716 CpG island hypermethylation of 35 tumour suppressor genes in endometrioid endometrial carcinomas

L. Catasus[1], J. Pena[1], C. Pons[1], J. Prat[1]. [1]*Hospital de la Santa Creu i Sant Pau, Department of Pathology, Barcelona, Spain*

Background: A subset of endometrial carcinomas has methylation of the CpG islands in the promoter region of several tumour suppressor genes. However, methylation patterns and pathophysiological consequences are not well characterized.
Material and Methods: Samples from 36 endometrioid endometrial carcinomas (EEC) were retrieved from the Tumour Bank and the Surgical Pathology files of Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. Genomic DNA was extracted from frozen tumour. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was used to assay CpG island methylation status of 35 different tumour suppressor genes (MRC-Holland®). This method is based on probes that recognize specific sequences in DNA containing a restriction site for a methylation sensitive HhaI enzyme. Methylation specific PCR (MSP) was performed to validate the methylation patterns. DNA was subjected to sodium bisulfite modification using the Methylamp One-Step DNA Modification kit (Epigentek®), and then was subjected to MSP using specific primers for detection of methylated and unmethylated DNA. Results were correlated to various genetic alterations including microsatellite instability (MI), *PTEN, PIK3CA, K-RAS*, CTNNB1, and p53, with the clinicopathologic parameters, and patients follow-up.
Results: Fifteen genes were found methylated in at least 10% of tumours. Promoter hypermethylation was found more frequently in *CDH13* (97%, 35/36), and *RASSF1A* (83%, 30/36) than *MGMT* (53%, 19/36), *MLH1* (50%, 18/36), *WT1* (47%, 17/36), *PAX5* (33%, 12/36), *TIMP3* (31%, 11/36), *TP73* (31%, 11/36), *APC* (28%, 10/36) or *MSH6* (25%, 9/13). Promoter hypermethylation of *TP53*, *GATA5*, *CHFR*, *CD44,* and *CASP8* was found in less than 20% of cases. A CpG Island Methylator Phenotype (CIMP) (methylation of at least 8 of 35 genes) occurred in 33% (12/36) of EEC. The *MLH1* methylation status divided the tumours in high (CIMP) versus low methylation groups ($p = 0.00$). Inactivation of *MHL1* by promoter hypermethylation was present in most carcinomas with MI ($p = 0.002$). Moreover, a trend of inverse correlation was found between *MLH1* promoter hypermethylation or CIMP tumours and *PIK3CA* mutations ($p = 0.060$, and $p = 0.115$ respectively).
Conclusions: *MLH1* methylation may be a general predictor of CIMP. Moreover, the inverse correlation between *MLH1* promoter hypermethylation or CIMP tumours and *PIK3CA* mutations might favor the occurrence of two subgroups of endometrioid endometrial carcinomas.

717 CDX2 regulation in intestinal metaplasia of the stomach

R. Barros[1], L. David[1], R. Almeida[1]. [1]*IPATIMUP – Institute of Molecular Pathol, Carcinogenesis, Porto, Portugal*

Background: Intestinal metaplasia (IM) of the stomach is a preneoplastic lesion that appears following *Helicobacter pylori* infection and that confers increased risk for gastric cancer development. IM is induced by *de novo* expression of the intestinal-specific transcription factor CDX2 in the gastric mucosa. However, the regulatory mechanisms and molecular pathways involved in the triggering and maintenance of CDX2 expression in the stomach are yet to be fully unravelled.
Methods: A cell culture and transfection approach was used, together with patient's tissue samples histopathological and immunohistochemical analysis.
Results: Following the demonstration of the importance of the BMP pathway in normal intestinal differentiation, we were able to show that key elements of this pathway not only co-localized with CDX2 in IM but also positively regulated CDX2 in an in vitro context. Further, we showed that in Juvenile Polyps, an intestinal differentiation phenotype somehow inverse to gastric IM, loss of BMP pathway activity related with decreased CDX2 expression and loss of intestinal differentiation, thus reinforcing our previous results. Since CDX2 appears ectopically expressed in the stomach following *H. pylori* infection, we tackle the hypothesis of a direct regulation of CDX2 expression by bacterial interaction with epithelial cells in an *in vitro* co-culture model. Lastly, a "regulatory haplo-insufficiency" as well as an autoregulatory mechanism for CDX2 had been suggested. These results, taken together with the apparent stability of the metaplastic phenotype, led us to hypothesize that CDX2 is regulating its own expression through an autoregulatory loop. We were indeed able to demonstrate that not only CDX2 binds to and transactivates its own promoter but also it positively regulates its own expression in gastrointestinal human carcinoma cell lines.
Conclusion: Altogether, our results put forward some of the regulatory mechanisms involved in CDX2 regulation in the gastric context, thus contributing to unveil molecular pathways implicated in the establishment/maintenance of the Intestinal Mataplasia of the stomach.

718 Selective activation of p53 target genes by depletion of various RNA polymerase I transcriptional factors

K. Nishimura[1], A. Murayama[1], T. Kuroda[1], K. Kimura[1], J. Yanagisawa[1]. [1]*University of Tsukuba, Graduate School of Life and Environmental Sciences, Ibaraki, Japan*

Background: The nucleolus is a key organelle that regulates the synthesis of ribosomal subunits. Recent report suggested that the function of the nucleolus is tightly linked to cell growth and apoptosis. A variety of cellular stresses induces nucleolar stress by disrupting nucleolar structure. We and other groups showed that abrogation of RNA polymerase I-dependent transcription in nucleoli induced p53 accumulation and its acetylation. The acetylated p53 then binds to the promoter region of p53 target genes and regulates their transcription.
The functional consequences of p53 acetylation suggested that the timing of acetylation of the different p53 regions may be important for accurate p53 regulation and cell fate determination. The activation of genes involved in cell cycle control requires partial acetylation, whereas the activation of proapoptotic genes requires full acetylation of p53.
Material and Methods: We screened for RNA polymerase I-transcription regulatory factors whose knockdown induce p53 activation in MCF-7 cells using siRNA library.
We generated siRNA against transcription initiation factor-IA (TIF-IA), UBF, TAF_I48, and CD3EAP.
The expression and acetylation levels of p53 were analyzed by immunoblot in TIF-1A, UBF, TAF_I48, or CD3EAP siRNA treated cells. Furthermore, we examined the induction levels of p53 target gene products by immunoblot and RT-qPCR.
Result and Conclusion: Because the RNA polymerase I-dependent transcription levels affects on p53 acetylation, we examined the relationship between the deficiency of RNA polymerase I-dependent transcription and p53 target gene selectivity. To investigate this, we knocked down several factors which are known to be involved in RNA polymerase I-dependent transcription: i.e. TIF-1A, UBF, TAF_I48, and CD3EAP. Interestingly, although depletion of these factors induced the defect of RNA polymerase I-dependent transcription, p53 accumulation, and its acetylation, there were differences in p53 target genes that were activated. The depletion of CD3EAP enhanced the expression of proapoptotic genes such as PUMA and NOXA. On the other hand, the depletion of TIF-IA increased the expression of p21, HDM2, and proapoptotic genes. Selective activation of p53 target genes caused by various nucleolar stresses may be due to the different way of inhibition of RNA polymerase I dependent transcription.

719 Conjugation of endogenous BRCA1 protein with SUMO-2/3 is cell cycle-dependent

P. Rio[1], A. Vialter[2], A. Vincent[3], N. Dalla Venezia[3]. [1]*INRA, UNH, Saint-Genès Champanelle, France*, [2]*INSERM, U484, Clermont-Ferrand, France,* [3]*Centre Léon Bérard, UMR 5201 CNRS/UBCL1, Clermont-Ferrand, France*

Background: BRCA1, the main breast and ovarian cancer susceptibility gene, has a key role in maintenance of genome stability, cell cycle and transcription regulation. Interestingly, some of the numerous proteins which interact with BRCA1 protein undergo conjugation with small ubiquitin-like modifiers (SUMO). This post-translational modification is related to transcription, DNA repair, nuclear transport, signal transduction, and to cell cycle stress response. These features of sumoylation mechanisms and of BRCA1 function lead us to test the hypothesis of BRCA1 sumoylation by SUMO-2/3 (mainly involved in cellular stress response).
Material and Methods: Protein sequence was analysed by the SUMOplot™ software. Nuclear extracts of MRC5 human embryo lung cell line, MCF7 breast cancer cells, DU145 prostate cancer cells were immunoprecipitated by anti BRCA1 antibodies and analysed by Western blot with anti SUMO2/3 antibodies. To reduce non specific interactions, the immune complexes had been washed in PBS plus 0.2M NaCl, and in PBS plus 0.1 M NaCl.
Results: Protein sequence analysis suggests that sumoylation target sites belong to the RING finger and BRCT domains (BRCA1 C-terminus), two crucial regions for BRCA1 function. Moreover SUMO interacting motifs are present in the sequence of almost all proteins of BRCA1 network. Immunoprecipitations and Western blotting show the conjugation of endogenous nuclear BRCA1 protein with SUMO-2/3. In DU145 cells, BRCA1 conjugation with SUMO-2/3 is linked to the cell cycle and seems to be related to the oxidative stress. No cell cycle dependence of sumoylation is observed in MCF7 cells.
Conclusions: Our preliminary data and a number of arguments are in favour of a conjugation between SUMO-2/3 and the RING finger and/or the C terminal BRCT repeat of BRCA1 protein. Depending on the cell line, this conjugation appears to be modulated by the cell cycle and seems to be related to the oxidative stress, although the mechanisms remain to be determined. BRCA1 sumoylation may have a general role in the building, stability and/or activity of macromolecular complexes, especially in the nucleus. BRCA1 conjugation with SUMO-2/3, its interaction with SUMO1 and SUMO modification of many

BRCA1-interacting proteins, and the possible existence of SUMO interacting sites both in BRCA1 and partners point to a intricate network encompassing several pathways related to stress response, disruption of which may contribute to carcinogenesis.

720 Simultaneous HER2/neu and PTEN deregulation correlates with aggressive phenotype in hepatocellular carcinoma: a tissue microarray analysis

E. Tsiambas[1], A. Karameris[1], G. Alexandrakis[2], L. Manaios[3], G. Vilaras[4], E. Patsouris[5], S.P. Dourakis[6]. *[1]417 Va Hospital, Pathology, Athens, Greece, [2]417 Va Hospital, Gastroenterology, Athens, Greece, [3]Bioclinic, Surgery, Athens, Greece, [4]417 NIMTS, Pathology, Athens, Greece, [5]Medical School University of Athens, Pathology, Athens, Greece, [6]Medical School University of Athens, Internal Medicine, Athens, Greece*

Background: Hepatocellular carcinoma (HCC) is a highly aggressive and chemo resistant type of cancer. Although novel anti-HER2/neu targeted therapeutic strategies have been developed and applied in some types of malignancies, specific mechanisms of deregulation in HER2/neu (17q21) depended – signaling transduction pathway remain under investigation in HCC. Our aim was to investigate the potential role of simultaneous HER2/neu and PTEN (10q21-suppressor gene) dysregulation in HCCs.
Materials and Methods: Using tissue microarray technology, fifty-two (n = 52) formalin fixed and paraffin embedded tissue samples of histologically confirmed primary HCCs were cored and re embedded in the final paraffin block (core diam 1.5 mm). Immunohistochemistry (IHC) was performed by applying anti-HER2/neu and anti-PTEN antibodies. Fluorescence in situ hybridization (FISH) analysis was also performed regarding those genes.
Results: Protein over expression was observed in 12/52 (23%) cases regarding HER2/neu, whereas PTEN decreased or loss of expression in 22/52 (43%) cases. HER2/neu gene amplification was confirmed in 7/52 (13%) cases, whereas no one of the examined cases demonstrated PTEN gene numerical imbalances. Combined HER2/neu and PTEN aberrant expression was observed in 9/52 cases associated to the grade of the examined tumours ($p = 0.01$).
Conclusions: HER2/neu up-regulation combined to PTEN down-regulation is a relatively frequent and critical genetic event in HCC correlated also with an aggressive phenotype. PTEN decreased expression maybe is a negative prognostic factor for applying anti-HER2/neu targeted monoclonal antibody therapy (high chemo-resistance levels) in patients with HCC, based on breast adenocarcinoma management experience.

721 Aromatase inhibitor resistance; a role for estrogen receptor and AIB1 in differential gene regulation

J. O'Hara[1], D. Vareslija[1], A.D.K. Hill[1], L.S. Young[1]. *[1]Royal College of Surgeons in Ireland, Surgery, Dublin 2, Ireland*

Background: Aromatase inhibitors (AIs) are fast becoming the first line treatment for post menopausal breast cancer patients. However, it is evident that AIs do not remove all estrogen and molecular studies suggest that this can result in adaptive estrogen hypersensitivity of the estrogen receptor-alpha (ERα) with consequent resistance to therapy. We hypothesised that, in the AI-resistant setting, ERα may have the capacity to recruit its coactivator protein AIB1 to drive transcription of ER sensitive genes and induce tumour proliferation.
Materials and Methods: MCF7 breast cancer cells were stably transfected with the aromatase enzyme to generate an AI-sensitive cell line (MCF7aro). To acquire an AI-resistant cell model, MCF7aro was treated long-term with the AI, letrozole, until it lost sensitivity to letrozole (MCF7aroR-Let).
Cellular proliferation was measured by crystal violet staining; ER target gene expression levels were analysed by PCR, real-time PCR and Western blotting; chromatin immunoprecipitation was used to determine recruitment of ER and AIB1 to the promoters of target genes; the expression of, and interactions between, ER and AIB1, were analyzed by co-immunoprecipitation and quantitative coassociation immunofluorescent microscopy, using cell lines.
Results: In the AI-sensitive cell line, increased proliferation and expression of ER target genes pS2, c-myc and cyclinD1 was observed in response to aromatase substrate androstenedione; this effect was inhibited by letrozole. In the AI-resistant cell line, letrozole failed to inhibit proliferation induced by androstenedione, nor expression of pS2 and c-myc. However, cyclinD1 expression remained sensitive to letrozole treatment.
Chromatin immunoprecipitation studies in these cells demonstrated that treatment with letrozole induced recruitment of both ERα and AIB1 to the promoter of ER target genes pS2 and c-myc in the AI-resistant cell line but not to the promoter of cyclinD1.
Co-immunoprecipitation and co-localisation of ER-α and AIB1 were increased in the AI-resistant cell line following treatment with letrozole in comparison with AI-sensitive MCF7aro.
Conclusions: These data suggest that in the AI-resistant setting ERα can utilise AIB1 to drive tumour progression in the presence of an AI, and that this occurs in a target gene-specific context. An alternative signalling network may be involved in regulating cyclinD1 gene expression and allowing AI-resistant cells to retain some sensitivity to AI treatment.

722 Characterisation of gene expression profiles in HeLa cells expressing BRCA1 missense variants

M. Caligo[1], V. Mariotti[2], E. Melissari[2], L. Guidugli[1], C. Iofrida[2], C. Rugani[1], G. Lombardi[1], S. Pellegrini[2]. *[1]Azienda Ospedaliero-Universitaria Pisana, Department of Oncology, Pisa, Italy, [2]Azienda Ospedaliero-Universitaria Pisana, Department of Experimental Pathology, Pisa, Italy*

Background: Most BRCA1 mutations originate non functional truncated proteins that predispose women to early-onset breast and ovarian cancer. A number of missense mutations whose role in the disease is often difficult to ascertain, however, have also been detected in hereditary breast cancer patients. To investigate the molecular mechanisms that may underlie a pathogenetic role for two missense variants located within the BRCT domain of BRCA1, the M1775R and the A1789T, we compared the expression profiles of HeLa cells transfected with these two BRCA1 variants and HeLa cells transfected with BRCA1 wild type. The M1775R variant has widely been described as deleterious by functional assays, but to date a characterization of its effects on gene expression in human cells has never been reported. The A1789T variant has never been studied before by other groups.
Materials and Methods: The gene expression profiles of five clones of HeLa cells transfected with plasmids expressing each of the two BRCA1 missense variants were compared by microarrays to those of five clones transfected with plasmids expressing BRCA1 wild-type. A reference design was adopted and the reference sample was obtained by pooling the mRNAs from the wild-type clones. Gene expression was investigated by two-colour microarray analysis, using the Whole Human Genome 4x44k Microarray G4112F (Agilent Technologies, Palo Alto, CA, USA).
Results: Compared to BRCA1wild-type, the M1775R variant showed 159 differentially expressed genes, 108 down-regulated and 51 up-regulated, while the A1789T variant showed 188 differentially expressed genes, 77 down-regulated and 111 up-regulated. Out of these genes 15 were differentially expressed with the same fold-change direction by both the mutations.
Pathway analyses mapped 33 out of the 159 and 33 out of the 188 differentially expressed genes in 54 and 55 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways respectively. For both variants many of the pathways with the highest values of impact factor were involved in cancer, including pathways implicated in cancer general network and those describing the co-regulation mechanisms which underlie different type of cancers.
Conclusion: Our findings indicate that the M1775R and the A1789T variants of BRCA1 gene affect the expression of many genes associated with known mechanisms of cancerogenesis and thus contribute to sustain the hypothesis that these two mutations have a role in the pathogenesis of familiar breast cancer.

723 Expression of HDAC1, 2, 3 and 7 as a prognostic markers in hepatocellular carcinoma

K. Quint[1], A. Agaimy[2], C. Hellerbrand[3], D. Neureiter[4], M. Ocker[1]. *[1]Philipps University of Marburg, Institute for Surgical Research, Marburg, Germany, [2]University Hospital Erlangen, Institute of Pathology, Erlangen, Germany, [3]University Hospital Regensburg, Department of Medicine 1, Regensburg, Germany, [4]Paracelsus Private Medical University, Institute of Pathology, Salzburg, Austria*

Background: Histone deacetylases (HDAC) are enzymes that are responsible for the transcriptional control of genes through modifications of histone proteins. Among others, they play a factor in the control of tumour suppressor genes. Hypoacetylated histone proteins have been associated with precancerous and malignant lesions and for some tumour entities, such as prostate cancer and colon cancer HDAC expression has been identified as an independent prognostic factor. Since inhibitors of histone deacetylases (HDACi) emerge as promising therapeutics in the management of solid tumours, including hepatocellular carcinoma, we analyze the importance of the expression of 4 HDAC isoenzymes as a prognostic marker in hepatocellular carcinoma.
Method: Tissue micro arrays of primary HCCs and adjacent normal tissue of 170 patients (male n = 145, 85.3%; female n = 25, 14.7%; mean age 61.9 ± 11.0 years) were evaluated immunohistochemically for the expression of HDAC isoenzymes 1, 2, 3, 7 and ki-67 antigen. Intensity and extensity of expression were evaluated by two independent blinded observers, a product score calculated (IRS, immunoreactivity score) and the data was correlated with histopathological and clinical criteria. Based on mean HDAC expression for each isoenzyme, patients were stratified into high and low expression groups and the groups compared in terms of clinical data.