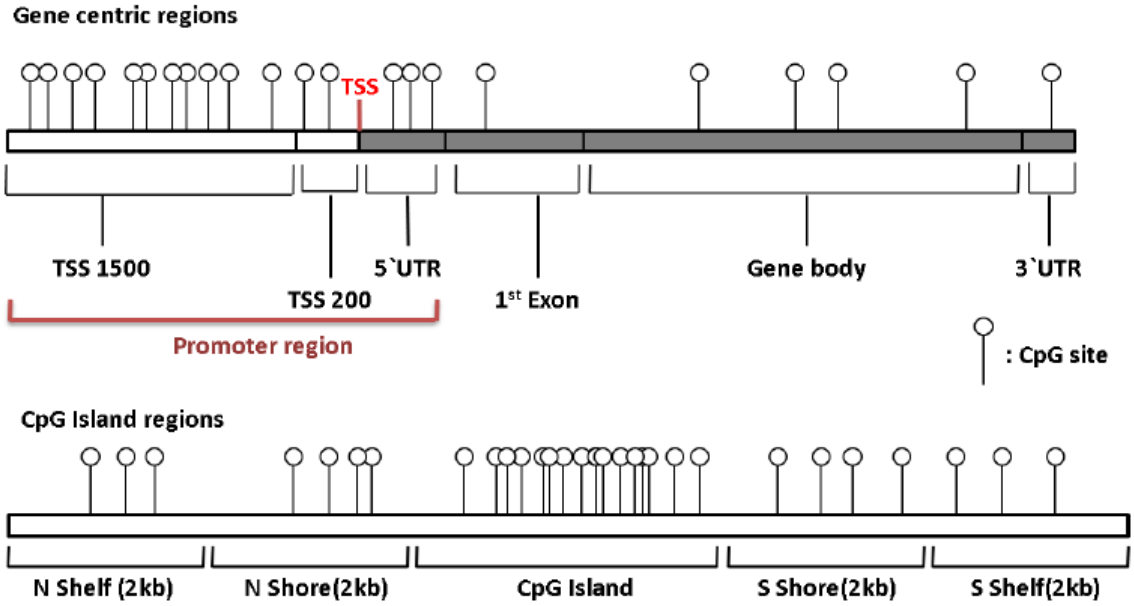
Figure : Conversion of cytosine into 5-methylcytosine through the action of DNA methyltransferase enzymes (*DNMT1*, 3a and 3b).

DNA methylation across the genome

DNA methylation could be present at **CpG sites** in **different genomic region** such as CpG islands of **gene promoters** (within transcription start sites), **gene bodies**, **intergenic regions** (such as repetitive sequences) and also at **regulatory elements** such as **enhancers** and **insulators** (Figure2).

DNA methylation patterns in different genomic regions could have **divergent effects on transcription** and this effect seems to vary with context. While CpG island promoter hypermethylation is usually reported to be associated with transcription silencing, DNA methylation at intergenic regions (IGR, a stretch of DNA sequence placed between genes), gene bodies, and transcription termination sites regions (TTSs) has been found to be either positively or negatively correlated with expression depending on specific genes and locations. DNA methylation

at gene bodies **blocks the transcription** of the **repetitive** and **transposal elements** (such as such as **retroviruses**, **LINE1 elements**, ***Alu* elements**) while allowing transcription of the host gene to run through them [121].



Main function of DNA methylation in healthy(normal) cells

1- Genomic imprinting

Genomic imprinting can be defined as the epigenetic marking of genomic loci on the basis of parental origin, leading to the **monoallelic expression** of the associated gene. DNA hypermethylation at one of the two parental alleles of a gene is required for genomic imprinting to ensure single allelic expression.

2- X-chromosome inactivation

A similar epigenetic **dosage reduction** effect has been described in X-chromosome inactivation in females to ensure that males and females have the same level of expression from X-chromosome-associated genes. Studies have shown that the DNA methylation of the inactivated X chromosomes has a significant impact on maintaining its inactive situation.

3-Suppression of mobile elements

Mobile elements (also called endo-parasitic sequences) are widespread genomic reparative sequences which have the ability (when they are activated) to replicate and insert themselves in different genomic regions. **To reduce transcriptional noise in normal cells**, heterochromatin and mobile elements are epigenetically silenced and inactivated by hypermethylation.

4- Chromosomal stability

The epigenetics deactivation of these repetitive elements suppresses their mobilization and thus has an essential role to ensure genomic stability and prevent the chromosomal instability associated with translocation-mediated

gene interruption. DNA global hypomethylation is one of the hallmarks of tumorigenesis that mainly occurs in the mobile repetitive regions.

5- Tissue-specific expression

All normal cells in the body **have identical DNA sequences**; however, these cells may have **different epigenetics marks** including DNA methylation and histone modifications. Accordingly, **tissue-specific gene expression** is partially regulated by DNA methylation at CpG

Histone modifications

Histones are **nucleoproteins** that form the structure of **chromatin (DNA+ protein)** in which 147 bp of DNA is wrapped on an octamer of four core histones. These include two molecules each of histones H3, H4, H2A and H2B. These core histones are globular proteins with N terminal tails that can be targeted by multiple different types of modification. Histones are not only DNA packaging molecules but are also crucial in the regulation of gene expression. Histones can undergo several post-transitional modifications, such as acetylation, methylation, phosphorylation, glycosylation, sumoylation and ubiquitylation. These modifications, which are collectively referred to as the "**histone code**", are central in controlling the structural configuration of **nucleosomes** and as a result regulating the accessibility of DNA for the binding of transcriptional factors to their target sequences.

Histone acetylation (hyper-or hypo-acetylation) changes the physicochemical features of the associated proteins and consequently affects the electrostatic attraction properties between negatively charged DNA and positively charged histones in the nucleosomes. Actively transcribed genes are usually associated with open nucleosomal configuration and **hyper-acetylated chromatin regions** that are readily accessible to transcription factors. The action of histone acetyltransferases (*HATs*) results in chromatin hyper-acetylation which leads to **relaxed and transcriptionally active chromatin,** whereas heterochromatin regions are generally associated with **closed chromatin structures** and the **repression of transcriptional activity**. Such regions exhibit hypo-acetylated nucleosomes, which is catalysed by the action of histone deacetylases (*HDACs*).

Interaction between DNA methylation and histone modification and configuration of the chromatin structure

Epigenetics modification involves the collaboration of all epigenetics modifiers with each other. DNA methylation and histone modifications interact together at specific chromatin regions, activating or repressing the transcription of a particular gene.

For example, *MeCP2* (methyl CpG binding protein 2) specifically recognises and binds to methylated CpG sites allowing the recruitment of multiple-protein compositions, including chromatin-remodelling proteins in addition to histone-modifying enzymes such as histone deacetylases (*HDACs*) and histone methyltransferases (*HMTs*) (Figure 4). Additionally, *DNMT3L* (a member of the DNA methyltransferase family) interacts particularly with histone H3 tails leading to *de novo* DNA methylation, and this reaction is shown to be intermediated by the

recruitment of *DNMT3A*. *H3K4*me histone modifier (a trimethylation of histone *H3 Lys 4*) is a mark that is associated with active genes and promotes transcription.

Comparison (and advantages) of DNA methylation with other potential 1.1.5biomarkers

Developing epigenetic biomarkers has so far concentrated almost exclusively on DNA methylation due to both **practical applications** and the known and consistent changes in DNA methylation found in cancer. DNA methylation is also the most studied epigenetic modification, as **it is:**

1- Stable (compare to gene expression) and can be obtained suing non-invasive procedures such as blood, stool, saliva or urine sample analysis have considerable advantages over invasive methods that may require surgical intervention, such as biopsies. Also it can be obtained from conserved tissue samples.

2- DNA methylation is more stable than RNA or proteins as well as easier to detect than histone modifications which tend to be more dynamic and whose detection depends on the utility of antibodies that are dissimilar in performance.

3-Methylation analysis also has some **advantages over genetic analysis**. The routine analysis of multiple genes for point mutations can **be time-consuming** since mutations for most genes can occur across the length of the gene. In comparison, DNA methylation-based gene inactivation is primarily related to the hypermethylation of promoter-associated CpG islands and can thus usually be identified by screening a single small region for each gene.

4-The occurrence **of high numbers of DNA methylation alterations** in malignancies, compared to **genetic aberrations**, has been seen to lead to **higher sensitivity**. The figure below (Figure 5) shows biomarkers discovery approaches.

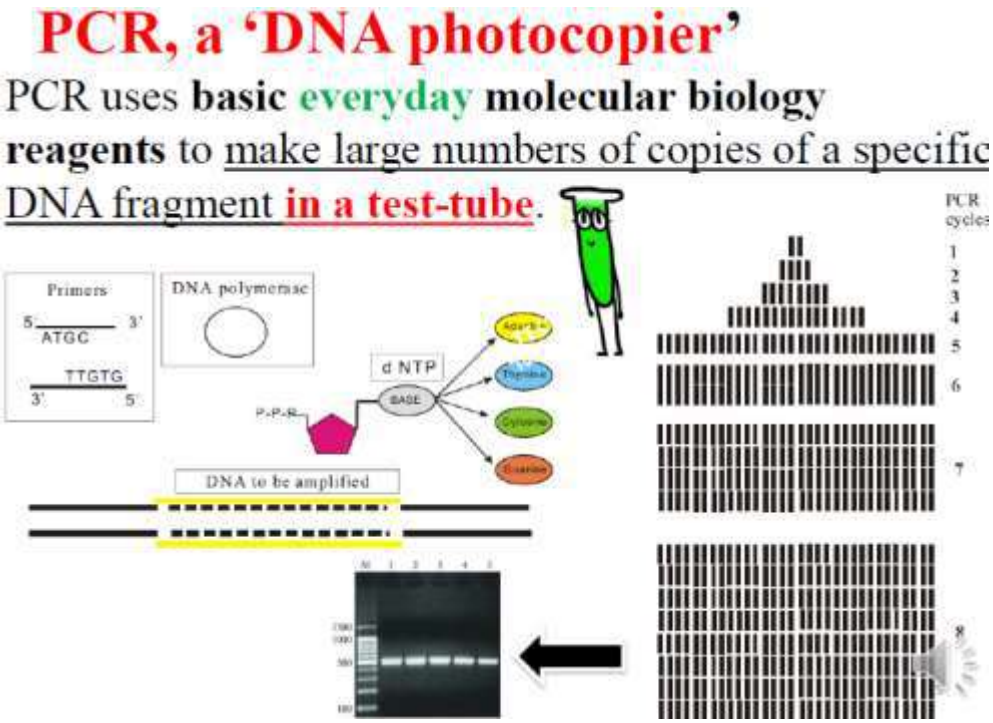
Other related topics of epigenetic

- Cancer epigenetics (inactivation (silencing) of tumor suppressor genes, activation of oncogenes and genomic instability.
- Pharmacoepigeneics (since epigenetic alterations are reversible, demethylation drugs and histone deacetylase Inhibitors have developed to
- Age-related epigenetics changes
- Environmentally induced epigenetic changes
- Epigenetic biomarkers (Detective, prognostic, predictive biomarkers) associated with different diseases.
- Epigenetics and immunity

Lecture 10: PCR as a Molecular Tool for Cancer Research

PCR Applications: What does PCR tell us?

1. **Detection and diagnosis** (viruses, bacteria, gene mutation, ….)
2. **Targeted Amplification** (cloning, sequencing, Genetic variations, SNP….)
3. **Quantification** (RT-PCR) , Gene expression (qPCR)
4. **Genotyping** (PCR-RFLP, ….)
5. CAN- Copy Number of Alterations
6. **Genetic marker** (RAPD, SSR , STR , AFLP **,** …)
7. **DNA methylation** (COBRA, Pyrosequencing, MSP)
8. **Solve criminal cases (DNA fingerprints)**
9. **Many others**



PCR Procedure

- **Initial Denaturation:** 95 °C /2–5 min double-stranded

  DNA (dsDNA) separated into single strands for amplification.

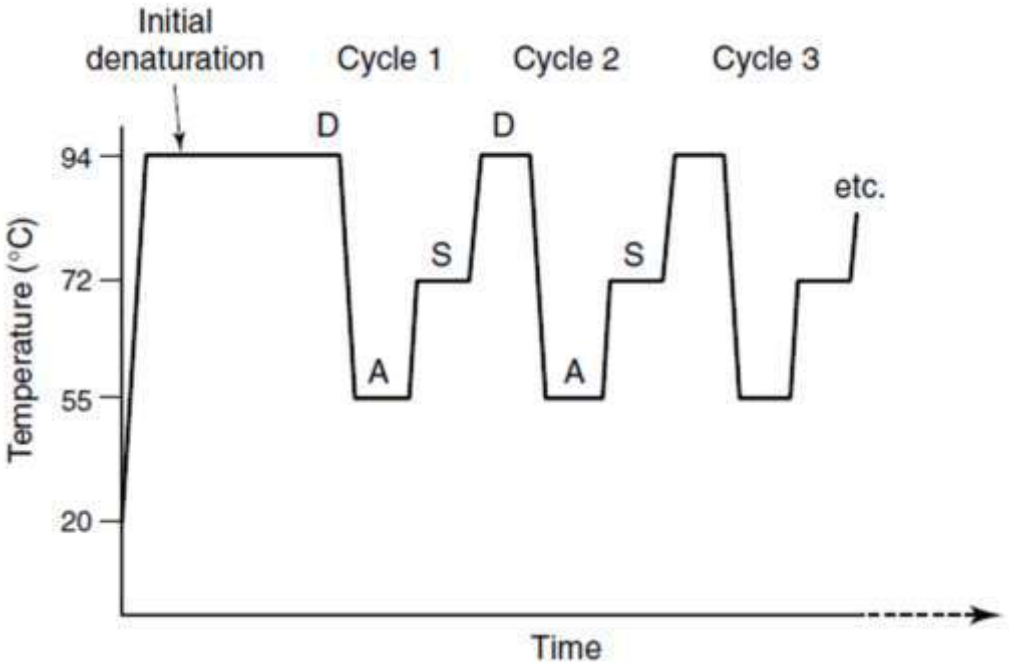
- **• Cycling:**

  1. **Denaturation:** 95 °C, all dsDNA into single stranded DNA (ssDNA).

  2. Annealing: (50–63 °C) to promote primer binding to the template.

  3.Synthesis (Extension): 72 °C, for **DNA polymerase activity** to allow the hybridized primers to be extended.

- **• Repeat:** Steps 1–3 are performed in a cyclical manner, resulting in exponential amplification of the amplicon.

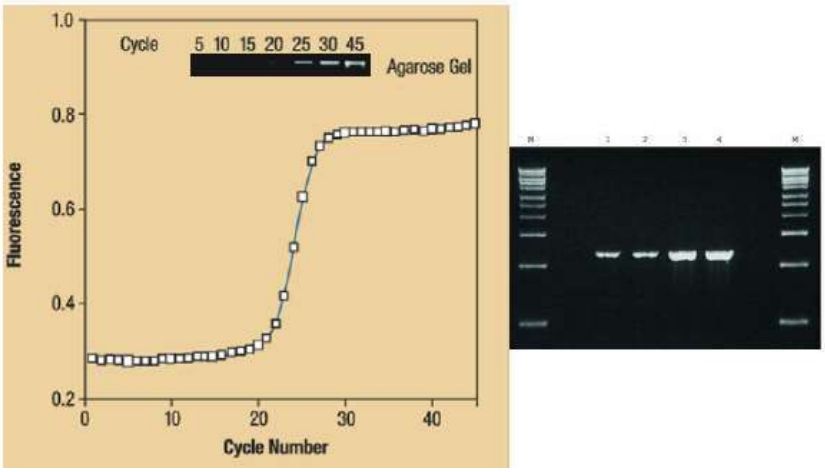


Amplicon detection, analysis &PCR evaluation

How do we know if the **PCR has worked**?
How should **we interpret the results**?

During the PCR reaction run:

Exponential accumulation of PCR amplicons to reach levels easily detectable by gel electrophoresis and/or fluorescence.



Inspection of gels

After electrophoresis and **staining of the gel** ☻, inspection on a UV trans-illuminator will allow one to determine:

1. **Whether there is an amplification product(s)**
2. **The quality of the product(s)**
3. **The specific size of the product(s)/ DNA ladder**

to avoid spending time, effort and money studying the wrong DNA fragment

Avoiding PCR contamination

a) Ideally, experiments should be set up in a laminar flow hood dedicated solely for PCR use.

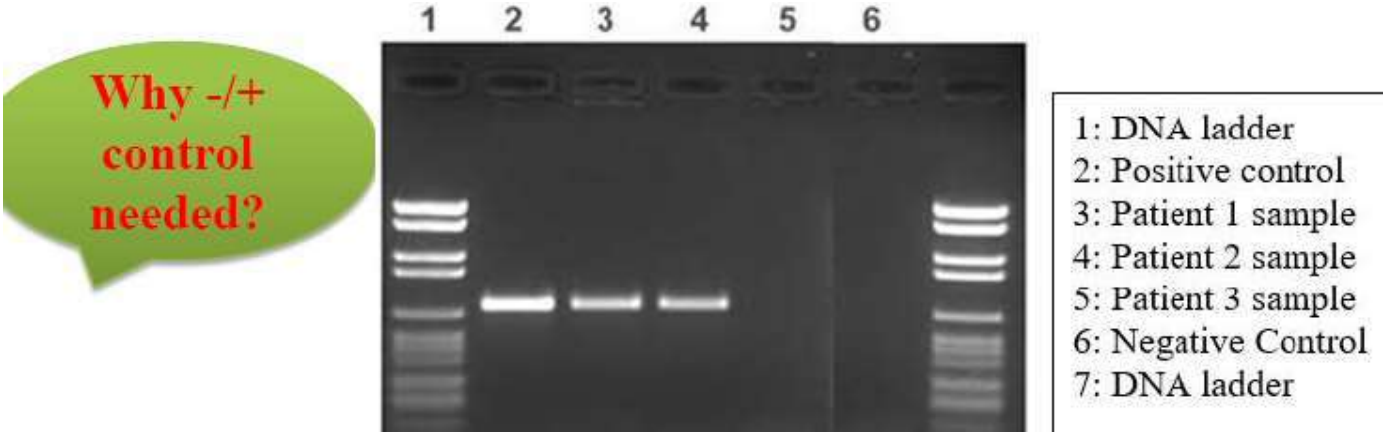
b) Work in separate lab completely isolated from **PCR product stores** or **plasmid clone preparation areas.**

c) Separate supplies **of pipettors, pipette tips, PCR tubes, and reagents should be kept specifically for PCR**

d)In general, **good laboratory practice** and rigorous attention to technique is required for any PCR-based strategy

e) **Positive control** sample DNA or RNA should be added to the reaction last,.

f) **Negative controls** containing **distilled water** instead of template should always include when performing PCR

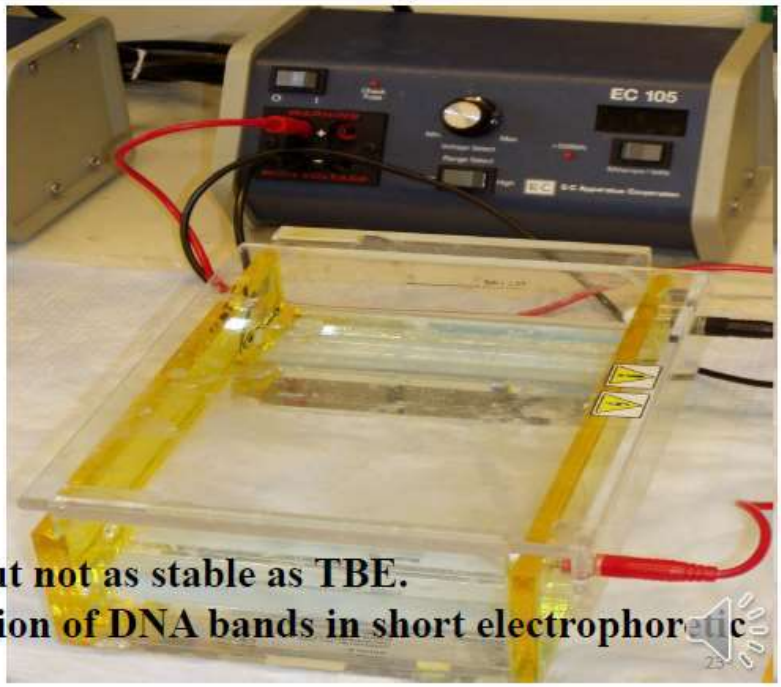


Continuous and discontinuous buffer systems

Effective separation of nucleic acids by gel electrophoresis depends upon the effective maintenance of **pH** within the matri

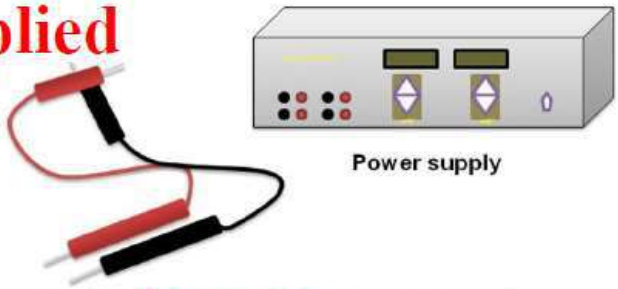
Tris-borate (TBE)

Trisacetate (TAE)



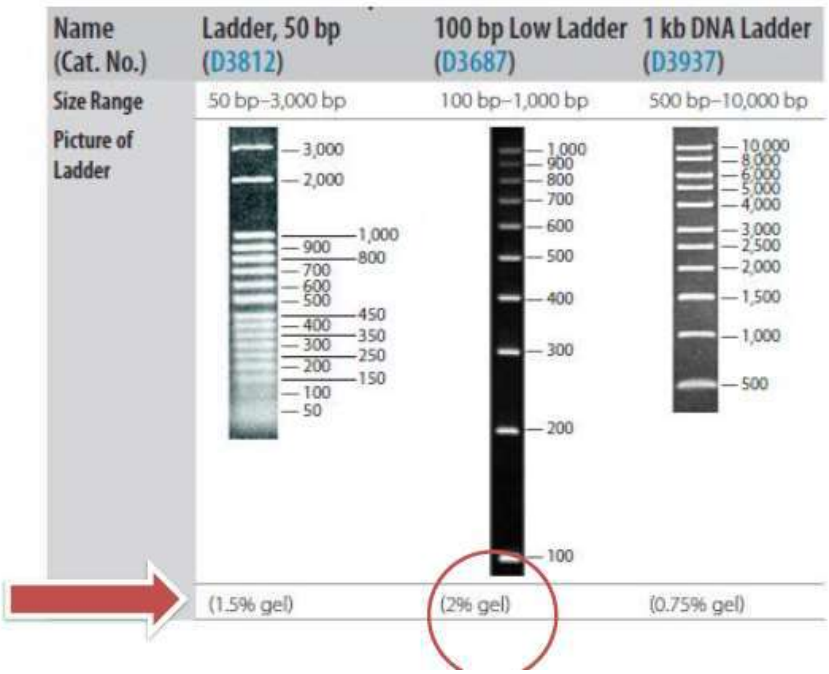
TAE is less expensive, but not as stable as TBE.
TAE gives better resolution of DNA bands in short electrophoretic separations

Voltage/current applied



- The **higher the voltage**/current, the **faster the** DNA migrates
- Voltage is too high, band streaking
- High voltage causes increase in buffer temperature
- **75- 100 mA for 45 mins**

Amplicon detection, analysis &PCR evaluation



General recommendation for setting a PCR reaction

1-Check the **primers' design** before the order.

2- Use **trusted** mastermix and components .

3- Work in **clean environment**.

4- Use new and **sterilized tubes and tips** .

5- Check your **micropipette accuracy**.

6- Prepare the **primers' solutions** properly.

7- **Store** properly .

8- **Work on ice** or use pre-chilled ceramic rack .

9- Use **suitable UPS** for your machine.

10- Pre-heat the thermocycler .

11- Use **hot lid** or **mineral oil** .

12- **Avoid** protocol that use additives .