

RNA in diagnostics



RNA in diagnostics

- 1.) direct RNA diagnostics – screening of whole coding region of given gene
- 2.) gene – expression analysis:
 - differential diagnostics of some tumours
 - detection of circulation tumour cells in blood and bone marrow
 - monitoring of course of therapy and detection of residual disease
 - control of graft before autologous transplantation
 - differential display, PTT test, functional tests...

RNA

Mammalian cell:

- 10 - 30 pg total RNA
 - rRNA (28S, 18S, 5S) 80-85%
 - tRNA, snRNA 15-20%
 - mRNA 1-5%
- 360 000 mRNA molecules/cell,
12 000 different transcripts
typical length of 1 transcript cca 2kb

RNA Unstability

- presence of ribonucleases (RNases) in cell
- RNase
 - very stable
 - do not need cofactors
 - efficient in low concentrations
 - difficult inactivation
 - contamination with RNases : human skin
dust particles (bacterias, fungi)
- isolation and analysis of RNA : special approach and methods

Stabilisation and storage of RNA

- gene-expression analysis: analysed RNA must represent *in vivo* expression of sample
- Complications - 1) reduction mRNA (downregulation of genes and enzymatic degradation of RNA), 2) expression induction of certain genes

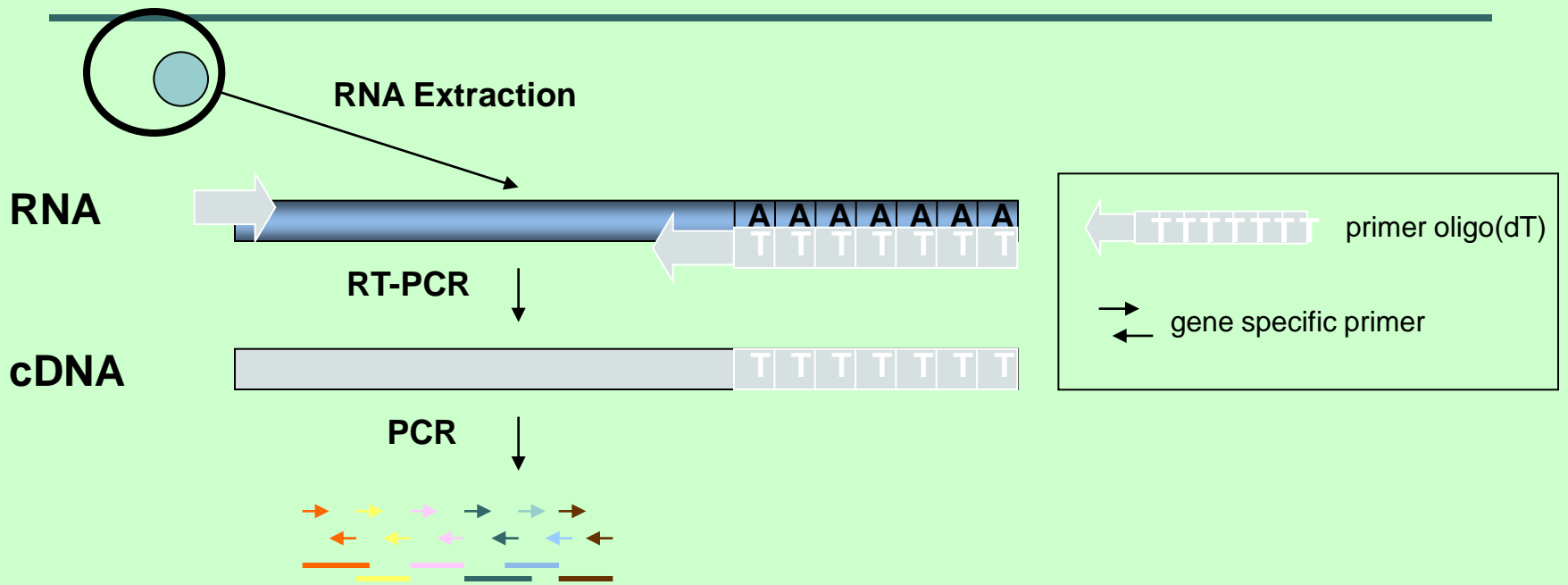
RNA stabilisation in sample:

- immediately frost in liquid nitrogen and store in -80°C
- stabilisation solutions: RNAlater, PAXgene

Contamination with DNA

PCR primers overlapping border intron/exon
digestion with DNases

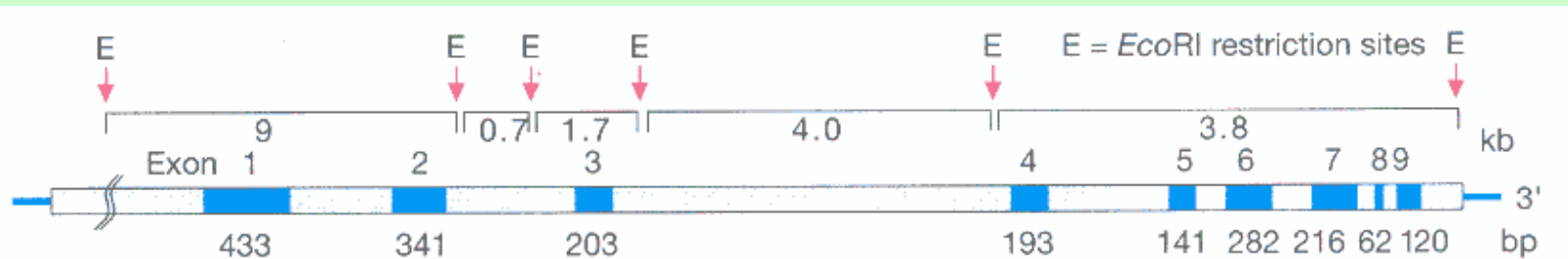
Direct RNA diagnostics



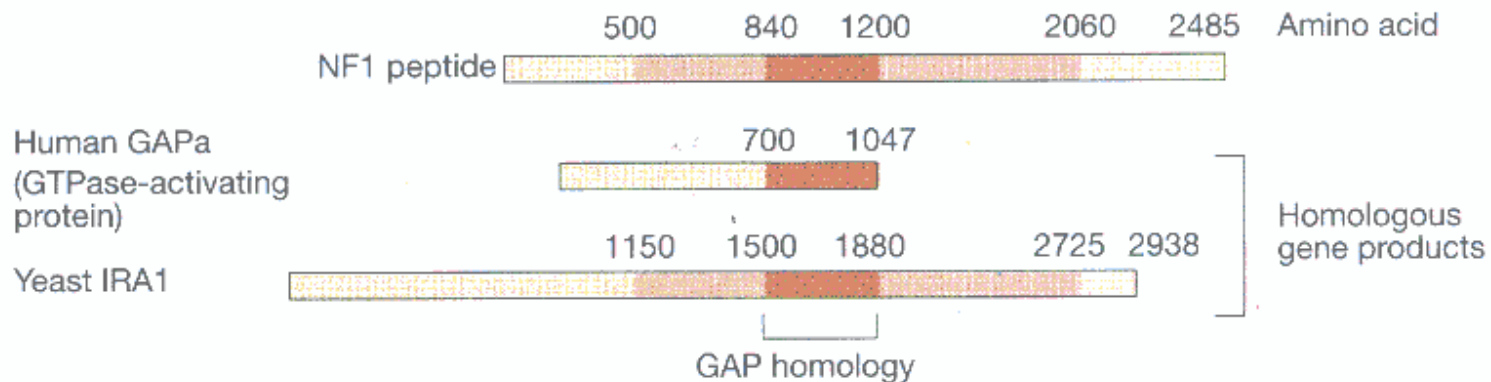
RNA diagnostics of NF1 gene

NF1 gene: 350 kb, 60 exons, 11 - 13 kb mRNA

- protein neurofibromin: 2818 aminoacids, probably tumour supresor



B. NF1 gene on chromosome 17



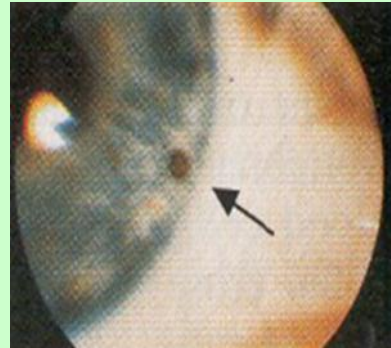
C. NF1 gene product (neurofibromin)

Neurofibromatosis type 1 von Recklinhausen disease

Autosomal dominant
Frequency 1:3000
Locus 17q

50% mutations de novo

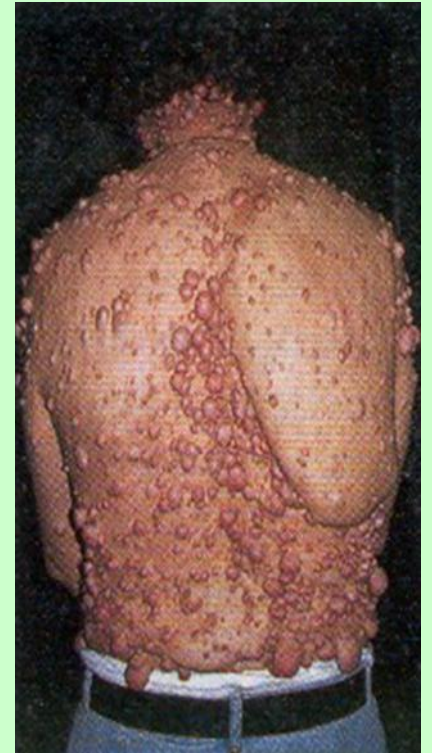
Predispositions to tumours of
neural system



Lisch nodule



Café-au- lait spots



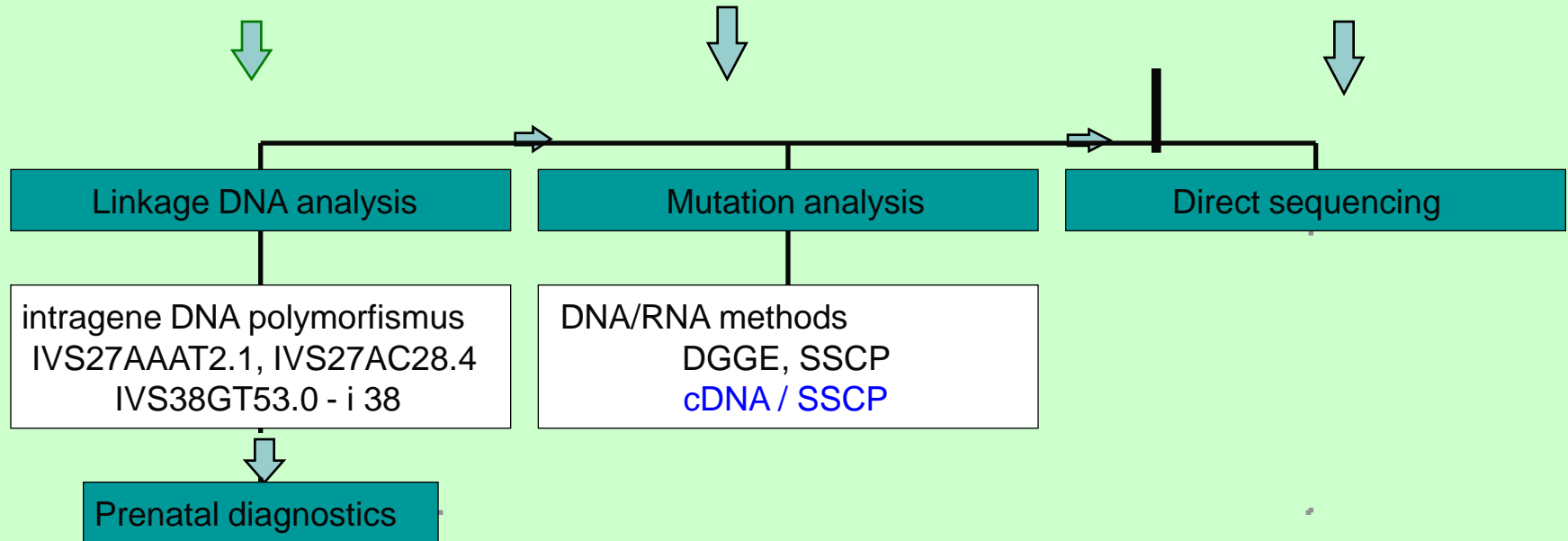
Neurofibromas

Complications in molecular diagnostics of NF1

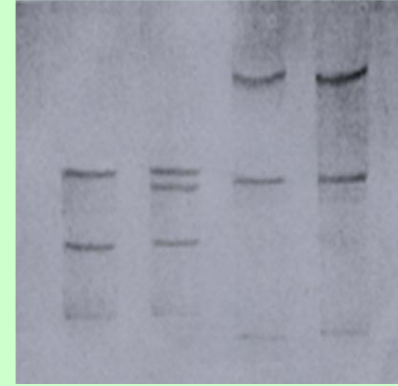
- problematic clinical diagnosis:
- up to 50 % cases *de novo*
- high mutation speed*
- length of gene* (350 kb, 60 exonů)
- absence of hot spots – need to search in whole gene
- unclear correlation between type of mutation and manifestation
- different clinical manifestation even in patients with the same mutation

Strategy of molecular – genetic testing of NF1 patients

NF1 DNA / RNA of patient



cDNA - SSCP analysis

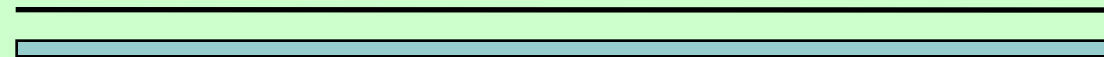


total RNA



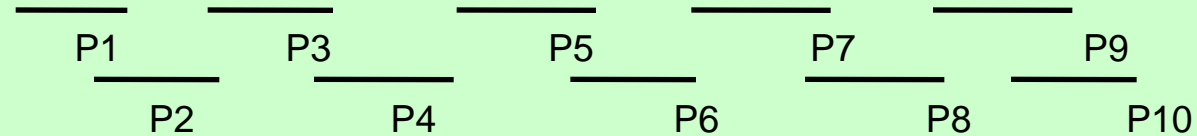
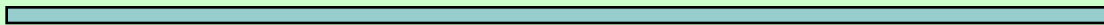
cDNA

RT ↓



PCR ↓

NF1 cDNA (60 exons)

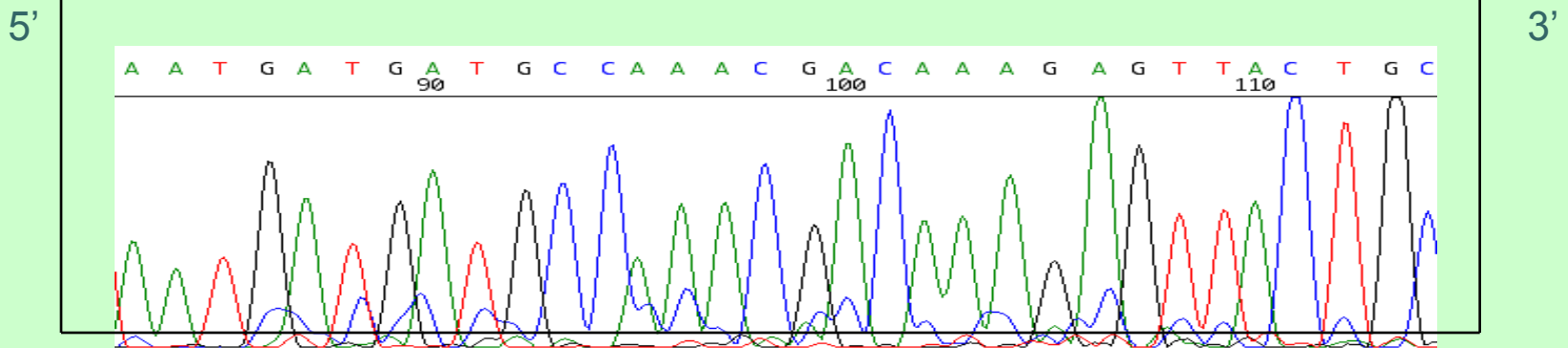


SSCP ↓

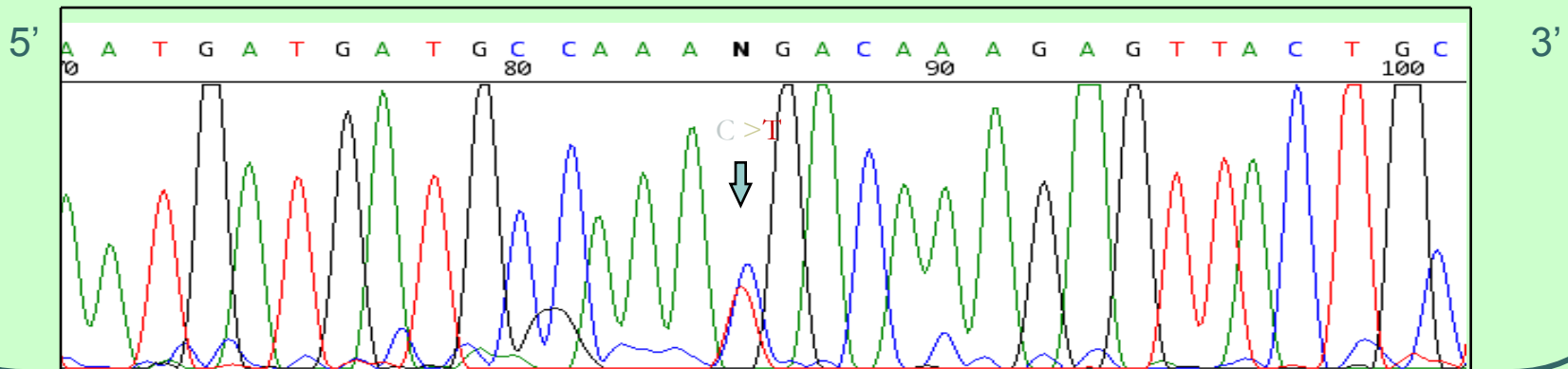
Sequencing analysis

Sequencing of cDNA segment P7 of NF1 gene (exons 28 -32/33)

standard cDNA



cDNA of NF1 patient, mt C5839T (Arg > STOP)



Advantages and disadvantages of RNA diagnostics

- Easier and faster screening of multiexonic gene (10 segments of cDNA instead of 60 exons), mRNA is without introns
- Capture of splice mutations in intrones
- Capture of deletion of whole exonu on one allele
- cheaper
- More difficult taking blood for RNA isolation
- Lower stability of RNA
- Longer segments – problems with electroforetic separation and sequencing
- Unclear effect of mutation on phenotype level

Gene expression analysis

Real-time PCR method

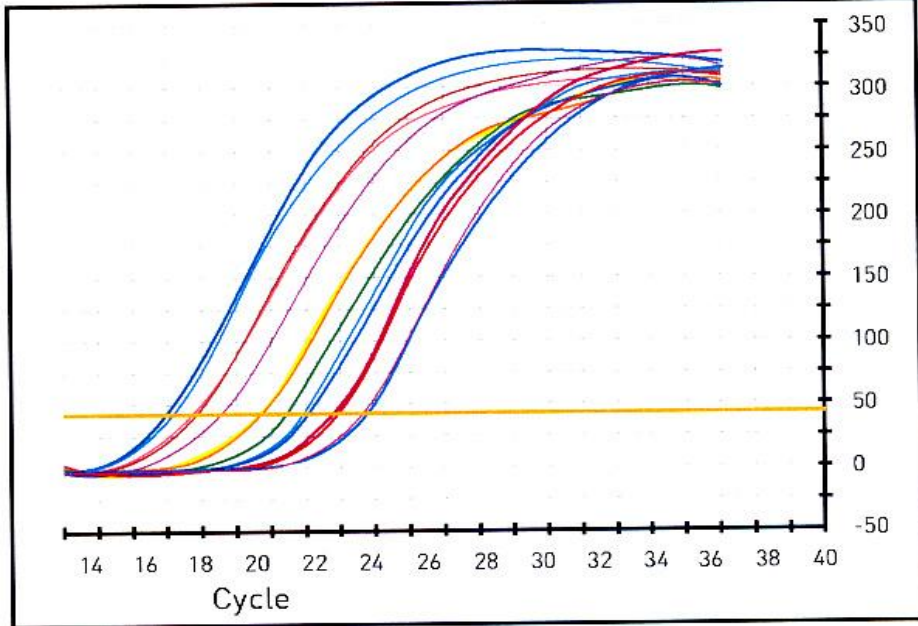
- Real-Time PCR combines DNA amplification with real time amplified product detection in a single tube
- Reliable
- Precise
- Fast
- Universal
- Variability of used probes
- Possibility of detection of more mutation during analysis
- Measurement of fluorescence

Determine differences in mRNA expression

- QUALITATIVE ANALYSIS
snp – single nucleotide polymorphism detection
- QUANTITATIVE ANALYSIS
amplicon amount detection

Detection

- Two types of Real-Time PCR
- 1. Specific Sequence Detection - Detects a specific sequence of intermediate products. Can be used to detect specific products (TaqMan)
- 2. Non-Specific Detection - Detects any dsDNA produced during the reaction (SYBR green)



Expression analysis in oncology

Tumour cells have different gene expression profile than healthy cells



Change in amount and spectrum of expressed mRNA

Relative quantification of expression relative to housekeeping gene

Housekeeping gene: control gene which is constant in the samples

Oncomarkers

A tumor marker is a substance found in the blood, urine, or body tissues that can be elevated in cancer. There are many different tumor markers, each indicative of a particular disease process, and they are used in oncology to help detect the presence of cancer.

From substances produced by normal cells they differ qualitatively – normal cells do not produce them, or quantitatively – they are produced by both types of cells

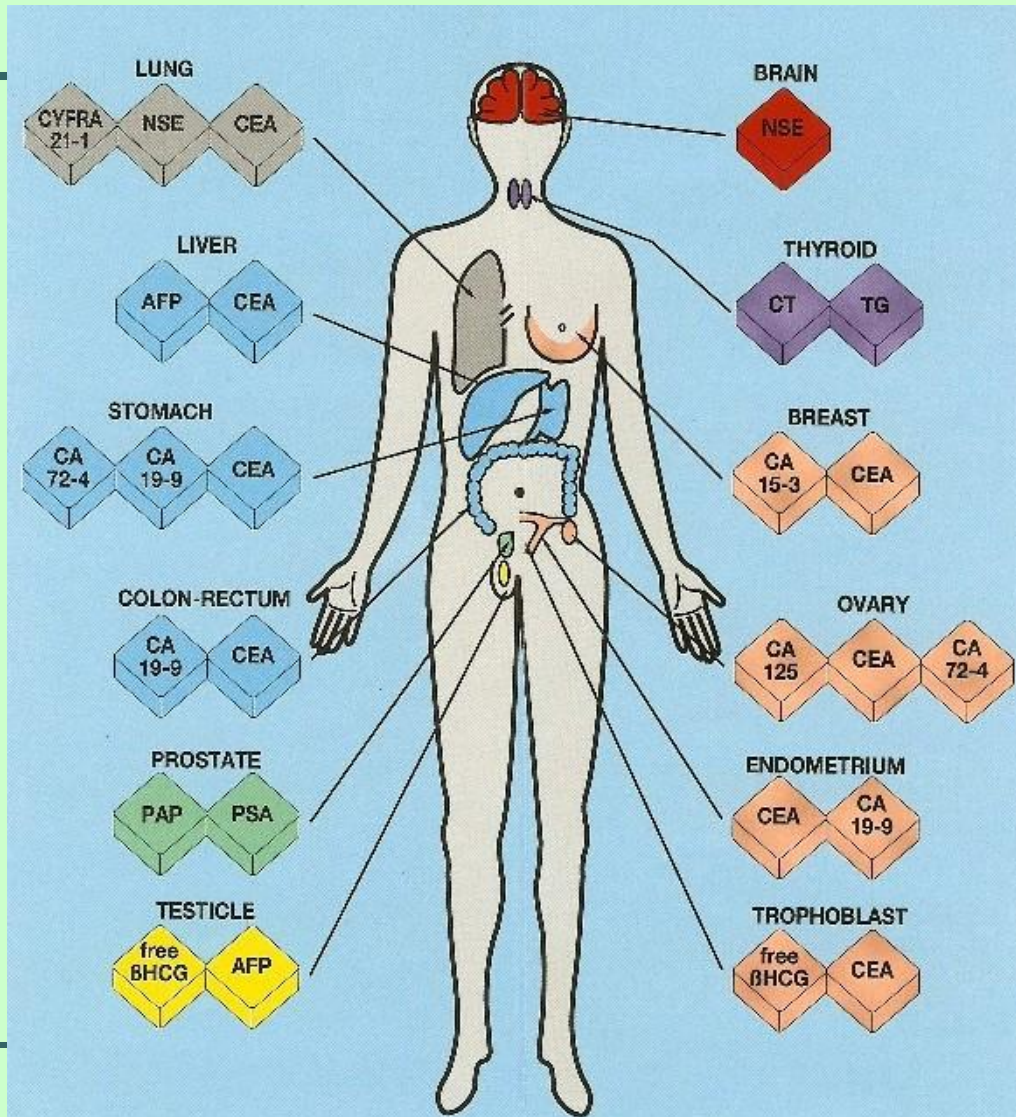
Ideal Oncomarker

- produced only in malignant cells
- organ specific
- in biological liquids in high concentrations
- level correlates with size of tumour, stage of disease, prognosis and therapy effect
- provides evidence of residual tumour tissue

Oncomarkes according to chemical structure or biological function

- oncofetal antigens
- enzymes
- hormones
- intracellular oncomarkers
- other non specified substances

Onkomarkers: indication according to organ



- **Prostate-Specific Antigen:** PSA is prostate-specific, not cancer-specific. A variety of conditions can raise PSA levels: prostatitis (prostate inflammation), benign prostatic hypertrophy (prostate enlargement), and prostate cancer. PSA levels can also be influenced by a number of other things.
- **Carcinoembryonic Antigen:** Although CEA was first identified in colon cancer, an abnormal CEA blood level is specific neither for colon cancer nor for malignancy in general. Elevated CEA levels are found in a variety of cancers other than colonic, including pancreatic, gastric, lung, and breast. It is also detected in benign conditions including cirrhosis, inflammatory bowel disease, chronic lung disease, and pancreatitis. The CEA was found to be elevated in up to 19 percent of smokers and in 3 percent of a healthy control population.

Thus, the test for oncomarker cannot substitute for a pathological diagnosis.

Detection of minimal residual disease

Detection of presence of isolated tumour cells in blood, bone marrow and lymphatic system - possible precursors of metastases

Imunohistochemistry - sensitivity 1 : 10 000

Flow cytometry - sensitivity 1 : 100 000

PCR - sensitivity 1: 1 000 000

Real- time PCR - sensitivity up 1 : 10 000 000