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Altered NDRG1 expression in breast cell lines influences apoptosis

E. Gjernes[1], H.A. Askautrud[1], G.L. Størvold[1], D.T. Ross[2], A.L. Børresen-Dale[3], C.M. Perou[4], E. Frengen[1]
[1]Ullevål University Hospital and Faculty of Medicine, Department of Medical Genetics, Oslo, Norway; [2] Applied Genomics Inc, Research and Development, Burlingame, USA; [3] Norwegian Radiumhospital Rikshospitalet University Hospital, Department of Genetics, Oslo, Norway; [4] Lineberger Comprehensive Cancer Center, Department of Genetics and Pathology, Chapel Hill, USA

Background: Several genes on chromosome 8q are amplified and show increased expression in breast tumours and breast cell lines. One of these genes, NDRG1, has been shown to be expressed at a higher level in cancerous than non-cancerous tissue of the same origin. However, increased NDRG1 expression has also been shown to inhibit tumour cell growth and NDRG1 has been suggested as a candidate metastasis suppressor gene in breast and prostate cancer. In addition, NDRG1 is induced in hypoxic conditions and results have suggested that in some cell lines NDRG1 expression is necessary for TP53-mediated apoptosis.

To gain more insight into the role of NDRG1 in tumour development, we have developed stable breast cell populations where the NDRG1 expression is reduced by RNAi and cell populations ectopically expressing the NDRG1 cDNA. In these studies we have selected cell lines with characteristics corresponding to the basal and luminal breast cancer subtypes.

Methods: We have used the two basal breast tumour models, SUM102 and ME16C2, which have high endogenous expression of NDRG1, and one luminal cancer subtype model, ZR-75-1, with low endogenous NDRG1 expression. Two shRNA constructs against independent target sequences on the NDRG1 transcript were introduced into the SUM102 and ME16C2 cell lines, resulting in a significant reduction in NDRG1 expression. The ectopic expression of the NDRG1 cDNA in ZR-75-1 gave a 100-fold increase in the NDRG1 protein level on western blots. Global gene expression analysis of the cell populations was also performed using microarrays.

Results: In the microarray experiments, SUM102 and ME16C2 cells transduced with the NDRG1 shRNAs were compared to cells transduced with empty vector. The differentially expressed genes identified suggested an enrichment of genes involved in cell adhesion and migration, which were generally increased in expression. Currently experiments are performed using the set of cell populations to investigate if NDRG1 has a role in cell migration and invasion. We have further treated the transduced cell populations with doxorubicin to induce TP53, and the results obtained by the TUNEL assay indicate that the induction of apoptosis is dependent of the level of NDRG1 expression. Experiments are also performed to assess how the cells with altered NDRG1 expression react to reduced oxygen levels ranging from hypoxic to anoxic conditions.

Conclusions: Our preliminary results indicate that NDRG1 is involved in cell migration and invasion. Our results further indicate that induction of apoptosis by doxorubicin is enhanced by increased levels of NDRG1 expression in the cell populations studied. This suggests that NDRG1 has a role in the apoptotic process both in the luminal and basal breast cancer cell models.

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Potentiation of antitumoral and antiangiogenic actions of docetaxel by docosahexaenoic acid (DHA): impact on micro- and macro-vascularization

S. Vibet[1], K. Mahéo[1], J. Goré[1], T. Hardy[2], P. Bougnoux[1], F. Tranquart[2], C. Goupille[1]
[1]INSERM U921, Nutrition Growth and Cancer, Tours, France; [2] INSERM U930, Explorations morphofonctionnelles cérébrales : développements et applications, Tours, France

Tumour sensitivity to anticancer agents is a key feature for cancer curability. In previous studies, we reported that marine origin long chain polyunsaturated n-3 fatty acids such as docosahexaenoic acid (DHA) increased tumour sensitivity to anthracyclines, both in human breast cancer cell lines and in autochthonous rat mammary tumours (Germain 1998, Colas 2005). Furthermore we found that this dietary DHA effect was associated to a reduction in tumour vascularization. Thus, tumour vascularization might have a pivotal role in chemosensitization induced by DHA. Taxanes such as docetaxel are major drugs in breast cancer treatment. Since this chemotherapy displayed antiangiogenic properties, we examined whether dietary DHA potentiates its antitumor action through a vascular-based effect.

Autochthonous mammary tumours were induced in Sprague-Dawley female rats by N-methylnitrosourea. Then rats were assigned to a control group or to a group with a fish oil-enriched diet (with a supplementation in DHA 2.5%). When the largest tumour reached 2 cm², docetaxel (6 mg/kg) was injected weekly during 6 weeks. Subsequent changes in the tumour surface were evaluated. Using microbubbles of Sonovue® as a contrast agent in ultrasonography, we measured dynamic parameters of the whole vascularization (contrast diffusion speed, contrast transit time) and the macrovascularization was discriminated from the microvascularization by using a MATLAB® script.

Under docetaxel treatment, we observed a stagnation of tumour size in control rats whereas dietary fish oil induced tumour regression as early as three weeks of chemotherapy (-65% at the end of treatment). Despite unchanged dynamic parameters of vascularization, we observed in the control group an antiangiogenic effect of docetaxel (-45 %) using contrast-enhanced sonography. Furthermore, the data we obtained in DHA supplemented rats suggested an enhanced antiangiogenic effect of docetaxel on mammary tumour vascularization (-63 %). In fact, after discrimination of macro- and micro-vascularizations, we observed only a decrease in macrovascularization (-52 %) in control rats, whereas DHA supplemented animals showed a decrease in both micro- and macro-vascularizations (-43 % and -75 % respectively).

Thus reduction in both micro and macrovascularizations by DHA may account for the enhancement of docetaxel antiangiogenic properties and might contribute to the increase in docetaxel efficacy.

www.n2c.univ-tours.fr - sophie.vibet@gmail.com

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Functional analysis of CDKN2A 5'UTR variants associated with family history of melanoma

A. Bisio[1], S. Nasti[2], S. Gargiulo[2], A. Provenzani[3], A. Quattrone[3], A. Inga[1], G. Bianchi-Scarrà[2]
[1]National Institute for Cancer Research, Molecular Mutagenesis and DNA Repair Unit, Genova, Italy; [2] University of Genoa, Department of Oncology Biology and Genetics, Genova, Italy; [3] University of Trento, Center of Integrative Biology, Trento, Italy

Malignant Melanoma derives from the neoplastic transformation of melanocytes and can arise de novo or from pre-existing benign nevi. Melanomas can occur in cancer prone families susceptible to a variety of cancers, such as in Li-Fraumeni syndrome families, or be the predominant cancer type in the case of Familial Cutaneous Malignant Melanoma (OMIM %155600). The CDKN2A gene is an established high-penetrance melanoma susceptibility gene. Germline CDKN2A mutations are observed in approximately 20–40% of melanoma-prone families from around the world. We have identified four Italian patients with established family history of melanoma that did not present mutations in the coding regions of CDKN2A (both p16 and p14arf were examined) nor of CDK4, but exhibited heterozygous variants in the 5'UTR of CDKN2A. To begin addressing the functional consequences of these novel variants we cloned the 271bp 5'UTR in different types of luciferase-based reporter vectors that can measure transcriptional as well as post-transcriptional effects. In a vector type, the UTR is cloned immediately upstream of the luciferase cDNA, which is transcribed from a minimal viral promoter. In a second vector the UTR sequence is placed upstream of a minimal promoter to evaluate its enhancer potential. We also constructed a bicistronic dual-luciferase vector that could test the effect of the variants on cap-independent translation.

To validate the reporter assays we examined a G>T transversion at the –34 position of the UTR that was previously identified in large melanoma-history family and shown to give rise to a novel AUG translation initiation codon out-of-frame with the canonical AUG, resulting in impaired translation of the gene. We also tested known single nucleotide polymorphisms (SNP) at the -33 and -191 positions. Unlike the two SNPs, the G-34T variant resulted in decreased activity of the luciferase reporters in the various plasmid types. Experiments are underway to examine the newly identified variants in these gene reporter assays. Quantitative PCR is also being used to further characterize the transcriptional/post-transcriptional effects of the variants. Finally, allele-specific qPCR is being attempted using lymphoblast cell lines derived from the heterozygous patients to confirm the impact of the 5'UTR variants at the endogenous gene.

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Tumor cell growth and metabolism at low growth factor and nutrient levels

A.M. Otto[1], C. Janzen[1], I. Szabados[1], U. Hopfner[1], S. Pritzlaff[1], B. Wolf[1]
[1]Munich University of Technology, Institute of Medical Electronics, Munich, Germany

The tumor microenvironment is characterized by deficiencies in the supply of oxygen and nutrients as well as by an acidic pH. To understand how these parameters affect energy metabolism and growth of tumor cells requires a systematic analysis of their interactions. Towards this aim we cultivated two breast cancer cell lines representing different degrees of malignancy (MCF-7, MDA-MB231) in medium containing 1% FCS

supplemented with various combinations of low glucose and glutamine concentrations at different pH-values. Cell growth was determined by cell count, while metabolism was monitored by the activities of pyruvate kinase, lactate dehydrogenase and mitochondrial dehydrogenases (XTT-assay). Moreover, a test system based on electronic sensor chips was used to monitor in parallel and in real-time the rate of extracellular acidification and oxygen consumption. Sensor chip measurements showed that a low serum concentration along with an acidic pH immediately reduced cellular acidification and respiratory activities, demonstrating the high plasticity of tumor cell metabolism. This metabolic reduction correlated with reduced cell growth. Cell growth was further attenuated when levels of glutamine and glucose fell below 0.5 and 1 mM, respectively; however, the specific activity (per cell) of XXT-conversion to formazan increased, indicating an inverse relationship between growth inhibition and the activity of mitochondrial dehydrogenases. On the other hand, the optimal concentration of glucose was highly dependent on the medium pH. When testing combinations of the metabolites at different concentrations, 0.1 mM glutamine with 2.5 mM glucose produced an extraordinary increase in formazan formation and pyruvate kinase activity, a key enzyme of the glycolytic pathway. In contrast, neither the activity of lactate dehydrogenase, of which pyruvate is a substrate, nor cell number showed a similar increase. Progressive doubling of the concentrations of this glucose and glutamine combination increased the cell number only by a factor of 1.3 during a 5-day incubation. Therefore, these parameters of energy metabolism do not correlate with tumor cell growth. This could mean that at low nutrient levels, cell proliferation is attenuated to ensure energy metabolism required for cell survival.

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Fra2 is an antagonist of p53

S. Piccinin[1], A. Grizzo[1], A. Zambon Bertoja[1], M. Garziera[1], M. Armellin[1], R. Maestro[1]
[1]CRO-National Cancer Institute, Experimental Oncology 1, Aviano, Italy

Repression of oncogene-induced apoptosis plays a key role in tumor development and progression. Through a phenotype screen in mammalian cells we have isolated Fra2 as a gene that confers protection from oncogene-induced apoptosis. Fra2 (Fos-related antigen 2) is member of the Fos family of transcription factors that, together with Jun proteins, participate in the formation of the AP-1 complex. Alterations of the AP-1 complex have been reported for a variety of cancers. Nevertheless, the specific role of Fra2 in this context has been only marginally investigated.

Here we provide evidence that Fra2 is an antagonist of the p53 pathway. Ectopic expression of Fra2 promotes the bypass of p53-dependent apoptosis and growth arrest of mouse embryo fibroblasts. Ectopic expression of Fra2 is accompanied by increased degradation of p53, which only in part depends on MDM2.

These data, together with the finding of elevated levels of Fra2 in a significant fraction of breast and colorectal cancers, support a role for Fra2 as important modulator of p53 in human cancer.

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The association of expression level of PRL-3 mRNA and liver metastasis in primary colorectal cancer

M. Baek[1], C. Chu[2], O. Song[2], E. Shin[2], D. Park[3]
[1]Soonchunhyang University College of Medicine, Surgery, Cheonan, Korea; [2] Soonchunhyang University Bucheon Hospital, Surgery, Bucheon, Korea; [3] Dankook University Hospital, Surgery, Cheonan, Korea

Liver metastasis occurs in 40-60% of all colorectal cancer patients, and main cause of deaths in colorectal cancer is liver metastasis. Studying the molecular basis of liver metastasis and identifying metastasis specific markers would provide critical information for the diagnosis and treatment of colorectal cancer. PRL-3 is a newly identified metastasis-related gene, which codes a 22 KDa non classical protein tyrosine phosphatase. In this study, we examined the relationship between PRL-3 expression and liver metastasis in colorectal carcinoma. Our purposes of the study are to asses PRL-3 mRNA expression in the primary colorectal cancers by quantitative analysis, investigate the correlation between clinicopathological features and PRL-3 expression and verify the utility of PRL-3 in predicting liver metastases in patients with colorectal cancer. From January of 2004 to December of 2006, the data of 86 patients who underwent surgical resection for colorectal cancer was collected. Resected specimens were cooled by liquid nitrogen and preserved at -80°C. RNA was extracted by usual manner from the preserved tissue. Real time RT-PCR using Light Cycler instrument (Roche Molecular Systems, Alameda, CA) was performed for quantitative analysis of PRL-3 mRNA. Retrospective analysis of correlation between PRL-3 mRNA expression and clinicopathologic factors (Gender, age, stage, cell differentiation, lympho-vascular invasion, and neural invasion). In our study, the association among PRL-3 mRNA expression and liver metastasis, and lympho-vascular invasion showed statistically significant correlation. High expression of PRL-3 was closely associated with extent of lymph node metastasis and tumor stage. These results suggest that high PRL-3 expression may participate in the liver metastasis of colorectal carcinoma. A close association between PRL-3 mRNA expression and liver metastasis of colorectal cancer suggests that PRL-3 assessment can be used to predict liver metastasis of patients with colorectal cancer. PRL-3 might be a novel molecular marker and a potential therapeutic target for colorectal cancer liver metastasis.

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Anti-metastatic therapeutic modalities based on the aged cancer patient as a model - a suggestion

J. Leibovici[1], T. Kaptzan[1], O. Itzhaki[1], E. Skutelsky[1], J. Sinai[1], A. Siegal[1], M. Michowitz[1]
[1]Tel Aviv University, Pathology Sackler Faculty of Medicine, Tel Aviv, Israel

Background: Metastasis is the most appalling aspect of cancer, responsible for the large majority of cancer- related deaths. However, practically almost no effective anti- metastatic treatment is available.

An apparently surprising phenomenon is the fact that while tumor incidence is known to increase with age, tumor growth and metastatic development often proceed at a slower rate in many cancers of elderly patients.

The question is what in the old organism reduces the aggressiveness of tumors. We reasoned that some information might be learned from this phenomenon by trying to identify the factors in the aged organism which are responsible for the reduced progression of tumors. Based on these mechanisms, we can then attempt to design anti- metastatic treatment modalities.

Materials and Methods: We compared in two experimental tumors, B16 melanoma and AKR lymphoma, in which we have shown a differential tumor growth in young and old mice (1), the degree of apoptosis (according to Apoptag staining and DNA flow cytometry), angiogenesis (microvessel density) and macrophage content in tumors from mice of different ages.

Results: We demonstrated that mechanisms responsible for the reduced tumor progression in the aged can be: increased apoptotic cell death, decreased angiogenesis and modification in immune response. The differences between tumors from young and those from old animals for the three properties were very marked: about 2 and 3 fold increase in apoptosis, according to cell morphology, in B16 melanoma and AKR lymphoma, respectively, 2 and 6 fold decrease in angiogenesis in B16 melanoma and AKR lymphoma, respectively, and about 15 fold increase in macrophage content in AKR lymphoma and a more modest increase, difficult to assess quantitatively, in B16 melanoma.

Conclusions: We suggest that drugs enforcing these mechanisms, namely inducers of apoptosis, anti- angiogenic drugs and immuno-modulators, might act as anti- metastatic drugs.

Moreover, anti- metastatic treatments based on the mechanisms responsible for the reduced tumor progression in the aged might be biologically more relevant and possibly less toxic than chemotherapeutic drugs which act against one and only cell property, tumor cell proliferation, a characteristic not necessarily relevant to the metastatic phenotype.

We can view the aging organism as a model of reduced metastatic spread. Studying the metastasis- inhibitory mechanisms of the aged may suggest new, so desperately needed, possibilities to treat metastatic disease, in old as well as in young patients. Studying the ways an elderly patient deals with metastatic spread could conduce to the discovery of treatment modalities which might be more relevant to physiological factors and possibly less offensive to the host.

Reference
Donin et al., Cancer Invest. 15: 416,1997

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Elevated apoptosis in tumors of aged as compared to those of young mice is more pronounced in primary than in metastatic tumors of AKR lymphoma

O. Itzhaki[1], T. Kaptzan[1], E. Skutelsky[1], J. Sinai[1], G. Schiby[1], M. Huszar[1], J. Leibovici[1]
[1]Tel Aviv University, Pathology Sackler Faculty of Medicine, Tel Aviv, Israel

Background: While incidence of neoplasia is known to increase with age, tumor growth and metastatic spread proceed, paradoxically, at a slower rate in aged as compared to young patients. Although not a general feature, this intriguing phenomenon is observed in many human and experimental tumors. We have shown this particular behavior in the AKR lymphoma and B16 melanoma.