tumor suppressor genes (TSG) in many types of cancers, including lung cancer. However, their expression can be restored by demethylating and histone deacetylating inhibiting drugs such 5Aza-dC-2deoxycytidine (5Aza-dC) and trichostatin A (TSA). Platinum-induced DNA hypermethylation may be involved in the development of drug-resistant phenotypes by inactivating genes required for drug cytotoxicity. We aim to identify the global profile of TSG silenced by epigenetic mechanisms in NSCLC cell lines after CDDP treatment, and therefore potentially involved in the development of chemotherapy resistance. The present study is based on an expression microarray analysis of genes reactivated in a set of CDDP-resistant and sensitive NSCLC cell lines after 5Aza-dC and TSA treatment. CDDP-resistant cells were established by treating two NSCLC cell lines, H-460 and H-23, with increasing concentrations of CDDP. Then, cells were exposed to 5Aza-dC (5μM) and TSA (500nM) before RNA extraction. Total RNA from the different cell lines was extracted, reverse-transcribed and hybridized into an array platform containing the whole human genome. We selected for validation those genes upregulated after 5Aza-dC and TSA treatment, which expression was previously downregulated in CDDP-resistant versus cisplatin-sensitive cell lines. Next, we confirmed the presence of CpG island in the promoter region, the expression in normal lung cells and excluded those genes located in imprinted areas. Gene expression changes were confirmed by semi-quantitative RT-PCR. Promoter methylation was validated by bisulfite sequencing. Finally, methylation of validated genes was analyzed by methylation specific PCR (MSP) in NSCLC specimens with known CDDP response. Epigenetic regulation of selected genes was further studied in the abstract presented by M Cortes (back to back Poster). We have identified, a panel of genes with altered expression as a result of CDDP and epigenetic reactivation treatments. After validation of five, we confirmed one gene as a potential clinical marker, able to detect with 80% specificity, sensitive versus CDDP-resistant tumors in a panel of 30 paraffin embedded NSCLC samples. This study provides information regarding de novo promoter hypermethylation of potential TSG involved in the development of resistance and their potential use as targets enabling the diagnosis and chemotherapy treatment of NSCLC to be approached at the molecular level.Supported by Health Investigation funding (FIS/ISCIII). Supprted by FIS project number: PI061234 and by an unrestricted educational grant by Fundación Mutua Madrileña

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Differential expression of DNAmethyltransferases in sensitive versus cisplatin resistant NSCLC cell lines

M. Cortés-Sempere[1], R. Perona[1], I. Ibanez de Caceres[1]
[1]Biomedical research Institute, Translational Oncology CSIC/H. La Paz., Madrid, Spain

Aberrant promoter hypermethylation is a common epigenetic mechanism for the silencing of tumor suppressor genes (TSG) in many types of cancers, including lung cancer. In eukaryotes there are three families of DNA methyltransferase enzymes (DNMT) that catalyzed the DNA methylation process. DNMT3 family is involved primarily in methylation of new sites and the DNMT3B member is more highly expressed in human cancer cell lines and primary tumors than in normal tissue. In addition it has been recently reported a new subfamily of DNMT3B (delta-DNMT3B) that are the predominant forms in non-small cell lung cancer (NSCLC), suggesting an important role in DNA methylation control in lung tumorogenesis. One of the main problems in this tumor type is the frequent development of acquired-chemotherapy resistance. Genetic and epigenetic alterations are known to underlie the initiation and progression of neoplasia, therefore, one of the possible reasons for the development of chemotherapy resistance in NSCLC might be the epigenetic inactivation of certain TSG as a consequence of chemotherapy treatment. In addition, cisplatin (CDDP), the paradigm of cytotoxic drugs for NSCLC treatment, has been reported to induce, de novo DNA hypermethylation in vivo.

We analyzed the potential role of the DNMT family in the development of chemotherapy resistance to CDDP in NSCLC. The study is based on an expression microarray analysis of genes reactivated in a set of CDDP-resistant and sensitive NSCLC cell lines after 5Aza-dC and TSA treatment. Resistant cells were established by treating two NSCLC cell lines, H-460 and H-23, with increasing concentrations of CDDP. Then, cells were exposed to 5Aza-dC (5μM) and TSA (500nM) before RNA extraction in order to reactivate those genes epigenetically silenced in the resistant cell lines. Total RNA from the different cell lines was extracted, reverse-transcribed and hybridized into an array platform containing the whole human genome. The first part of this study is accessible in the abstract presented by I Ibanez de Caceres, in which we show very promising results regarding de novo promoter hypermethylation of specific genes and their relevance in the development of chemo-resistance to CDDP in NSCLC. In order to confirm a possible role of DNMT members silencing the selected genes in chemoresistance, we analyzed the differential expression of the DNMT family on sensitive versus CDDP-resistant cell lines. We found a marked increased expression of DNMT3B in the resistant cell lines compared with the parental ones. We confirmed this result by RT-PCR in both NSCLC cell lines, and in the ovarian human cancer cell lines 41M and 41MR, sensitive and resistant to CDDP respectively. Those results indicate a possible role of DNMT3B on the epigenetic regulation of specific genes responsible of the CDDP-acquire-resistance process; defining possible innovative treatment strategies for platinum resistant tumors.

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EGFR mutation in renal cell carcinoma confers sensitivity to gefitinib treatment

A. Sokolenko[1], A. Iyevleva[1], A. Novik[2], A. Ivantsov[3], V. Moiseyenko[2], E. Imyanitov[1]
[1]N.N. Petrov Scientific Research Institute of Oncolo; [2] N.N. Petrov Scientific Research Institute of Oncology, Clinical Department, St-Petersburg, Russian Federation; [3] N.N. Petrov Scientific Research Institute of Oncology, Department of Pathomorphology, St-Petersburg, Russian Federation

Intragenic microdeletions and selected missense mutations located within tyrosine domain of epidermal growth factor receptor (EGFR) are known to be associated with the pronounced response to low molecular weight EGFR tyrosine kinase inhibitors (TKI), gefitinib or erlotinib. Unfortunately, these TKI-sensitizing mutations have been detected almost exclusively in lung adenocarcinomas, while their occurrence in tumors of other histological types or other organs is exceptionally rare. We applied EGFR mutation test as the last hope option to a heavily pretreated patient G., 60 years old, who suffered from the progression of renal cell carcinoma (RCC) and was administered to the hospital due to life-threatening condition. Unexpectedly, PCR and sequencing analysis revealed “lung-type” 15 base pair deletion, so the therapy by gefitinib was applied. This treatment led to a dramatic symptomatic response within the first week of therapy; the reduction of dyspnea was so evident that allowed the patient to return to the work. Clinical examination demonstrated complete disappearance of extensive pleuritis and pericarditis. Computed tomography measurement of metastatic lesions revealed minor response which could be classified as disease stabilization (RECIST criteria). The duration of clinical benefit was 4 months. The above observation led to a question, whether EGFR mutations occur at a noticeable frequency in RCC and whether their testing has to be considered in the routine clinical setting. Available literature indicates that only 38 RCC samples have been tested for the presence of EGFR mutations up to now, and one of those contained “lung-type” EGFR deletion. Therefore, we collected 118 RCC cases and subjected the tumor material to molecular analysis. However, none of these tumors contained TKI-sensitizing EGFR mutation. Taken together with published data, the following conclusions can be drawn from this study: 1) By now, 3 cases of gefitinib treatment of EGFR mutation-containing non-lung tumors (1 thymoma, 1 ovarian carcinoma, and 1 RCC from this study) have been reported, and all of them demonstrated evident clinical benefit from the therapy. Therefore, intragenic deletions of EGFR confer sensitivity to TKI treatment independently of the tumor type. 2) EGFR mutations in RCC are rare, thus the utility of the appropriate test for clinical management of kidney cancer remains questionable.

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MLH1 promotor hypermethylation, BRAF and K-ras mutation analysis on tumours suspected from Lynch Syndrome to prioritize mismatch repair gene testing

E. Meulemans[1], G. Roemen[1], A. Rüland[1], J. Peeters[1], M. Van Engeland[2], A. De Bruïne[1], E. Gomez-Garcia[3], F. Bot[1]
[1]University Hospital Maastricht, Department of Pathology, Maastricht, The Netherlands; [2] University Maastricht, Department of Pathology, Maastricht, The Netherlands; [3] University Hospital Maastricht, Department of Clinical Genetics, Maastricht, The Netherlands

Background: Microsatellite Instability (MSI) testing and immuno-histochemistry (IHC) are powerful tools that help identify individuals at risk for having LS and current diagnostic strategies can detect almost all highly penetrant Mismatch Repair (MMR) gene mutations. Our goals were to compare the performance of two panels of microsatellite markers in relation to IHC, as well as BRAF V600E mutation- and MLH1 promotor hypermethylation assays, and the determination of KRAS mutations in the Microsatellite Stable tumours (MSS).

Methods: Patients with a family history suggestive of Lynch syndrome (n=524) in the time period of 2005 till 2007 were tested for MSI and IHC staining of the MMR proteins MLH1, MSH2, MSH6 and PMS2. MSI-high tumours without expression of MLH1 in IHC, were tested for BRAF V600E

mutation and hypermethylation of hMLH1 promotor. MSS tumours from patients < 50, were tested for K-ras mutations in codon 12/13.

Results: MSI-high tumours (75/524) were negative in at least one of the four MMR IHC staining (14%). MSI-high tumours negative in MLH1 IHC (n=37), contained in 16% the BRAF V600E mutation and in 27% hypermethylation of the MLH1 promotor. BRAF mutation together with MLH1 methylation was present in 20 % of the cases. Most of the tumours showed MSS and were positive in the four MMR IHC (83%), In 85 MSS and MMR positive IHC tumours (patients < 50), K-ras mutations were detected in 26 cases (30,5%). We identified six different K-ras mutations and most of the DNA changes (80%) occurred at the second base of codon 12 and codon 13 (21/26).

Conclusions: A combination of both MSI and IHC provides the most optimal selection for mutation analysis. MLH1 methylation analysis in MSI-H CRC is a valuable molecular tool to distinguish between HNPCC and sporadic MSI-H CRC and the detection of a BRAF V600E mutation further supports the exclusion of HNPCC. A somatic transversions G>T in K-ras is associated with a biallelic mutation of MYH and was found in 34%. A K-ras G>A transition is associated with MGMT epigenetic silencing in sporadic CRC lesions and was found in 46 %.

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Cholangiocarcinoma associated blood transcriptome

C. Wongkham[1], C. Subimerb[1], V. Lulitanond[2], S. Pinlaor[3], C. Leelayuwat[4], M.S. McGrath[5], S. Wongkham[1]
[1]Liver Fluke and Cholangiocarcinoma Research Center-Khon Kaen University, Biochemistry, Khon Kaen, Thailand; [2] Khon Kaen University, Microbiology, Khon Kaen, Thailand; [3] Liver Fluke and Cholangiocarcinoma Resarch Center-Khon Kaen University, Parasitology, Khon Kaen, Thailand; [4] Faculty of Associated Medical Science-Khon Kaen University, Immunology, Khon Kaen, Thailand; [5] Faculty of Medicine University of California San Francisco, Pathology, San Francisco, USA

Background: Risk factor of cholangiocarcinoma (CCA), a major cause of cancer death in the northeast of Thailand, is disclosed to be the chronic inflammation related to liver fluke (Opisthorchis viverrini) infection. Association of inflammatory cells, particularly the monocyte-macrophage, with pathogenesis of chronic inflammation diseases and cancer is repeatedly reported. Here we report the gene expression profiling of the circulating peripheral blood cells from CCA patients compared to those of healthy persons.

Material and Methods: RNA was extracted from heparinized blood using TRIzol reagent and purified by Purelink kit. Biotinylated cRNA was synthesized and probed with Oligonucleotide array, HG_U133 plus 2. RNA expression levels were quantitated by measuring the Fluorescence intensity using Affymetrix scanner 3000. Eight differential expressed genes between CCA and healthy subjects were validated by real-time PCR.

Results: Expression profiles of peripheral blood from 9 CCA and 8 healthy subjects were compared. Principle component analysis indicated 49.2% difference between transcriptome of peripheral blood from CCA and those from healthy subjects. Ingenuity Pathway Analysis indicated the involvement in immune response and cancer for blood transcriptome from CCA patients, and the association with immune and hematological development for blood transcriptome from healthy subjects. A gene set coordinately up-regulated by the existence of CCA was obtained. Eight up-regulated genes were verified in homogenates of peripheral blood cells from 18 CCA patients. The obtained gene profiles were grouped according to the pattern to be tumor signature of CCA.

Conclusion: Our findings demonstrated that peripheral blood cells expressed genes which responded to CCA. Blood cells from CCA patients expressed genes involving in cell proliferation, angiogenesis, chemotaxis and anti-apoptosis pathways which may support growth and progression of CCA. In addition, the unique patterns of blood transcriptome may be surrogate transcriptional markers for CCA.

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Relevance of breast carcinoma extracellular matrix composition in drug sensitivity

T. Triulzi[1], P. Aiello[1], F. Castiglioni[1], G. Piloni[1], A. Bergamaschi[2], R. Orlandi[1], S. Ménard[1], E. Tagliabue[1]
[1]Fondazione IRCCS-Istituto Nazionale dei Tumori, Experimental Oncology, Milan, Italy; [2] Institute for Cancer Research, Genetics, Oslo, Norway

A new view of tumors as functional tissue intimately connected with its microenvironment has recently been presented. It is well-established that tumor cells must reorganize the extracellular matrix (ECM) to facilitate communication and escape the homeostatic control exerted by the microenvironment. The identification of the tumor matrix composition represents an important step toward developing response prediction tests and toward the goal of increasing cancer cell sensitivity by modifying their interaction with the microenvironment.

A statistical analysis of microarray (SAM), performed on a dataset obtained from a cohort of breast carcinoma patients treated with docetaxel as neoadjuvant therapy, using sensitivity as supervised variable among a gene list enriched in ECM molecules, resulted in a list of 47 genes that consist of several collagens, laminin chains, matrix-associated proteins as SPARC, and proteolytic enzymes as ADAMs and Cathepsins significantly downmodulated in resistant tumors. Furthermore SAM method using as supervised variable the disease progression resulted in 2 genes significantly downmodulated in tumors with a residual disease major than 100%: PRSS22, a serine protease and CTSC, a cysteine protease that appears to be a central coordinator for activation of many serine proteases, and in 4 upmodulated genes among which serpinB5, an inhibitor of serine proteases, presented a fold increase of 13.4. Based on these data, we hypothesized that tumor protease levels through remodeling of mammary tissue, could play a critical role in drug response. To test this hypothesis, drug response of MDA-MB-361 breast carcinoma cells xenotransplanted in nude mice was evaluated in presence or absence of doxycicline, an inhibitor of collagenolitic enzymes. Taxol or doxorubicin activity was impaired in doxycicline treated mice by 73% and 61%, respectively. Evaluation of collagenases levels in animal plasma and xenotransplants showed that doxycycline significantly reduced both plasma and tumor enzymes level providing evidence that the blocking of tumor proteases expression leads to drug resistance.

The secreted proteolytic enzymes (from both the cancer and stromal environment) are belived to degrade the extracellular matrix, thus facilitating drug diffusion. (Partially supported by a grant from AIRC)

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Re-assessment of Smad-interacting protein 1 expression in human tumor tissues

T. Yagci[1], E. Oztas[1], A.E. Sayan[2], A. Ozcan[3], E. Tulchinsky[2]
[1]Bilkent University, Molecular Biology and Genetics, Ankara, Turkey; [2] Leicester University, Cancer Studies and Molecular Medicine, Leicester, United Kingdom; [3] Gulhane Military Medical Academy, Pathology, Ankara, Turkey

Smad interacting protein 1 (SIP1, also known as ZEB2) encoded by ZFHX1B is a member of ZEB family of transcriptional repressors. SIP1 has been reported to mediate epithelial-to-mesenchymal transition (EMT) in developmental processes and during tumor cell invasion and metastasis by attenuating E-cadherin expression. However, due to the lack of human SIP1-specific antibodies, the expression of SIP1 in human tumor tissues has never been studied. Hence, by immunizing BALB/c mice with a partial human SIP1 recombinant protein (aa 1-400), we generated two monoclonal antibodies, clones 1C9 and 6E5, with IgG1 and IgG2a isotypes, respectively. A squamous epidermoid carcinoma cell line A431 with Tet-on doxycycline-inducible wild-type SIP1 expression (A431/WTSIP1) was used to assess the specificity of generated MAbs. Using both antibodies, nuclear expression of SIP1 in A431/WTSIP1 cells maintained in the presences of doxycycline for 24 hours was detected by immunofluorescence. By using MAb 6E5 we evaluated the tissue expression of SIP1 in paraffin-embedded tissue microarrays consisting of normal and tumoral tissues of kidney, colon, stomach, lung, bladder, esophagus, uterus, rectum and liver. Interestingly, SIP1 mainly displayed a granular cytoplasmic expression and to a lesser extent nuclear localization. The examination of normal tissues revealed a strong expression of SIP1 in distal tubules of kidney, parietal cells of the basal layer of stomach and lobular hepatocytes, implicating a co-expression of SIP1 and E-cadherin. Squamous epithelium of the esophagus and surface epithelium of the bladder, colon and rectum were faintly stained. Normal uterus and lung tissues remained completely negative. By comparison with normal tissues, we observed SIP1 overexpression in renal cell carcinoma, stomach adenocarcinoma, squamous carcinoma of the lung and adenocarcinoma of the uterus. SIP1 was found to be down-regulated in hepatocellular carcinoma and bladder transitional cell carcinoma, and no differential expression was found between normal and tumor tissues of colon and rectum. To our best knowledge, this is the first immunohistochemical study of the expression of SIP1 in human cancers. Our finding that SIP1 is not exclusively localized to nuclei suggests that the subcellular localization of SIP1 is regulated in normal and tumor tissues. This observation adds another level of complexity to the control of EMT programs in tumors progressing towards metastatic state.