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Dihydroartemisinin-induced apoptosis depends on the presence of proapoptotic Bax or Bak and increases efficacy of ionizing radiation and targeted chemotherapy in vitro

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Introduction: Molecular signaling of apoptosis involves intermediate formation of reactive oxygen species (ROS). Properties of endoperoxides as mediators of ROS provide a rationale for their application in tumor treatment. The radical forming antimalaria drug artemisinin exerts promising cytotoxic effects. Aim of our present study was to evaluate the antineoplastic potential of the derivative dihydroartemisinin (DHA) alone and in combination with ionizing radiation or chemotherapy (TRAIL) focusing on its proapoptotic action.

Methods: Cell death in Jurkat T-lymphoma, HCT116, DU145 was analyzed by IF-microscopy, flow cytometry and immunoblotting. To elucidate molecular signaling, clones with alterations in the receptor pathway (FADD-, Caspase-8 neg.) or deficient mitochondrial pathway (lack of Bak, overexpression of Bcl-2, Bcl-xL, caspase-9DN) and DU145 reexpressing Bax as well as HCT116 clones lacking either Bax, Bak or Bax/Bak and RNAi approache were used. Activation state of proteins was analyzed by activation specific antibodies. Lipid peroxidation was measured by flow cytometry (Bodipy®).

Results: DHA induced apoptosis in Jurkat, HCT116 and DU145 in a time- and dose-dependent manner yielding 60% apoptotic cells after 24h (12.5-25μM). Cyt c release, breakdown of $\Delta\psi$m, caspase activation, PARP cleavage and DNA fragmentation were observed. Inhibition by glutathione and N-acetylcysteine support a ROS dependent mechanism. Moreover, alterations in the emission spectrum of Bodipy® suggest membrane oxidizing potential. Over-expression of caspase-9DN or of antiapoptotic Bcl-xL or Bcl-2 decreased mitochondrial alterations and DNA-fragmentation while absence of FADD or Caspase-8 did not alter apoptosis rates. While cellular levels of pro- and antiapoptotic proteins remained rather constant, the amount of Bax/Bak in an active conformation was increased by DHA. Deficiency of Bax/Bak or siRNA-mediated downregulation of Bak almost abrogated DHA-induced apoptosis.

DHA improved radiation-/TRAIL-induced apoptosis in a concentration-dependent manner, exhibiting at least additive effects.

Conclusions: Data implicate that DHA induces apoptosis via mitochondrial death pathway. While Bak expression is sufficient to mediate the effects in the Bax-/p53-deficient Jurkat model, Bax may substitute for Bak in regard to apoptosis execution in solide tumor cells. Our findings suggest that DHA may be a promising antitumor agent when it used alone or in combination with TRAIL or XRT.

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Protein tyrosine phosphatase PTPN13/PTPL1 regulates aggressiveness of breast cancer cells

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Background. PTPL1/PTPN13 gene presents the genetic characteristics of a tumour suppressor gene. It is located on chromosome 4q21, a region frequently deleted in ovarian and liver cancers, its expression was frequently down-regulated or silenced through promoter hypermethylation within several tumour types, and a mutational analysis of colorectal cancers identifieddifferent somatic mutations in PTPL1. At the mechanistic level, we and other have recently evidenced its ability to inhibit signal transduction driven by the tyrosine kinase receptors IGF-1R and HER2. Materials and methods. In this study, we explore the phenotypic properties of PTPL1/PTPN13 by assessing its effects on the aggressiveness of a noninvasive human breast cancer cell MCF-7 using short interfering RNA or short hairpin RNA. Results. We show that knockdown of the phosphatase significantly reduced cell-matrix adhesion on human fibronectin and collagen 4 which are components of basal lamina and/or extracellular matrix. Same result was obtained when cells were coated on Matrigel, a reconstituted basement membrane. In accordance with the negative effect of the inhibition of PTPL1 expression on cell-matrix adhesion, when coated on fibronectin, PTPL1 siRNA-transfected cells exhibited a loss of focal adhesion structures compared to control cells. This inhibition was correlated with an increase in in vitro cell invasiveness, measured in Matrigel coated Boyden Chambers, and with an enhanced outgrowth of cells embedded in Matrigel. We have then generated stable MCF-7 cells expressing a short hairpin RNA to measure longer biological effects. Two clones that exhibited 80-90% reduction in PTPL1/PTPN13 protein expression formed larger colonies in soft agar and presented a significantly increased tumour growth in nude mice compared with control clones.

Conclusions. These studies demonstrate that loss of PTPL1 expression induces phenotypic changes of breast cancer cells in agreement with its role as a potential tumour suppressor gene in this disease.

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O6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation in neoplastic and normal mucosa in patients with colorectal carcinoma(CRC)

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Purpose: Colorectal cancer is a complex and heterogeneous disease in which genomic instability and epigenetic alterations play important roles. Hypermethylation of cytosine residues in the CpG islands of certain DNA repair genes is a distinct epigenetic alteration in CRC. The process contributes to cancer formation through the transcriptional silencing of genes. MGMT gene removes mutagenic adducts from the O6-position of guanine in DNA. Loss of its function leads to the inactivation of DNA repair and to microsatellite instability. Experimental procedures: 31pts with CRCs (18M/ 13F, mean age 67.67 years, 3 of them less than 50 years old) were included. The tumors, 13 of the right and 18 of the left colon, were staged according to Astler- Coller classification. The analysis of methylation status in the promoter region of the MGMT gene was performed on isolated genomic DNA, which was obtained from 31 paraffin-embedded colon cancer samples and from their normal mucosa. Finally, a total of 62 samples were examined using modified protocols for bisulfite treatment and methylation-specific “hot-start” PCR (MSP) followed by detection on agarose gel. Our results on promoter methylation of MGMT gene were correlated with known clinicopathological parameters. Results: MGMT promoter methylation was present in 29% of the tumors (9 out of 31 pts). Five out of 9 pts whose cancer had MGMT promoter methylation also had substantial MGMT promoter methylation in their normal mucosa. All pts with promoter methylation showed low differentiated CRCs with proximal mostly location of the tumors (6/9), while no age- related correlation was noted. Conclusion: MGMT gene is frequently methylated in CRC. It appears that the methylation plays a more important role in proximal colon cancer development than in distal colon cancer and is correlated with low differentiated CRC. In our study no patient, without promoter methylation of MGMT gene in cancer tissue, showed this epigenetic alteration in the corresponding normal mucosa. Further studies with more pts are now carried out in order to assess if the above observations may ultimately be useful in CRC studies.

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ZEB-1 can repress SEMA3F semaphorin - a tumor suppressor gene in lung cancer

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SEMA3F, a class-3 semaphorin gene, encodes a potent tumor suppressor protein with effects on both tumor and endothelial cells. Downregulation of SEMA3F is frequent in lung cancer and correlates with advanced stage disease, while the reexpression of SEMA3F in tumor cells inhibits multiple signaling components, HIF-1alpha protein translation and VEGF mRNA. Therefore, understanding how SEMA3F expression is inhibited is important. We previously defined the promoter organization of SEMA3F and found that while promoter methylation correlated with transcriptional repression, chromatin remodeling by a histone deacetylase inhibitor (HDACi) was sufficient to activate SEMA3F expression. In lung cancer, we have shown that ZEB-1, an E-box transcription repressor, is predominantly responsible for loss of E-Cadherin associated with a poor prognosis and resistance to EGFR inhibitors.

In the present study, we found that ZEB-1 is also responsible for SEMA3F repression. Levels of ZEB-1, but not Snail or Slug, significantly correlate with SEMA3F inhibition in lung cancer cell lines. Similarly, overexpression or inhibition of ZEB-1 correspondingly affected SEMA3F expression. Four conserved candidate E-box sites were identified in the SEMA3F gene. ZEB-1 binding was confirmed by chromatin immunoprecipitation assays for two of these: site 1 in the CpG-island promoter region and site 4 in the third intron. In addition, ZEB-1 binding to these sites was reduced when cells were treated with a HDACi.

In conclusion, these results demonstrate that ZEB-1 directly inhibits SEMA3F expression in lung cancer cells. Together with its effects on E-Cadherin, these data indicate that ZEB-1 plays a critical role in the pathogenesis or progression of this disease.

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Polymorphic microRNA binding sites within candidate genes are associated with the risk of colorectal cancer

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Introduction: The individual risk to develop colorectal cancer (CRC) is hypothesized to be modulated, at least in part, by common polymorphisms within specific candidate genes. They are involved in the carcinogenic processes, through the regulation of the cell growth, differentiation, apoptosis, and the maintenance of genome stability. Recent evidences indicate that small non-coding RNA molecules, called micro-RNAs (miRNAs), bind to the 3'UTRs of mRNAs and interfere with their translation, thereby regulating cell growth, differentiation, apoptosis, and tumorigenesis. Therefore, we hypothesized that polymorphic miRNA binding sites at the 3'UTRs of cancer candidate genes could modulate the individual risk of cancer.

Materials and Methods: To confirm our hypothesis, we selected 129 genes that, according to published data and various online resources (e.g. BioCarta and KEGG pathways; http://cgap.nci.nih.gov/Pathways) are candidate genes for CRC. Fifty-one genes are involved in inflammatory processes, 37 belong to synthesis of prostaglandins and thromboxanes, 16 genes are connected with obesity and insulin resistance, and 25 genes are involved in early and late stage of this type of tumour. We identified putative microRNAs binding sites by means of specialized algorithms (PicTar, DianaMicroT, miRBase, miRanda, TargetScan, and microInspector). Then, we found 79 SNPs within the putative binding sites for their ability to affect or impair the binding with the miRNA, by assessing the variation of ΔG (Gibbs free energy) (defined as $\Delta\Delta G$) comparing the "wild-type" and their correspondent variant alleles. Considering the validation status of the SNPs and their frequencies (MAF>0.10), we found at least 15 candidate polymorphisms of biological relevance that could be investigated by performing case-control association studies on a series of samples from Czech Republic.

Results: We found statistically significant associations between risk of CRC and variant alleles of CD86 (OR=2.74 95%CI=1.24-6.04, for the variant homozygotes) and INSR genes (OR=1.94; 95%CI=1.03-3.66, for the variant homozygotes).

Conclusion: This study suggests that SNPs in miRNA binding sites may be important in the modulation of the individual risk of cancer and encouraged to undertake future works. Moreover, since the genotyping allows the screening of a relatively large number of polymorphisms in short time, the proposed study suggested also a way to restrict the number of miRNA targets to be actually experimented using time-consuming molecular biology techniques.

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HIF-1alpha is a novel target of the SWI/SNF chromatin remodelling complex

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Background: Hypoxia inducible factor (HIF-1) is a master regulator of the transcriptional responses to hypoxic stress. The majority of HIF-1alpha control happens at the protein level and mRNA changes in response to hypoxia are not readily observed. The SWI/SNF chromatin remodelling complex is important for activation and repression of transcription, and acts by modulating chromatin structure. Despite the importance of this complex, only a few direct targets have been identified.

Methods: Using mRNA, Western Blot and promoter analysis we have investigated how chromatin remodelling complexes regulate HIF-1alpha.

Results: We demonstrate that the HIF-1alpha is a direct target of SWI/SNF. SWI/SNF components are found associated with the HIF-1alpha promoter and their depletion results in a reduction of HIF-1alpha expression and its ability to transactivate target genes. Importantly, depletion of BAF57 (a conserved subunits of SWI/SNF) results in reduced recruitment of other SWI/SNF components as well as impaired polymerase II recruitment.

Conclusions: These results reveal a previously uncharacterized dependence of HIF-1alpha on the SWI/SNF complex, demonstrating a new level of control over the HIF-1alpha system. In addition, these studies identify BAF57, as the main targeting subunit of SWI/SNF to the HIF-1alpha promoter.

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Convergent mechanisms that activate MYB transcription in colon and breast cancer which provide a therapeutic opportunity to target metastatic disease

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Background: MYB is over-expressed in the majority of colo-rectal cancers1 (CRC) and ERalpha positive breast cancers. Activation of MYB transcription occurs at the earliest stages of adenoma formation in the colon and progressively increases in primary and finally metastatic adenocarcinoma. In CRC mutations that affect the transcriptional elongation of the gene are frequent and have been functionally validated2. Conversely in breast cancer mutations in this sequence are rare but estradiol induces ERalpha mediated induction of MYB transcription obviating the need for the mutations inherent in CRC3. Materials and Methods: Experimental mouse models have bee used to identify the interplay between c-Myb and the adenomatous polyposis coli gene in synergistically driving the c-Myc gene expression and that c-Myb over-expression increases in metastatic CRC and mammary cancer. With this in mind a DNA fusion vaccine has been devised to generate a c-Myb specific immune response to potentially treat these two common cancers. This has been achieved even though c-Myb, like many tumor antigens, is weakly immunogenic as it is a "self" antigen and thus subject to tolerance. To break tolerance, a DNA fusion vaccine was generated comprising wild-type c-Myb cDNA flanked by two potent Th epitopes derived from tetanus toxin. Vaccination was performed targeting a highly aggressive, weakly immunogenic, subcutaneous, syngeneic, colon adenocarcinoma cell line MC38 which highly expresses c-Myb. Results: Prophylactic intravenous vaccination significantly suppressed tumor growth, through the induction of c-Myb specific anti-tumor immunity for which the tetanus epitopes were essential. Vaccination generated anti-tumor immunity mediated by both CD4+ and CD8+ T cells and increased infiltration of immune effector cells at the tumor site. Importantly, no evidence of autoimmune pathology in endogenous c-Myb expressing tissues was detected 4. Conclusions: These data highlight the role of MYB in 2 common epithelial cancers and establish c-Myb as a viable antigen for immune targeting and serve to provide proof of principle for the continuing development of DNA vaccines targeting c-Myb. As c-Myb is expressed at its highest in metastatic CRC we propose that a vaccine against c-Myb may have a place in patients post-surgery and adjuvant therapy.

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Identification of proteins implicated in Kit receptor signalling

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The receptor tyrosine kinase Kit is required for the development of germ cells, melanoblasts, interstitial cells of Cajal, erythroblasts and mast cells. Gain-of-function mutations of Kit are found in human proliferative pathologies such as mastocytosis, gastrointestinal stromal tumour (GIST), acute myeloid leukaemia (AML) of the CBF class or testicular germ cell tumours. Different kinds of mutations lead to ligand-independent activation of the receptor. The substitution of the aspartate 816 in the kinase domain occurs in 80% of the cases of mastocytosis. Substitutions or deletions in the regulatory juxtamembrane domain also induce constitutive receptor activation and subsequent cellular transformation.

Following stimulation by its ligand, Kit undergoes transphosphorylation on tyrosine residues, thus creating docking sites for signalling molecules. The JM domain of Kit contains 6 tyrosines of which Y568 and Y570 are autophosphorylation sites. We work with HMC-1 cells (a human mastocytoma cell line carrying the mutation D816V) as a model of Kit