NEUROBLASTOMA IN MUSCULOSKELETAL SYSTEM, TUMOR HYPOXIA, HIF-1 AND CHEMORESISTANCE

21th Prague-Lublin-Sydney-St. Petersburg Symposium

5th September 2019, Humpolec

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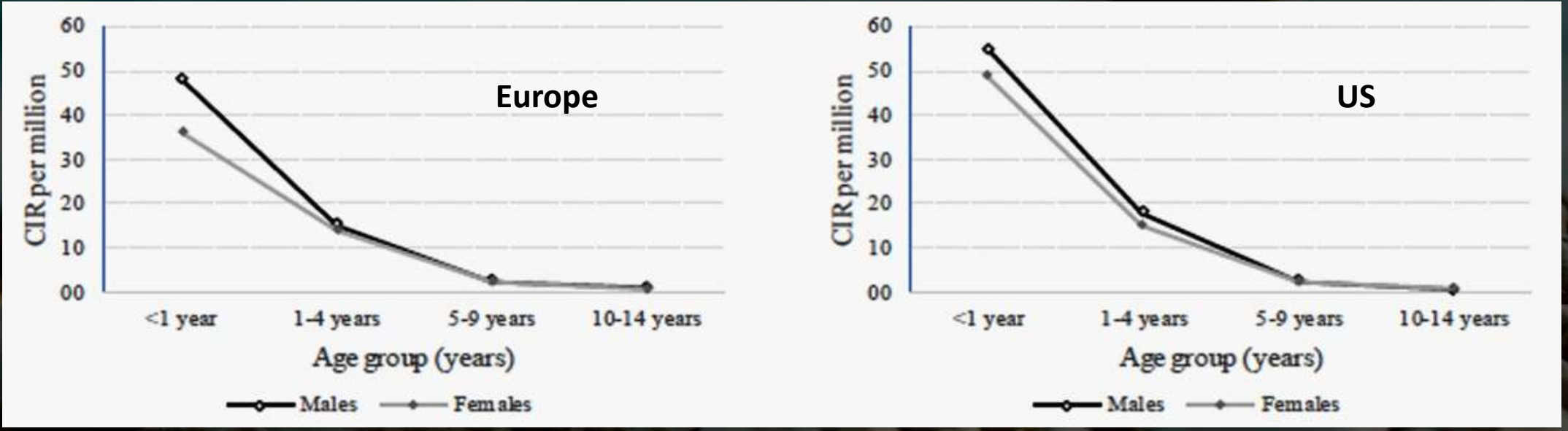
Introduction

1. Neuroblastoma (NB)
   - Symptoms
   - Staging
   - High-risk NB: indicators, therapy and treatment
2. Hypoxia in tumors
3. HIF-1α
4. Our work: HIF-1 in chemoresistance
5. Inhibition options
6. Results
7. Other factors involved in hypoxia-induced chemorezistance
8. Conclusions

NEUROBLASTOMA

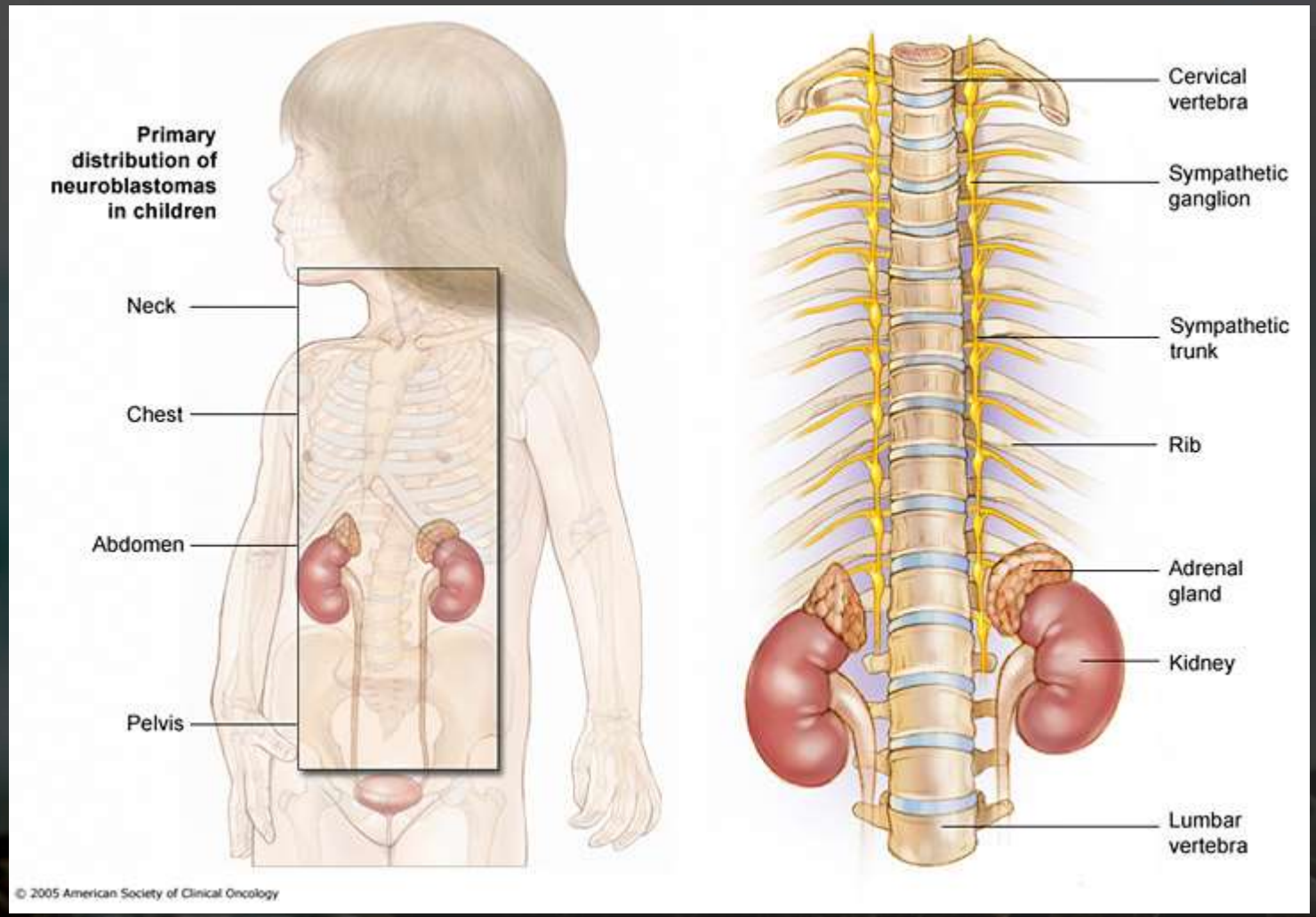
- Common and a frequently deadly cancer, almost exclusively seen in children
- The prevalence is about 1 case per 70 000 live births. This incidence is fairly uniform throughout the world
- It accounts for about 7–8 % of all cancers in children and the most common cancer in infants
- Nearly 90% of cases are diagnosed by age 5 **X** rare over the age of 10 years



Neuroblastoma among children in Southern and Eastern European cancer registries: Variations in incidence and temporal trends compared to US, Georgakis M. K. et al. , 2017

- Solid embryonal tumor

- Can arise anywhere throughout the sympathetic nervous system
- Most common primary site is adrenal gland found in the abdomen
- May also begin in nerve tissue in the neck, chest, or spinal cord.



- The clinical course of patients with neuroblastoma is highly heterogeneous

Symptoms

- The “enigmatic tumor”
  - broad spectrum of clinical presentation, biological features and prognosis that vary greatly depending on size, location, and spread of the tumor
- Widespread bone and bone marrow disease causes bone pain -> limping or irritability
- Bone marrow replacement
- Anemia, bleeding, or infection
- Presenting symptoms of neuroblastoma depend on the location of the primary tumor, presence of metastatic lesions
- Weight loss and fever
- Metastases:
  - lymph nodes, bone marrow, bones, liver, skin
  - rarely to lungs or brain
  - multiple bone metastases in the vertebrae, metaphysis of long bones and skull

NB STAGING AND PROGNOSIS

- The **International Neuroblastoma Risk Group Staging System (INRGSS)** - results from imaging tests (such as CT or MRI and MIBG scan)

- The **International Neuroblastoma Staging System (INSS)** - results from the surgery

Stage 1:	Localized tumor* with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive).
Stage 2A:	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically.
Stage 2B:	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
Stage 3:	Unresectable tumor infiltrating across the midline** with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement.
Stage 4:	Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined in Stage 4S).
Stage 4S:	Localized primary tumor (as defined for Stage 1, 2A, or 2B), with dissemination limited to skin, liver, and/or bone marrow*** (limited to infants less than one year of age).

The INRGSS has 4 stages:

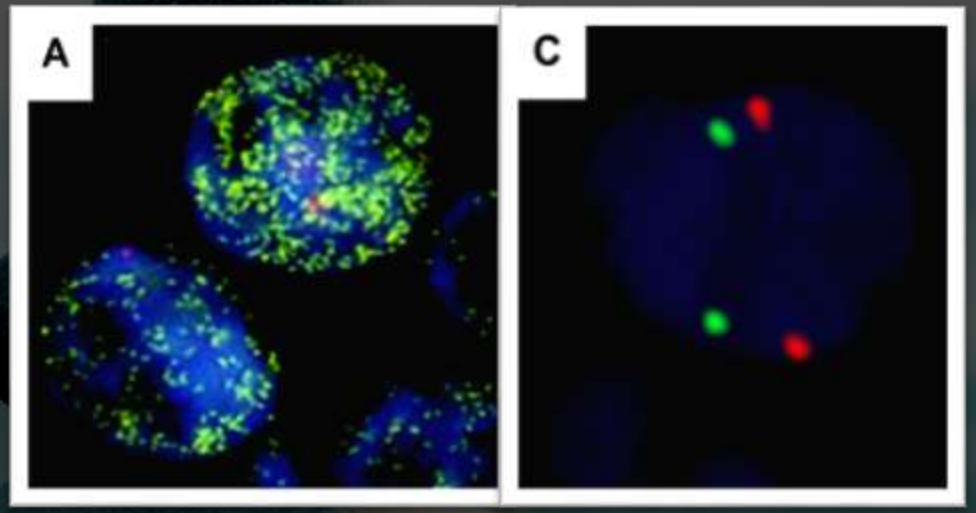
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors[b] and confined to one body compartment
L2	Locoregional tumor with presence of one or more image-defined risk factors[b]
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow

The INSS

HIGH-RISK TUMOR INDICATORS

Myc proteins:

- *MYCN* – amplification of *MYCN* oncogene is an established marker indicating aggressive tumor progression of NB
- c-Myc



Ref. Wang et al., Diagnostic pathology, 2013.: Representative FISH image of neuroblastic tumor cells displaying MYCN gene status. A: Amplification:The number of MYCN signals (green) is more than 10 copies of the CEP2 probe signals (red). C: No Alteration: Cells with MYCN signals (green) showing the same numbers of the CEP2 probe signals. (DAPI counterstain, original magnification ×1000).

DNA ploidy / DNA index:

- the higher the better prognosis in children under 2 years of age

Therapy of High-Risk NB

- Multi-agent chemotherapy
- Radiation therapy
- Stem-cell transplantation
- Surgery (if possible)

Problems in treatment:

- Late diagnosis
- Most NBs are radioresistant
- Chemoresistance

After treatment

- only 40–50 % survive 5 years after diagnosis
- NB can cause long-lasting side effects:
  - problems with bones and muscles – scoliosis, osteoporosis
  - chronic pain
  - problems with growth and development
  - metastases, bone marrow infiltration

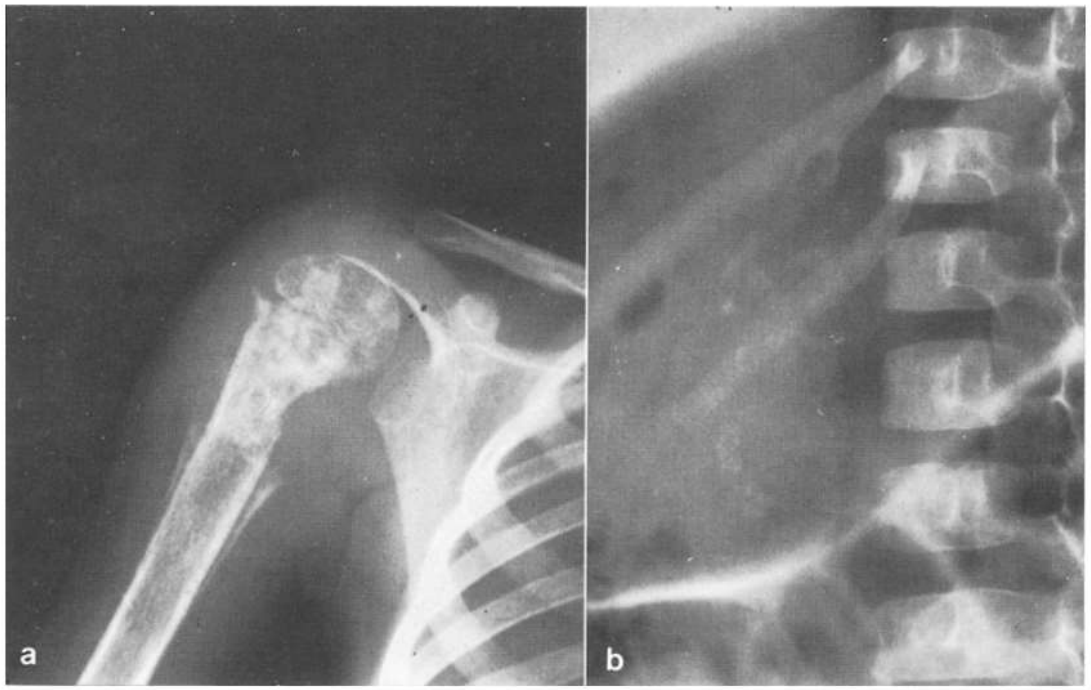
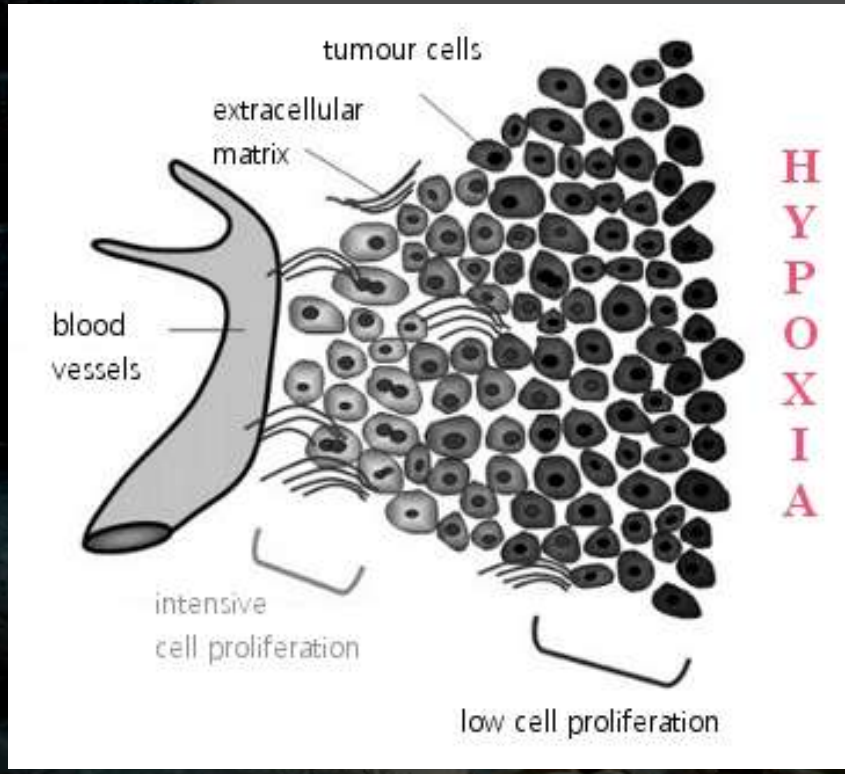


Fig. 578. - Radiograms of the same case as Fig. 577. (a) Metastasis in the proximal humerus. (b) Calcification in retroperitoneal primary tumor.

Campanacci M. et al., from book: Bone and soft tissue tumors, 1990

Hypoxia in tumors

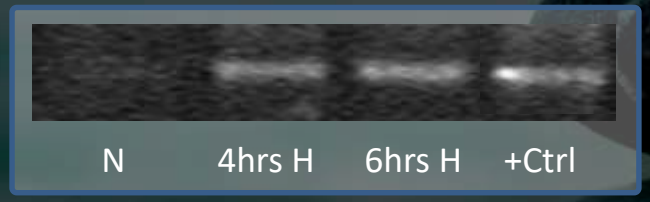
- The uncontrolled proliferation results in an imbalance between tumor cell oxygen consumption and tumor angiogenesis -> most parts of solid tumors are hypoxic
- Most tumor cells have adapted to these conditions
- Dramatic shift towards anaerobic metabolism -> increased expression of genes coding for glycolytic enzymes and glucose transporters
- Pro-angiogenic growth factors (VEGF, PDGF, angiopoietins)

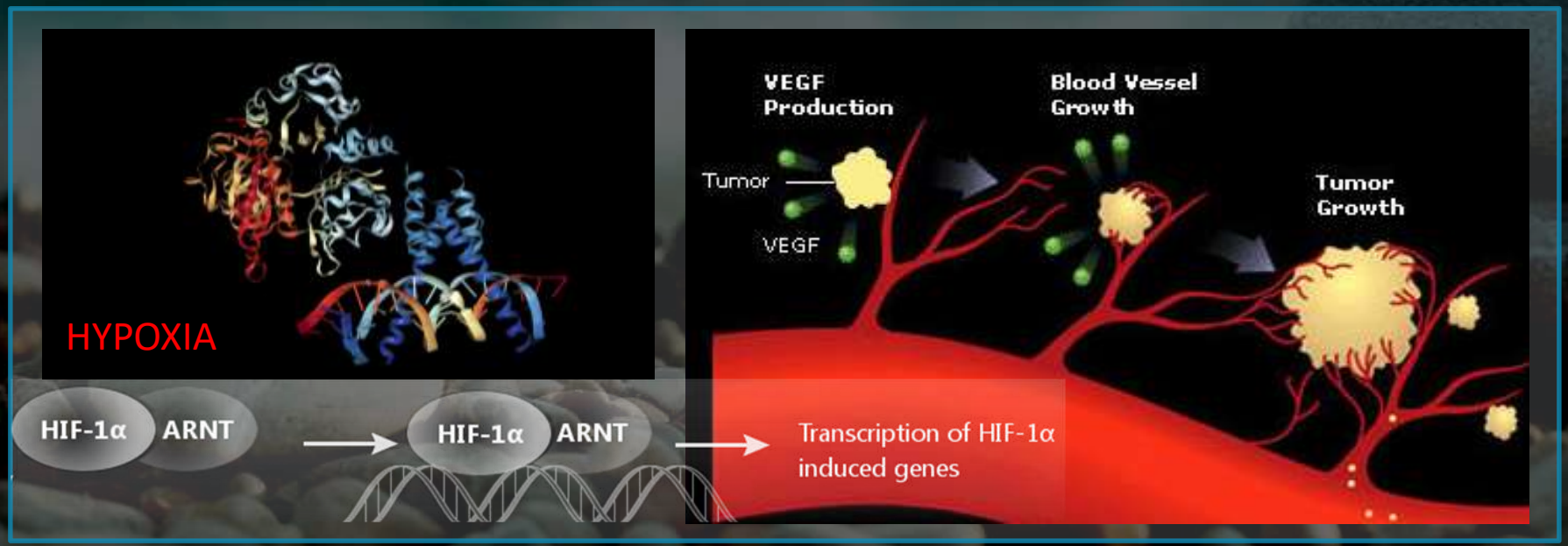


- Tumor hypoxia is the main driving force behind vascularization and neoangiogenesis
- Metastases

Hypoxia inducible factor-1α / HIF-1α

- Hypoxia-induced **α subunit** creates the transcriptional complex with the constitutively expressed **β/ARNT subunit** -> HIF-1 transcription factor
- Normoxic degradation
- Hypoxic stabilization





HIF-1 regulates the expression of over 100 of genes that mediate the adaptive response of tumor cells to hypoxia

Hypoxia inducible factor-1α / HIF-1α

- Tab.1 Classification of the examples of HIF-1 regulated genes *(Ref. Doktorova H. et al., Biomedical Papers, 2013)*

BIOLOGICAL FUNCTIONS	GENE SYMBOL/ALIAS
Transcription factors	TWIST1, Snail, ZEB1, ZEB2, ID2, SMAD7, PPARγ, GATA1
Histone modifiers	JMJD2B, JMJD2C, MLL1
Enzymes	MMP1, MMP3, LOX, ADAMTS1, ACE, ACE2, XPA, HK1, HK2
Receptors, receptor-associated kinase	CXCR4, CX3CR1, uPAR, PAI-1, 67-kDa laminin receptor, c-Met
Small GTPases, intracellular signalling molecules	**VEGF**, TGF-α, TGF-β3, IGF2, Cdc42, Rac1, RhoE, IRS-2
Transporters	**glut-1, glut-3**, MDR1, VDAC1, transferrin, ceruloplasmin, IGF-BP1 - 3
Membrane proteins	ANGPTL4, L1CAM, α5 integrin, CD151, CD24, CD147, Galectin-1 and 3, MUC1, Semaphorin 4D, Caveolin-1
Scaffold protein, cytoplasmic protein	HEF1, Lipirin-α4
Matricellular proteins	CYR61, NOV

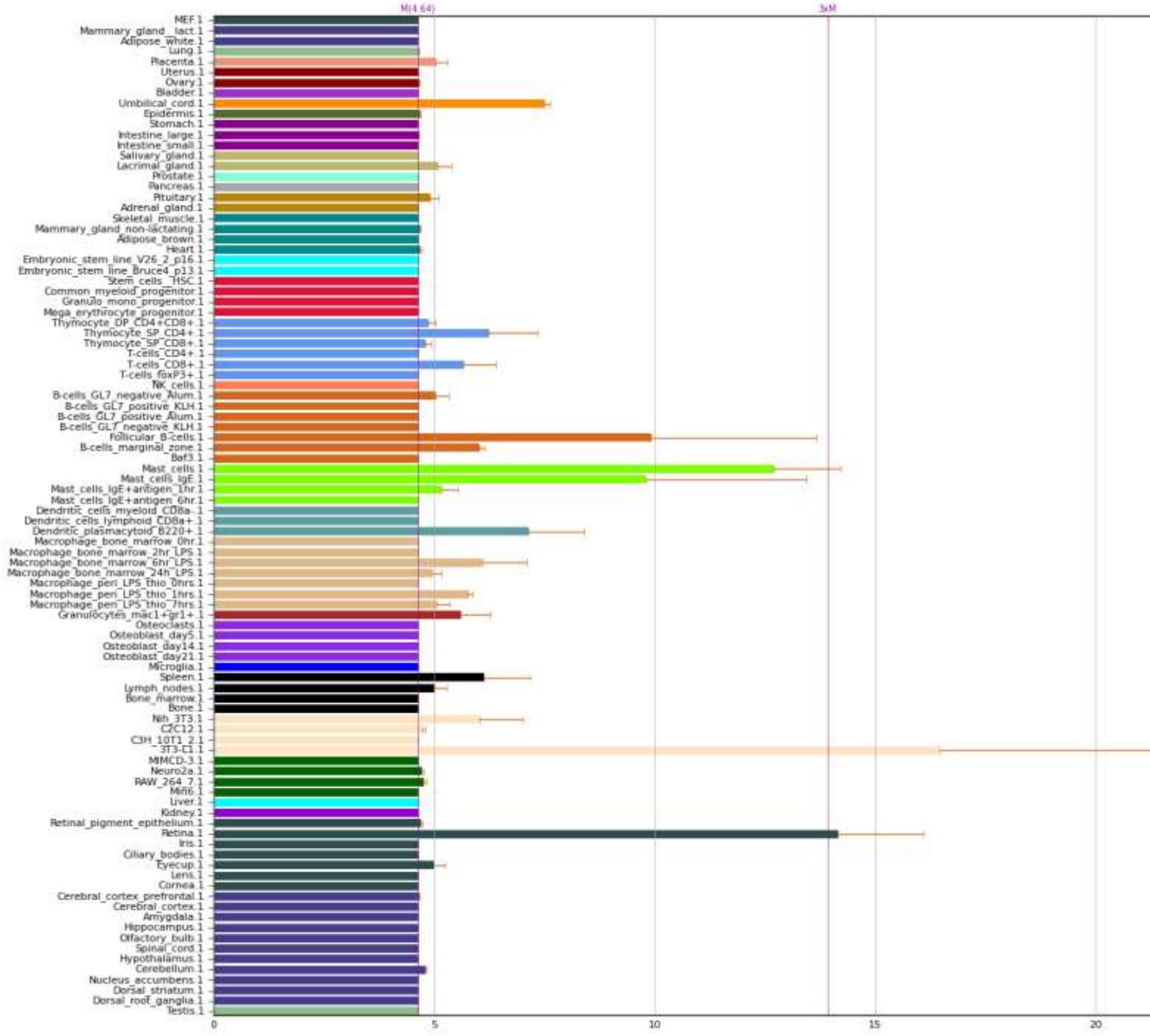
- There were described also several mechanisms in some types of tumors that regulate the stability and activity of HIF-1 in an oxygen-independent manner
  - > consequence of growth factor signalling
  - > in cells with defects in pVHL

Hif1a (hypoxia inducible factor 1, alpha subunit):

Gene expression/activity chart (mouse), physiological state

http://biogps.org

- Umbilical cells
- T- cells
- Follicular B-cells
- Mastocytes, Mast cells IgE
- Granulocytes
- 3T3-L1 preadipocytes
- Retina
- Bone marrow macrophage



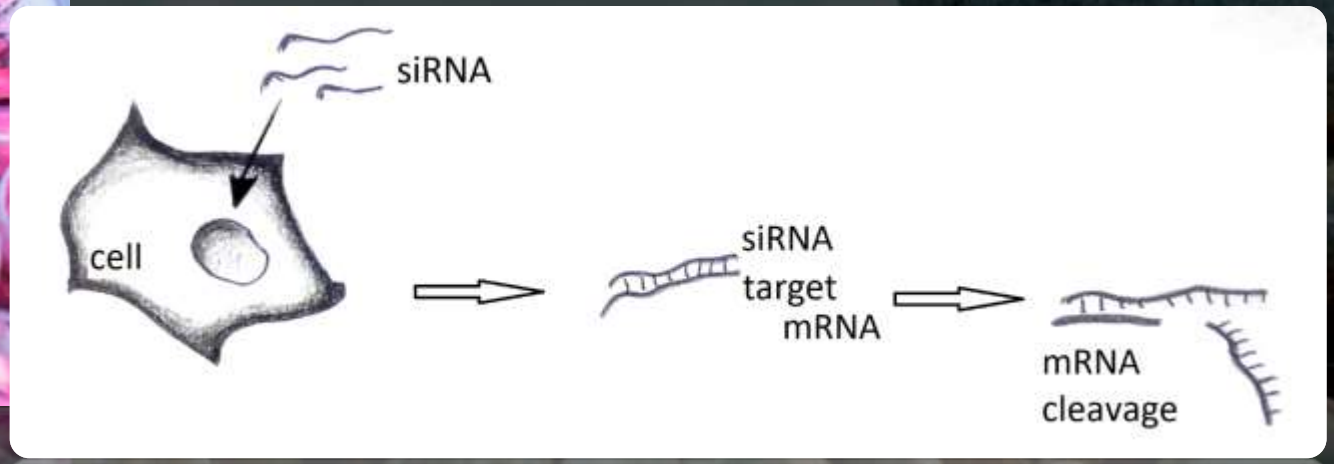
Our work: HIF-1 and chemoresistance

- The overexpression of HIF-1 in cells results in higher resistance to the therapy and poor prognosis
- Targeting the HIF-1 pathway is as an important area for cancer therapy research
- We focus on *in vitro* study of high-risk neuroblastoma derived cell lines and their hypoxia-induced resistance to commonly used cytostatics, which is a major cause of anti-tumor failure in these children
- We have reported resistance of NB cell lines to cisplatine and ellipticine
- Other experiments: resistance to other commonly used cytostatics like doxorubicin, etoposide, melpahalan, 5-flouoruracil, gemcitabine and docetaxel

Inhibition options

- small molecule inhibitors (YC-1, LW6, Topotecan, Bisphenol A, Vorinostat etc. – different mechanisms of inhibition)

- gene knockdown, e.g. RNA interference (siRNA, shRNA)



Results

- HIF-1 inhibition is responsible for the growth supression

  (used cell lines: UKF-NB-4, Kelly, SK-N-AS)

- Lower hypoxia-induced resistance to cisplatin after HIF-1 inhibition

- Other factors have to be responsible for hypoxia-induced chemoresistance in NB cells

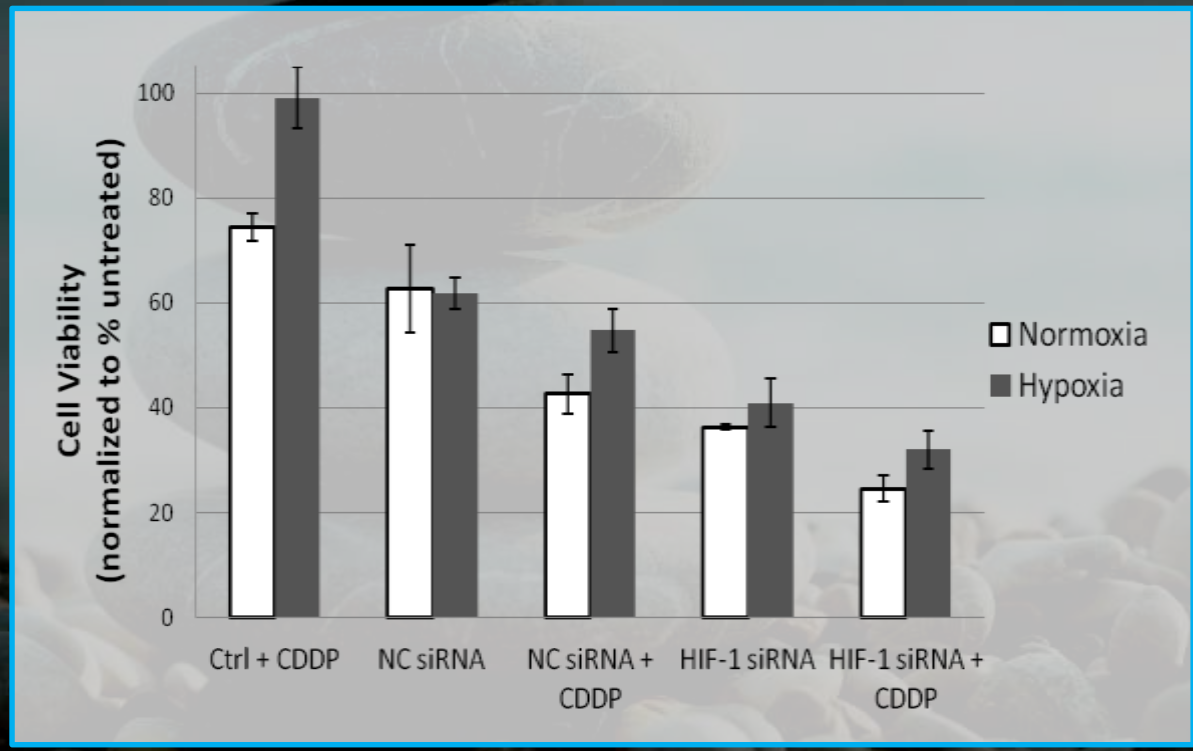


Fig. UKF-NB-4 cells were treated 48 h with 20μM cisplatin (CDDP) in normoxia or hypoxia (1 % O_2) and then analyzed by alamarBlue® - Cell Viability Assay. Cells were transfected with non-coding siRNA (NC siRNA) or HIF-1 siRNA, 24 h prior treatment. Similar results were observed in other studied NB cell lines. HIF-1 silencing has been verified by flow cytometry and Western blot analysis (data not shown).

Other factors involved in hypoxia-induced chemorezistance

- HIF-1α independent
- factors that can somehow cooperate with HIF-1

Tab.2 Examples of other factors participating on chemoresistance due to the hypoxic conditions in cells	
Anti-apoptotic factors (IAP3, Bcl-2 family)	HIF-1 independent
Cyclooxygenase-2 (COX-2), sphingosine kinase 2 (SphK2)	HIF-1 independent
Pim serine/threonine kinase-1 (Pim1)	HIF-1 independent
Nuclear factor kB (NF-kB)	can interact with HIF-1
Protein survival kinases PI3K and Akt	both dependent or independent on HIF-1
Signal transducer and activator of transcription 3 (STAT3)	both dependent or independent on HIF-1
Carbonic anhydrases (CA IX)	acts via HIF-1 pathway
V-ATPase	stabilises HIF-1α in normoxia

Conclusions

- Cancer is still the leading cause of death. Prompt medical attention and aggressive therapy in high-risk NB treatment are important for the best prognosis.
- Continuous follow-up care is essential:
  - Side effects of radiation and chemotherapy, recurrence of the disease.
- New methods are continually being discovered to improve treatment and to decrease side effects.
- The reason for unsuccesfull chemotherapy is the hypoxic environment:
  - Hypoxia-induced chemoresistance has been reported in a number of experiments
  - HIF-1 inhibition may have an impact on more efficient treatment of malignant tumors, it can suppress the growth of NB cells and their chemoresistance
- The detection of other factors can provide important prognostic features allowing subsequent individualization of anticancer therapy
- We still have more and better opportunities in research and so, there is hope…

Team and support: Hrabeta Jan, Groh Tomas, Mohamed Abdel Rahman, Feriancikova Barbara, Cerna Tereza, Podhorska Natalia, Prof. Eckschlager Tomas

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Thank you for your attention!