

Poster sessions

A.01

DNA PLOIDY AND PROLIFERATIVE ACTIVITY IN PROSTATIC ADENOCARCINOMA

A.M. Amira Raafat¹, A.E. Ali El Hendawi²,
M. Abeerl, H. Ahmed El²

¹National Cancer Institute, Cairo University, Cairo, Egypt, ²Faculty of Medicine, Cairo University, Cairo, Egypt

The biologic behaviour of prostatic adenocarcinoma is influenced by many factors. The proliferative activity of the tumour can be one of these factors, which serves as the basis to estimate prognosis and design treatment. In the present study, DNA content and S phase fraction (SPF) of prostatic adenocarcinoma specimens obtained from 25 patients were related to other tumour characteristics (grade and stage). Nuclei from paraffin embedded material were isolated, DNA content and SPF were determined using the flow cytometer. Fifty two percent of cases were diploid and 48% were aneuploid. Fifty percent of diploid cases showed low SPF, 25% showed moderate SPF and 25% showed high SPF. On the other hand, only 18.2% of aneuploid cases showed low SPF, 18.2% showed moderate SPF and 63.6% showed high SPF. There was a directly proportionate relationship between flow cytometric parameters and histopathologic grade, as aneuploid patterns and high SPF were found in high grade more than low grade tumours. No obvious correlation was found between flowcytometric parameters and tumour stage.

A.02

APOCRINE CHANGE IN FNAB: NUCLEAR MORPHOMETRY AND DNA IMAGE CYTOMETRY

A.I. Elzagheid¹, Y. Collan², T. Kuopio³

¹University of Turku, Turku, Finland, ²Pathology Department, Turku, Finland, ³Jyvaskyla Central Hospital, Jyvaskyla, Finland

The aim of this study to investigate the potential of nuclear morphometry and DNA cytometry in supporting the decision on apocrine change in fine needle aspiration biopsy (FNAB). Nuclear size was estimated with computerized morphometry and DNA con-

tent with DNA cytometry. The effect of methods of preparation on the results were also studied. On average, the smears had larger nuclei than the ethanol fixed samples. Mean nuclear areas in smears ranged between 59.0 μm^2 and 151.0 μm^2 , in ethanol fixed samples between 32.3 μm^2 and 63.4 μm^2 . The DNA histograms were not affected by methods of preparation. Apocrine change typically showed a diploid peak, but a limited number of proliferating cells. One case was tetraploid suggesting atypical apocrine change. After histological investigation this sample was diagnosed as infiltrating carcinoma. The patient had had X-ray irradiation earlier for a mediastinal lymphoma.

DNA cytometry and nuclear morphometry findings in apocrine metaplasia were described in a systematic study for the first time. The data suggested that with these methods premalignant or malignant apocrine lesion can be distinguished from the more conventional typical apocrine metaplasia.

A.03

ANALYSIS OF ANNEXIN II PROMOTOR HYPERMETHYLATION IN PROSTATE CARCINOMA

C. Markert-Hahn¹, D. Geiger-Schönach¹, G. Kagerer¹,
H. Munz¹, C. Ott¹, P. Ploog¹, D. Rehn², J. Kleiber¹
¹Roche Diagnostics GmbH Penzberg, Penzberg, Germany, ²F. Hoffmann-La Roche AG, Basel, Switzerland

There are many genes known from literature that are regulated by promotor methylation; in clinical oncology one of the best described is the GSTP1 gene; there are some more candidate genes however, one being Annexin II, whose downregulation in prostate carcinoma (PCa) on protein and RNA level was described recently.

The goal of our study was to analyse if promotor hypermethylation could be responsible for this downregulation.

First we analysed the methylation status of Annexin II promotor sequences in clinical samples by methylation independent PCR followed by direct bisulfite sequencing.

We examined fresh frozen microdissected tissues from 16 benign prostate hyperplasia (BPHs) and 17 PCas of different stages. As a control we used LNCap cells. The result was that all CpG sites analysed

showed a 100% methylation in LNCap cells but only in 1/17 PCAs there was a partial methylation of 4 CpG sites; 0/16 BPH samples showed methylation.

For confirmation we analysed the same tissues as used for bisulfite sequencing by different methylation specific PCRs (MSP). 16/16 BPHs and 16/19 PCAs did not show any growth curve or crossing point. 3/19 PCAs showed at least one positive growth curve in the different MSPs.

Our results based on direct bisulfite sequencing as well as MSP analysis show that there is no significant promoter hypermethylation present in Annexin II gene that would allow a discrimination of the clinical status of tumor and benign prostatic tissues. The hypothesis of Chetcuti et al. that downregulation of Annexin II expression in PCa patients is due to its promoter hypermethylation could not be confirmed. There must be some other mechanism that counts for their results. Annexin II does not seem to be a promising candidate marker for a methylation based screening assay for prostate carcinoma.

A.04

DOWN-REGULATION OF ID4 BY PROMOTER HYPERMETHYLATION IN GASTRIC ADENOCARCINOMA

A. Chan¹, W.Y. Tsui¹, X. Chen², K.M. Chu¹, T.L. Chan¹, A. Chan¹, R. Li², S. So², S.T. Yuen¹, S.Y. Leung¹

¹The University of Hong Kong, Hong Kong, Hong Kong, ²Stanford University, Stanford, USA

Promoter hypermethylation has become apparent as a common mechanism of gene silencing in cancer. Based on our published microarray expression data, we noticed a prominent down-regulation of ID4 in gastric adenocarcinoma. The dense 5' CpG island covering the previously mapped upstream promoter of ID4 has prompted us to relate its down-regulation to promoter hypermethylation. ID proteins are distinct members in the helix-loop-helix family of transcriptional regulators, which modulate various key developmental processes. Emerging data have suggested the involvement of ID genes in tumorigenesis. In this study using bisulphite genomic sequencing, we have found hypermethylation of ID4 promoter in most gastric cancer cell lines and 30% of primary tumors. This correlated with decreased level of ID4 expression. Restoration of ID4 expression in various gastric cancer cell lines was achieved by treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine, which at

times required the synergistic action of the histone deacetylase inhibitor trichostatin A, but not with trichostatin A alone. Re-expression was accompanied by the corresponding ID4 promoter demethylation. Furthermore, we have found significant association of ID4 promoter methylation with hMLH1 promoter methylation ($p = 0.008$) and microsatellite instability ($p = 0.006$). Overall, our results have shown that transcriptional silencing of ID4 is related to the aberrant methylation of its promoter in gastric cancer. The significant association of ID4 and hMLH1 promoter hypermethylation suggested that ID4 may also be among the genes being targeted in the CpG island methylator phenotype tumorigenic pathway.

A.07

DNA AMPLIFICATION OF THE 11Q13 REGION INVOLVES EMSY, MYEOV, CYCLIN D1 AND CORTACTIN

E. Schuurin¹, A. van Rossum², J. Gibcus¹, L. Menkema¹, W. Moolenaar³, C. Theillet⁴, J. Janssen⁵, L. Hughes-Davies⁶, P.M. Kluijn¹

¹University Medical Center Groningen, Groningen, The Netherlands, ²LUMC, Leiden, The Netherlands, ³Cell Biology, NKI, Amsterdam, Amsterdam, The Netherlands, ⁴CRLC, Montpellier, France, ⁵Genetic Ruprecht-Karls Universität, Heidelberg, Germany, ⁶CRC/Wellcome Institute, Cambridge, UK

Amplification of the 11q13 region is found in various carcinomas such as those of the breast (13–20%), bladder (15%), ovary (20%) and head/neck region (36%). At present, in the amplified 11q13 region at least 4 genes have been found that are overexpressed: (1) cyclin D1, involved in cell-cycle regulation; (2) cortactin, an actin-binding protein involved in regulating actin-polymerization, cell adhesion and migration; (3) myeov, identified in the NIH/3T3-tumorigenicity assay; and (4) EMSY, identified by its binding to the breast cancer-susceptibility gene BRCA-2. In a series of 946 breast carcinomas, we found that cases with 11q13 amplification show amplification of EMSY only in 23% of the cases, cyclin D1/cortactin co-amplification only in 35%, myeov amplification only 13% and co-amplification of all 4 genes in 29%. In head/neck carcinomas amplification of myeov, cyclin D1 and cortactin is observed, but no amplification of EMSY. In contrast, in ovarian cancer only EMSY is amplified. The difference in the amplification patterns between the three malignancies implies that the 11q13 amplicon in fact represents independent amplicons.

This finding is not novel except that we have identified a candidate overexpressed gene in each amplicon. To illustrate possible involvement of these genes in these malignancies with 11q13 amplification, we have started to investigate the effect of overexpression of these genes on biological properties such as radiore-sistance, cell migration and tumorigenesis. We will discuss our recent achievements regarding the characterization of EMSY and cortactin in detail and speculate on their possible role in carcinomas with 11q13 amplification. Our findings show that cyclin D1 is not the only involved gene in the 11q13 amplicon.

**A.08
CHROMOSOMAL INSTABILITY IN HUMAN
SPORADIC COLORECTAL CANCER. IS
THERE A LINK WITH ADENOMATOUS
POLYPOSIS COLI GENE MUTATIONS?**

C. Prevosto¹, T. Venesio², F. Lombardo², A. Sciutto¹,
E. Geido¹, M. Risio², W. Giaretti¹

¹National Institute for Cancer Research, Genova, Italy,

²Institute Cancer Research-Treatment, Candiolo (TO),
Italy

Background & aims: Aneuploidy and adenomatous polyposis coli (APC) gene mutations are present in the majority of human sporadic colorectal cancers and in their precursor adenomas. Recently, *in vitro* experiments with mouse embryonic stem cells suggested that APC truncated proteins, loosing their kinetochore and microtubule binding sites, may be at the origin of chromosomal instability and aneuploidy. With the aim to verify this hypothesis, we evaluated aneuploidy and the APC mutation spectrum among 61 human sporadic colorectal adenomas.

Methods: The degree of aneuploidy was evaluated by the flow cytometric DNA Index and APC mutation spectrum was detected by the protein truncation test followed by DNA sequencing using epithelial nuclei sorted by flow cytometry. Additionally, microsatellite instability and allelic loss at APC loci were investigated.

Results: Frequencies microsatellite instability, DNA aneuploidy (DNA Index lower/higher than 1), and APC mutations were respectively 7%, 33% and 31%. DNA aneuploidy, mainly in the near-diploid region, was not associated with APC mutations ($P = 0.25$). In particular, 6 of the 7 APC mutations which occurred upstream the mutation cluster region, roughly defined by codons 1200–1500, were associated with DNA diploidy (DNA Index = 1). On the contrary, 8 of

the 12 APC mutations within and downstream the mutation cluster region, which conserved a minimum of 3 beta-catenin binding sites and additional beta-catenin degradation and axin sites, were associated to DNA aneuploidy.

Conclusions: We suggest that specific APC mutations associated with beta-catenin functions cooperate toward chromosomal instability and aneuploidy in human sporadic colorectal adenomas.

**A.09
NEAR-DIPLOID AND NEAR-TRIPLOID
HUMAN SPORADIC COLORECTAL
ADENOCARCINOMAS DIFFER FOR KRAS2
AND TP53 MUTATIONAL STATUS**

C. Prevosto¹, A. Sciutto¹, E. Geido¹, T. Venesio²,
M. Risio², W. Giaretti¹

¹National Institute for Cancer Research, Genova, Italy,

²Institute Cancer Research-Treatment, Candiolo (TO),
Italy

Mutations of the KRAS2 protooncogene and inactivation of the TP53 oncosuppressor gene were suggested to contribute to chromosomal instability (CIN) and aneuploidy in colorectal cancer (CRC). Previous work has also shown that the degree of DNA aneuploidy as obtained by flow cytometry (DI) in CRC is non-randomly distributed and, in particular, that DI near-diploid and near-triploid values are well separated by a low probability valley region. At present, it is not known if a relationship exists between DI and the mutational status of KRAS2 and TP53. Multiple samples obtained from 35 human sporadic CRCs have been used to provide nuclei suspensions for flow cytometric analysis and sorting of specific DI subpopulations. Sorted nuclei were then used to analyse the high microsatellite instability (MSI-H) phenotype and the mutation spectrum of KRAS2 and TP53 genes. A single MSI-H case was detected. There were 6 DNA diploid (DI = 1) and 29 aneuploid (DI different from 1) CRCs, with the DI aneuploid cases non-randomly subdivided in 9 near-diploid (DI different from 1 and $DI \leq 1.4$), 8 near-triploid ($1.4 < DI < 1.6$), and 12 high-aneuploid ($DI \geq 1.6$) cases. Proximal CRCs were more often DNA diploid and near-diploid than distal ones, and Dukes' C cases were more commonly high-aneuploid than Dukes' B. Moreover, the incidence of mutations of the KRAS2 and TP53 genes was lowest among the DNA near-triploid subpopulations and highest among the near-diploid ones. We suggest that DNA near-diploid and near-triploid subpopulations in human sporadic CRC reflect different genetic mechanisms of CIN and have potentially different clinical behaviour.

A.10 METHYLATION OF HISTONE H3 LYS 4 AND GENE ACTIVITY IN HUMAN LEUKEMIC CELLS UNDERGOING DIFFERENTIATION

E. Bartova, S. Kozubek

Institute of Biophysics AS CR, Brno, Czech Republic

Post-translational modifications of histones are considered as functionally important events, that have an ability to influence package of eukaryotic DNA into repeating nucleosomal structures folded into higher-order chromatin fibres (summary Jenuwein, *Science* (2001), 1074–1080). Processes such as histone methylation, hypoacetylation and/or phosphorylation are involved in epigenetic mechanisms regulating not only general chromatin structure but also gene activity. Recently, it has been found that methylation of H3 lysine 9 (K9) occurs in facultative heterochromatin of inactive X chromosome of differentiating murine cells (Mermoud et al., *Current Biology* (2002), 247–251). This type of epigenetic modification is also associated with constitutive pericentric heterochromatin (Maison et al., *Nature Genetics* (2002), 329–334). On the other hand, dimethylation of H3 lysine 4 (K4) in coding regions and histone acetylation in promoters facilitate transcription (Bernstein et al., *PNAS* (2002), 8695–8700).

In our experiments we studied nuclear location of dimethyl H3 (K4) regions during two differentiation pathways induced in human leukemic cells. We observed differences in mH3 (K4) interphase arrangement during selected processes of cell maturations. Apoptosis accompanied monocytic differentiation of human leukemic HL-60 cells was characterised by certain compartmentalization of mH3 (K4) regions. Our results indicate that interphase nuclear localization as well as number and volume of nucleoli could influence general higher order chromatin structure including distribution of dimethyl H3 (K4) regions. Mentioned histone modification frequently co-localized with nuclear positioning of active copies of chimeric Bcr/Abl genes forming Ph chromosome in human leukaemia cell line K-562. Studied active loci were positioned in close proximity to the interchromatin DAPI negative spaces. On the other hand, centromeric regions of chromosome 9 were located out of mH3 (K4) areas studied in the identical interphase nuclei. During megakaryocytic differentiation of K-562 cells we determined down regulation of chimeric Bcr/Abl genes and co-localization of studied loci with mH3 (K4) regions was not observed. Our data support the idea that methylation of

histones is an important posttranslational modification taking parts in regulation of transcription during cell differentiation.

A.11 CENTROSOME MORPHOLOGY IN PROSTATE CANCER – CORRELATION WITH CLINICO-PATHOLOGICAL FEATURES AND DNA-PLOIDY

M.I. Toma, K. Friedrich, W. Meyer, G. Baretton

Institute of Pathology TU Dresden, Dresden, Germany

Introduction: Prostate cancer is the second leading cause of cancer death in men. A major prerequisite for tumor initiation and progression is the acquisition of chromosomal aberrations. Centrosomes play an important role in the normal segregation of chromosomes as the major microtubule organizing centre. Thus, centrosome abnormalities may contribute to the acquisition of chromosomal aberrations leading to changes in cell morphology and function. The aim of this study was to analyse changes in centrosome number and morphology in association to clinico-pathological features (especially Gleason score) and DNA ploidy.

Material and methods: Paraffin sections of 60 prostate cancers (20 Gleason score 5–6, 20 Gleason score 7, 20 Gleason score 8–9) were studied for centrosome number and morphology by immunohistochemistry with a monoclonal antibody against gamma tubulin (clone GTU-88; Sigma). The brightfield microscopy and image analysis were performed by Spectracube SD-200H (Applied Spectral Imaging) and the software package SpectraView. Studied features were number, area and shape of the centrosomes. DNA image cytometry was performed on Feulgen stained disintegrated 50 μm paraffin sections by an OPTIMAS based image cytometry workstation. For the statistical analyses, the *T*-test according to Student (significance level $p < 0.05$) was used.

Results: There was no significant difference in the number of centrosomes between euploid (peridiploid and peritetraploid) and aneuploid cases. There was also no difference in centrosome number between the groups with well differentiated (Gleason score 5–6) and poorly differentiated (Gleason score 8–9) tumors.

The aneuploid tumors with Gleason score 8–9 showed significantly larger centrosomes than tumors with Gleason score 5–6 or euploidy, respectively. Centrosome shape differed significantly between the high and low Gleason score group as well as between aneuploid and euploid cases.

Conclusion: Changes in centrosome shape and size indicate a disturbed centrosome function and are significantly correlated with aneuploidy and loss of differentiation in prostate cancer. The results of this study confirm the role of centrosome defects in the development of chromosomal aberrations in prostate cancer.

A.12 COMPARISON OF GENOMIC INSTABILITY IN BLADDER CANCER TISSUES AND THEIR CORRESPONDING URINARY SEDIMENTS

B. Schlichtholz, J. Turyn, M. Matuszewski
Medical University of Gdansk, Gdansk, Poland

Genomic instability is a characteristic molecular feature of bladder cancer. In this study, we investigated the microsatellite alterations: microsatellite instability (MSI) and loss of heterozygosity (LOH) at chromosomes 2p, 3p, 8q, 9p, 9q, 12q, 13q, 18q and 17p in 12 patients with the stage T1 tumor (lamina propria invasive) and in 28 patients with the stage T2–4 tumor (muscle invasive). The microsatellite alterations in tumor tissue and exfoliated cells from the urine were detected using twelve microsatellite markers. PCR products were separated on denaturing gels for allele sizing. The overall frequency of LOH or MSI was 67.5% (27/40) and 55.6% (15/27) patients had instability in more than 2 microsatellite loci. LOH or MSI was detected in 75% (9/12) T1 tumors and in 64.3% (18/28) T2–4 tumors. Interestingly two microsatellites D9S242 and D9S252 were unstable in 74.1% (20/27) of all LOH/MSI positive patients. Twenty eight of fifty three (52.8%) alterations were detected in both urinary sediment and corresponding tumor tissue. Moreover, microsatellite alteration not matching those in tumor were detected at one locus in T1 tumor and at seven loci in five T2–T4 tumors. There was no relationship between the microsatellite alterations and overall survival. Univariate analysis by the Cox proportional hazards model identified only stage ($P = 0.01$) as independent predictor of decreased survival for stage T2–T4 tumor. The results of present study indicate that two markers D9S242 and D9S252 are especially useful in analysis of microsatellite alterations in bladder cancer patients. Moreover, the low frequency of microsatellite instability in urine sediments suggests that this type of analysis should be considered carefully in noninvasive diagnosis of the bladder cancer.

A.13 IMPLEMENTATION OF IMAGE DNA CYTOMETRY IN FNAB DIAGNOSTICS OF BREAST DISEASE

Y.U.I. Collan¹, A. Elzagheid²
¹*University of Turku, Turku, Finland,* ²*Dept. of Pathology, Turku, Finland*

68 breast lesions were studied with a diagnostic protocol including fine needle aspiration biopsy cytology, and DNA measurements from restrained samples. Nuclei of cell groups and free cells were studied separately, which improves sensitivity (Elzagheid et al., 2002, 2003).

Follow up of patients showed two carcinomas among 17 samples classified as definitely benign, and one carcinoma among 11 samples classified as atypical but benign. Corresponding samples showed diploid/peridiploid histograms, without improving diagnostic accuracy for carcinoma. Among slightly suspect samples ($n = 17$) 8 carcinomas were found. DNA histograms showed aneuploid or tetraploid peaks, and/or $>5c$ cells in 5 cases, offering help in 62.5% of cases. Of 12 highly suspect cases histological cancer diagnosis was available for 10 cases, of which 9 were supported by DNA cytometry. One case showed a diploid/peridiploid histogram. All definitely malignant cases showed histograms suggestive of malignancy. The results suggested that the methodology could improve the sensitivity cancer diagnosis, especially among slightly or clearly suspect cases, but had little value among samples categorized as benign.

However, the interpretation of histograms was problematic. Among definitely benign samples there was one case with DNA histograms suggesting carcinoma. Histology showed fibrocystic disease. Among atypical but benign samples 5 samples showed an atypical histogram. One presented breast hamartoma, one fibrocystic disease, and 3 did not show evidence of carcinoma after five year follow up. These findings suggest that benign breast lesions like fibrocystic disease may harbour cells which can cause slight DNA histogram abnormalities. This is why the criteria of histogram interpretation should be defined carefully to avoid false positives.

The results confirm the hypothesis that DNA cytometry as a diagnostic procedure can be helpful if the interpretation criteria are carefully defined. The criteria should preserve the sensitivity of the methodology and avoid false positives. Definition of interpretation criteria will need a carefully planned prospective study, possibly on international basis.

A.14 CARDIAC CELL POLYPOIDY: EVALUATION AND SIGNIFICANCE

D. Cesselli¹, L. Pulpito¹, L. Castriota¹, N. Finato¹,
U. Livi², L. Mariuzzi¹, R. Bussani³, F. Silvestri³,
C.A. Beltrami¹, A.P. Beltrami¹

¹University of Udine, Udine, Italy, ²Dept. Cardio-
surgery, Udine, Italy, ³University of Trieste, Trieste,
Italy

Human cell polyploidy is a quite rare phenomenon both in physiological (megakaryocytes) and pathological conditions (hepatocytes). Cardiomyocyte ploidy has been extensively studied under normal and pathological settings using stoichiometrical methods (i.e.: Feulgen) or cytofluorimetric approaches.

We decided to address this problem with a novel approach, consisting of *in situ* hybridization of fluorescent labeled probes (FISH) able to recognize the sexual chromosomes, with the assumption that a variation in the number of those chromosomes could represent a modification of the total chromosome number. Moreover, with this approach, we have been able to correlate on the same cell: (1) chromosome number, (2) cell volume, (3) expression of senescence associated cell markers, (4) proliferation associated markers, (5) differentiation associated markers. We also correlated the chromosome number calculated with the FISH method with the DNA content measured by the fluorescence intensity of DAPI staining. Conclusion: this method allows us to separate two major classes of myocytes undistinguishable from a morphological point of view, but different with regard to chromosome number and cell volume.

Within the class of the smallest cells we can identify the youngest cells, still able to proliferate and divide.

Interestingly, we have found a gender difference in the proportion between diploid and polyploid cells, suggesting a higher growth reserve of the female heart.

A.15 METHYLATION OF THE HUMAN PAX3 UPSTREAM REGION IN RHABDOMYOSARCOMAS

R.T. Kurmasheval, C.A. Peteson¹, C.A. Cooney¹,
D.M. Parham²

¹University of Arkansas for Medical Sc., Little Rock,
USA, ²Arkansas Children's Hospital, Little Rock, USA

Rhabdomyosarcomas (RMS) exhibit abnormal methylation in key gene promoter regions. In order to

determine whether altered PAX3 promoter CpG island methylation is present in RMS, and whether differential methylation of the PAX3 CpG island occurs in alveolar and embryonal subtypes, we examined methylation status of the human PAX3 5'-CpG island and compared it to that of normal muscle.

Methylation was tested in 27 rhabdomyosarcomas (12 alveolar, 15 embryonal) and 6 normal muscle samples using semi-quantitative analysis of DNA methylation. DNA templates were amplified by PCR following digestion with methylation-sensitive restriction enzymes Bss H II, Eag I and Hae II compared to undigested. A 8-kb CpG island immediately upstream of the PAX3 coding sequence, surrounding the transcription start site and including the first untranslated exon, was analyzed. Our results showed that CpG island in the promoter region of the human PAX3 gene is abnormally methylated in rhabdomyosarcomas. Further study is currently underway to determine the distinguishing characteristics in DNA methylation of alveolar and embryonal rhabdomyosarcomas.

A.16 CENTROMERIC BREAKAGE IN SPORADIC AND FANCONI ANEMIA RELATED ORAL SQUAMOUS CELL CARCINOMA

R.I. Klein Geltink, D. Cremers, M.A.J.A. Hermsen,
G.A. Meijer

VUMC, Amsterdam, The Netherlands

Background: In oral squamous cell carcinoma (SCC), centromeres have been frequently observed to be involved in translocations. Although centromeric translocations appear to be random in the sense that all chromosomes seem to be involved, its high frequency indicates that it is an important aberration in the molecular pathogenesis of oral SCC.

Oral SCC also occurs in patients with Fanconi Anemia (FA), a hereditary genetic disorder characterized by hypersensitivity to DNA cross-linking agents like Mytomycin-C (MMC). Also in these tumors, centromeric translocations are observed frequently. FA genes could therefore play a role in the creation of centromeric breaks in oral SCC carcinogenesis. The aim of the present study was to study the contribution of centromeres to the spectrum of breaks induced by MMC, and to compare this to the spectrum of breaks induced by aphidicolin a compound known to induce fragile sites.

Material and methods: Six sporadic oral SCC cell lines and one FA related oral SCC cell line were in-

cluded in this study. After exposure to MMC or aphidicolin, cells were fixed and metaphase spreads were prepared. The metaphase chromosomes were stained with DAPI and analyzed using ASI Bandview software.

Results and discussion: Centromeric breaks, especially of chromosome 1 and 9 were frequently observed in all cell lines (sporadic and FA related) after exposure to MMC. Cells exposed to aphidicolin showed a comparable spectrum of breaks, although the centromeres were less frequently involved. The overall percentage of centromeric breaks was higher in the FA related oral SCC cell line upon exposure to MMC while aphidicolin did not yield any significant differences.

Conclusion: Centromeric breakage in oral SCC cell lines can both be induced by cross-linking agents and aphidicolin, suggesting that centromeric regions could be regarded as fragile sites in oral SCC. In addition, FA-related oral SCC showed a higher involvement of centromeric breaks, which may suggest a role for (defective) FA genes in the acquisition of centromeric translocations.

A.17 GENOMIC INSTABILITY – HOW TO DESCRIBE?

G. Haroske¹, K. Friedrich², W. Meyer²

¹Dresden-Friedrichstadt General Hospital, Dresden, Germany, ²Dresden University Hospital, Dresden, Germany

Genomic instability is a key issue in understanding the evolution of malignant tumours. Although often used the term is not defined precisely. It seems to depend on the field of interest whether a precondition of malignant transformation or its result or both is meant. Furthermore indicators for instability are used very arbitrarily. The much often cited DNA aneuploidy needs by far not to be identical with instability. Might a tumour cell population be really unstable, if it is in equilibrium demonstrated by the existence of a stemline?

The paper was intended to study the relation between genomic aberrations and departures from normal nuclear DNA distributions in breast cancers. From 106 primary tumors with a median follow-up of 31 months (1 to 93 months) genomic alterations were investigated by CGH. Tumour imprints or fine needle aspirates were used for DNA image cytometry. For most of the tumours also p53-, WAF-1, and Mib-1-

immunohistochemistry as well as some FISH results were available.

The data show that there are statistically significant correlations between DNA ploidy and the Average Number of Copy Alterations (ANCA), but a considerable part of DNA aneuploid tumours had very few, another part of DNA diploid tumours had many ANCA's as well. DNA tetraploid tumours were found to have equal proportions of many and few ANCA's. That means that either DNA aneuploidy or the ANCA or both are not appropriate descriptors for instability. A more detailed analysis of DNA histograms leads to histogram descriptors more closely linked to ANCA status than the ploidy type. However, at least breast cancers seem to be genomically stable at a large extent.

B.01 CHRONIC HELICOBACTER PYLORI INFECTION MAY RESULT IN GASTRIC CANCER IN HYPERGASTRINEMIC MICE BUT NOT IN GASTRIN-DEFICIENT MICE

D. Chen¹, C.-M. Zhao¹, X. Wang², L. Friis-Hansen³, H. Waldum¹, J. Halgunset¹, T. Wadstrom²

¹Norwegian University of Science and Technology, Trondheim, Norway, ²University of Lund, Lund, Sweden, ³Rigshospitalet, Copenhagen, Denmark

Clinically, it is well established that *Helicobacter pylori* infection leads to gastric cancer or duodenal ulcer, but the mechanism by which single strain of *Helicobacter pylori* may cause the two distinct clinical outcomes is still poorly understood. Recent study in transgenic mice that are hypergastrinemic revealed spontaneous gastric cancer over a period of 1–2 years and accelerated development of the cancer (within 7 months) by *Helicobacter pylori* infection. The present study in gastrin-deficient (gene knock-out) (gastrin^{-/-}) vs. wild-type (gastrin^{+/+}) mice was undertaken to identify a possible role of gastrin in determining the different responses of the parietal cells and ECL cells in the stomach to chronic *Helicobacter pylori* infection. Gastrin^{+/+} and gastrin^{-/-} mice were infected with *Helicobacter pylori* (CagA positive type I and vacuolating toxin-producing) for 9 months. The acid output was measured 4 h after pylorus ligation (known to cause vagal excitation). The gastric mucosa was examined by immunocytochemistry with antisera to H^+/K^+ -ATPase for the parietal cells, and to histamine and vesicle monoamine transporter-2 for the ECL cells, and by quantitative electron microscopy. In infected gastrin^{+/+} mice, the acid output

and the percentage of secreting parietal cells (freely fed state) were 20–30% of the values in uninfected controls, while the density and ultrastructure of parietal cells were normal. The infected mice had hypergastrinemia and displayed hypertrophy and hyperplasia of ECL cells. Although uninfected gastrin^{-/-} mice had lower the acid output than uninfected gastrin^{+/+} mice, there was a higher acid output (~3-times) in infected gastrin^{-/-} mice than their uninfected homologues. The numbers of parietal cells and ECL cells remained unchanged in infected gastrin^{-/-} mice. In conclusion, chronic *Helicobacter pylori* infection results to impaired parietal-cell function (acid hyposecretion), hypergastrinemia and hyperplasia of ECL cells in wild-type mice but leads to vagally induced hypersecretion in gastrin-deficient mice. We propose that gastric acidity and gastrin are host factors to determine the pathological phenotypes in the stomach.

B.02

MONITORING CELL CYCLE DISTRIBUTIONS AND APOPTOSIS IN LIVING CELLS BY VIDEOMICROFLUOROMETRY AND DISCRIMINANT FACTORIAL ANALYSIS

J. Savatier, J. Vigo, J.-M. Salmon
University of Perpignan, Perpignan, France

Single living cells are labelled with three fluorescent markers: Hoechst 33342 for nuclear DNA, Rhodamine 123 for mitochondria and Nile Red for plasma membrane. Numerical image analysis allows us to obtain, for each cell, morphological parameters (cell and nuclear sizes, nucleo-cytoplasmic ratio, shape factor) and functional information by fluorescence intensity (nuclear DNA content, level of mitochondria energetic state, amount and properties of plasma membrane) with total value, mean and standard error. These parameters are used in a typological analysis (dendrogram method) which separates control cells into four groups. A discriminant factorial analysis confirms these groups: G0-G1, S, G2+M and polyploid cells called Gn. Discriminant factorial analysis indicates which parameters are the most discriminant. It gives the mean value of each parameter for each group and the cell probability to belong to each phase. These control cells define a learning population. The populations of treated cells (with several concentrations and times of incubation with adriamycin) are analysed as supplementary individuals in a discriminant factorial analysis using control cells as learning population. They are classified into G0-G1, S, G2+M and Gn groups. Fur-

thermore such an approach allows to accurately evidence, after 5 days treatment, the changes of some cellular parameters values such as those induced during apoptosis, combining shape factor and size of the nucleus, and total and standard error of Hoechst 33342 fluorescence intensity (DNA amount and DNA clusterisation, respectively). Apoptotic cells make up a new group that does not exist in standard conditions and allows to follow the apoptotic process induced by adriamycin treatment.

B.03

DIFFERENTIATION PROMOTING AND ANTIMITOTIC ACTIVITIES OF SODIUM VALPROATE ON HUMAN NEUROBLASTOMA CELLS

A.M. Ferreri, P. Rocchi
Cancerologia, Bologna, Italy

Histone acetyltransferases and histone deacetylases (HDAC) determine the acetylation status of histones. This acetylation affects the regulation of gene expression, and inhibitors of HDAC have been found to inhibit the growth of a variety of tumor cells in culture. Valproic acid is a HDAC inhibitor which belongs to the group of simple branched-chain fatty acids. The present study was aimed at evaluating the effect of sodium valproate on differentiation and proliferation in the human neuroblastoma cell lines AF8 and TS12.

Neuronal differentiation was assessed by means of morphological and cytochemical parameters, i.e. neurite outgrowth and acetylcholinesterase specific activity. Growth curves and colony-forming assay were performed in order to determine cell growth inhibition.

Sodium valproate induced inhibition of cell growth (demonstrated by growth curves) and reduced the colony-forming ability in a dose dependent manner in both cell lines. Inhibition of cell proliferation was accompanied by morphological features of neuronal differentiation: on both cell lines valproate induced neurogenesis in a dose dependent fashion. Biochemical differentiation was demonstrated by the increase in the acetylcholinesterase specific activity, an enzyme widely used as biochemical marker for neuronal differentiation in neuroblastoma cells.

These results underline the role of HDACs inhibitors as new cancer drugs and suggest the evaluation of sodium valproate for cytodifferentiation therapy in the treatment of neuroblastoma.

B.04**PROFILING OF A TUMOR CELL PHENOTYPE MULTIRESISTANT TO APOPTOSIS**

A. Sokolovskaya¹, A. Mikhailov², O. Melin²,
D. Blokhin³, J. Eriksson²

¹Russian Cancer Research Center of RAMS, Moscow, Russian Federation, ²Center for Biotechnology, Turku, Finland, ³Russian Cancer Research Center, Moscow, Russian Federation

Fas (CD95/APO-1) signaling pathway perhaps plays a significant role in drug-induced apoptosis and the absence or diminished expression of Fas-receptor on the surface of tumor cells is associated with resistance to cytotoxicity drug treatment.

A Fas-receptor deficient cell line (A4) was generated from the parental wild-type Jurkat (human lymphoma) cell line by continuous treatment with an agonistic anti-CD95 mAb. Surprisingly, the generated A4 cell line was not only resistant to Fas-mediated apoptosis, but also displayed high resistance to apoptosis induced by another death ligand, the TRAIL-ligand, as well as anti-cancer drugs (doxorubicin, etoposide, cis-DDP), X-rays, UV-light, staurosporin, hydrogen peroxide, and menadione. Immunochemical screening revealed only minor differences in the expression of most established components of apoptotic machinery, its regulatory signal transduction molecules, and the heat shock response.

RNA of multiresistant (A4) and parental (Jurkat) cell lines were purified and subjected to oligonucleotide DNA microarray (Affymetrix GeneChip). The assay reveals a surprisingly small set of the genes possibly involved in multiresistance program. The candidate genes include enzymes involved in specific metabolic chains, transcription factors, and antigens of immune recognition. A selection of the candidate genes was analyzed for protein levels. The expression of the selected genes showed moderate to good correlation with the protein levels.

Development of the multiresistant clone A4 was promoted by continuous cells stimulation of Fas. The model can reflect usual clinical situation when microevolution of cancer cells being under pressure of the immune system yield highly malignant multiresistant tumors.

In conclusion, our data suggest that the multiple resistance of A4 is not only based on the deficiency of Fas expression, but mainly arises from the defects of other molecular targets, which are interfering with the execution phase of apoptosis. Resistance to apoptosis

of A4 cells is not connected with expression of any obvious antiapoptotic proteins like Bcl-XL and Bcl-2, or p53 dysfunction.

B.05**GENE EXPRESSION ANALYSIS OF A HUMAN BREAST CANCER CELL LINE CONTAINING A BRCA1 MUTATION AND WITH EXTREME SENSITIVITY TO POLYAMINE DEPLETION**

C. Hegardt, S. Oredsson, Borg
Lund University, Lund, Sweden

Breast cancer is the most common malignancy among women in the Western world. A number of different strategies including radiotherapy and chemotherapy are used but new treatment strategies based on knowledge about the specific genetic defect of the tumour are needed. A new group of chemotherapeutic compounds include drugs that deplete cells of their polyamine pools. We have treated the human breast cancer cell lines MCF-7, SK-BR-3, HCC1937 and L56Br-C1 with the spermine analogue N1, N11-diethylnorspermine (DENSPM). DENSPM induces a rapid depletion of all polyamines by downregulating the activity of the biosynthetic enzymes and upregulating the activity of the catabolic enzyme spermidine/spermine N1-acetyltransferase. An initial growth inhibition followed by a delayed apoptotic response was found in MCF-7, SK-BR-3 and HCC1937 but L56Br-C1 showed an extensive growth inhibition and cell death within 48 hours of DENSPM treatment. L56Br-C1 was established from malignant tissue of a woman with a germ-line mutation in the BRCA1 tumour suppressor gene, the most commonly detected alteration in hereditary breast cancer. In addition, the cells carry a somatic p53 mutation. The rapid cell death after DENSPM treatment in the L56Br-C1 cell line was confirmed to be apoptotic by analysis of DNA fragmentation and caspase activity. After 48 hours of treatment more than fifty percent of the cells were found in the sub-G1 region, as determined by flow cytometry, and caspase-3, -8 and -9 were significantly induced. The high sensitivity to DENSPM is interesting in the light of the fact that the tumour in the patient was highly refractive to various anticancer treatment regimens. To investigate the effect of DENSPM treatment on gene expression in the different cell lines, cDNA microarray analysis was performed. Extracted RNA from control and treated samples was labelled and co-hybridised to microarray slides containing 27000 cDNA clones. The gene expression patterns of the different cell lines were compared in order to find a genetic marker for sensitivity to polyamine depletion.

B.06**VARIATION IN THE HUMAN TP53 GENE AFFECTS OLD AGE SURVIVAL AND CANCER MORTALITY**

M. Beekman, D. van Heemst, S.P. Mooijaart, S. van Gerwen, J. Schreuder, B.W. Brandt, P.E. Slagboom, R.G.J. Westendorp
LUMC, Leiden, The Netherlands

p53 mediated induction of apoptosis and senescence plays a key role in protection against cancer. Mice deficient for p53 are highly susceptible to cancer, while mice with constitutively activated p53 (p53 +/m) are almost cancer free. However, despite their cancer resistance, the longevity of p53 +/m mice is reduced and accompanied by early tissue and organ atrophy. These mouse models demonstrate that longevity may depend on a delicate balance between tumour suppression and tissue renewal mechanisms. Recently, it has been shown in human cell lines that replacement of Arginine (Arg) by Proline (Pro) at position 72 decreases the apoptotic potential of p53 at least five-fold. Here we show that in people aged 85 years and over, the Pro/Pro genotype is associated with a 41% increase in old age survival ($p < 0.05$), with similar risk estimates in two independent cohorts. Among these Pro/Pro carriers however, proportional mortality from cancer is 2.54 fold increased ($p < 0.05$). Taken together, our data show that for Pro/Pro carriers in old age the beneficial effects of survival outweigh the harmful effects of mortality from cancer.

B.07**NITRIC OXIDE INHIBITS ANTIPROLIFERATIVE EFFECT OF ADRIAMYCIN AND APOPTOSIS OF TUMOR CELLS**

I.V. Kondakova, G.V. Zagrebelya, M.V. Perepechaeva, E. Choinzonov
Research Institute of Oncology, Tomsk, Russian Federation

Antiproliferative effect and apoptosis induction has been implicated in the adriamycin cytotoxicity. Nitric oxide (NO) was recently identified as inhibitor of apoptosis and cell protective factor against DNA damage. We studied the involvement of NO in the protection of adriamycin treated Ehrlich's carcinoma cells, that may be important in the development of tumor resistance to antineoplastic drugs. The present study demonstrates that a combination of chemically gener-

ated NO or L-arginine – substrate of NO-synthase reaction, with adriamycin failed or impaired toxic effects of adriamycin.

Our data showed that the realization of tumor-toxic effect of adriamycin occurred both through the inhibition of proliferation processes and through the increase in the level of apoptosis. The combination of NO-generating compounds with adriamycin led to a significant increase in the [3H]-thymidine incorporation in DNA of tumor cells in comparison when adriamycin was used alone. The study of apoptosis death using chromatin cleavage assay showed that addition of NO-generating compounds to incubating medium significantly reduced the apoptosis induction of adriamycin-treated tumor cells. The important mechanism of NO-protective effect may be the activation of DNA-dependent protein kinase which is one of the key enzymes of DNA repair. Exposure of tumor cells to NO led to the increase in the expression of this enzyme.

Thus, the data obtained show that NO is the factor which protects DNA of tumor cells from the damaged action of adriamycin and can contribute to the development of tumor resistance to anthracycline antibiotics.

B.08**NEOADJUVANT PERCUTANEOUS-4-HYDROXYTAMOXIFEN DECREASES BREAST CANCER CELL PROLIFERATION: A PROSPECTIVE RANDOMIZED IMAGE ANALYSIS STUDY**

I. Simony-Lafontaine¹, M. Esslimani¹, S. Gourgou¹, S. Pouljol¹, P. Rouanet¹, E. Le Nestour², A. Kramar¹, G. Linarez-Cruz³
¹Centre Val d'Aurelle, Montpellier CEDEX 5, France, ²Laboratoires Besins-Int., Paris, France, ³Pharmacologie IFR Saint-Louis, Paris, France

Aim of this study: to determine by means of Image Analysis the capacity of a percutaneous gel of 4-hydroxytamoxifen (4OHT) (a potent metabolite of tamoxifen) to inhibit malignant cell proliferation *in situ*.

Patients and methods: 49 patients were diagnosed with local invasive estrogen-receptors positive cancer by Tru-Cut biopsy. They were then randomized either to 4OHT gel 0.5 mg ($n = 8$), 1.0 mg ($n = 9$) or 2.0 mg ($n = 10$) applied daily to breasts, or oral tamoxifen (20 mg/d) ($n = 11$) or to no treatment ($n = 11$) during 14 to 21 days. The tumor was then surgically removed. Proliferation-associated antigens Ki67 and PCNA expressions were assayed by immunohistochemistry on

formalin fixed paraffin embedded sections of the pre- and posttreatment samples. Bcl-2 antiapoptotic gene expression and the TUNEL technique were used on the same samples to assess the apoptotic status of the tumor cells. For each immunolabeling assay Image Analysis was performed with a computer assisted system (SAMBA-Alcatel, France) determining a series of parameters (Mean Optical Density, stained nuclear area . . .). A labeling index (LI) score was then determined. Stromal proliferating lymphocytes served as internal positive controls. Data analysis was performed with Kruskal-Wallis test and Fisher's exact test.

Results: tumor tissue Ki67 and PCNA LI scores after treatment differed significantly among the different groups: treated patients (4 OHT gel and oral tamoxifen) exhibited significantly lower mean Ki67 ($P = 0.0054$) and PCNA ($P = 0.002$) LI scores compared to those of the untreated patients. A trend for dose-dependence was observed for 4-OHT gel. The inhibition of cellular proliferation in the 4-OHT gel treated patients was equivalent to that seen in the Tamoxifen treated patients. No difference was demonstrated for TUNEL and Bcl-2 expression.

Conclusion: previous studies have shown tamoxifen treatment results in a significant reduction in the incidence of invasive as well as non-invasive breast cancer. However adverse effects remain a problem for preventive therapy. In the study Image Analysis has demonstrated the biological and potentially clinical efficiency of a topical 4OHT gel.

B.09

EXTRACELLULAR MATRIX BIOPOLYMERS AS NEW TARGETS IN THE THERAPY OF HUMAN OSTEOSARCOMA

R. Harisi¹, J. Dudas², F. Timar², I. Kovalszky², M. Szendroi³, A. Jeney²

¹Semmelweis University, Budapest, Hungary, ²1st Department of Pathology, Budapest, Hungary, ³Department of Orthopaedics, Budapest, Hungary

Introduction: The proliferation and metastatic potential of human osteosarcoma may also depend on extracellular matrix (ECM) related signal transduction pathways. Since the ECM construction is principally different in tumours from normal tissue, this issue may contribute to the understanding of growth properties of this malignancy.

Aims: In this study involvement of ECM and its biopolymers in osteosarcoma cell growth was investigated.

Materials and methods: A stabile osteosarcoma cell line (OSCORT) was established from the biopsy fragments of a 17-year-old boy. Osteosarcoma cells were cultured conventionally, or on extracellular matrix gel (ECM-gel), as well as on the matrix produced by them. Cell proliferation, cell cycle, expression of proliferation-related proteins and collagenase IV activity was compared within these culture conditions.

Results: ECM increased cell proliferation, designated also by increased cyclin D1 and PCNA protein synthesis. Among ECM biopolymers heparan sulfate proteoglycan (HSPG) and fibronectin was the most responsible for these effects. On the other hand, laminin did not change, collagen IV definitively decreased cell proliferation. Cells in S-phase has doubled in number when they were kept on ECM-gel or treated with HSPG or fibronectin. Collagen IV decreased G1 and S-phase and increased G2-phase counts. Synthesis of cyclin B1 decreased, while PCNA and Ki-67 increased in cells cultured on ECM-gel. All studied ECM biopolymers caused the decrease of cyclin B1 and cdc2 as well. Cyclin D1 expression increased by ECM-gel, HSPG and fibronectin, did not change by laminin, and decreased by collagen IV. ECM-gel induced a 92kDa collagenase IV activity, which was most enhanced for HSPG.

Conclusion: ECM has a significant role in maintenance of the malignant phenotype of human osteosarcoma, HSPG produced by osteosarcoma cells may partly be responsible for this effect, providing a potential therapeutic target in the future.

B.10

ADENOVIRAL-MEDIATED MODULATION OF HEPATIC LAT1/CD98 LIGHT CHAIN EXPRESSION

N.I. Thompson¹, B.T. Storey², C.A. Vaslet², C. Fugere³

¹Providence, RI, USA, ²Brown University, Providence, RI, USA, ³RI Hospital, Providence, RI, USA

LAT1 is a light chain component of the CD98 heterodimeric amino acid transporter, cell activation and integrin linked molecule. Our laboratory originally cloned this molecule as 'TA1' based on its increased expression and loss of regulation in rat hepatocellular carcinoma. In previous work, we have demonstrated increased expression is associated with a variety of human primary cancers and that LAT1 expression in normal hepatic cells is responsive to the amino acid environment. We hypothesize that constitutive expression in tumor cells provides an adaptive growth or

survival advantage particularly under limited nutrient conditions. To test this hypothesis and assess its relationship to the tumor phenotype, we have constructed tetracycline-regulated adenoviral LAT1 sense and antisense viral vectors and utilized them in a rat model system of normal and transformed hepatic cells. Viral induced gene expression can be detected in these cells and results in modulation of LAT1 *in vitro*. We have assessed cellular growth rates, viability and cell cycle parameters relative to adenoviral modulation of LAT1 expression under conditions where CD98 heavy chain/4F2 is constitutive. We also examine relative surface LAT1/CD98 expression in cells infected with sense and antisense virus compared to cells infected with beta-galactosidase control virus. In aggregate, these studies are expected to reveal whether altered expression of the LAT1 tumor associated amino acid transporter is a cause or consequence of neoplastic transformation.

B.11
TARGETING THE FAS RECEPTOR
ENHANCES 5-FLUOROURACIL- AND
ANTIFOLATE-INDUCED CELL DEATH

W.I. Allen, D.B. Longley, T. Wilson, U. McDermott, J. Boyer, P.G. Johnston
Queens University Belfast, Belfast, Northern Ireland

The Fas (CD95/Apo-1) death receptor is a member of the tumour necrosis factor cell surface receptor family. Receptor binding results in formation of the death inducing signalling complex (DISC), at which procaspase 8 molecules are activated leading in turn to activation of pro-apoptotic downstream molecules including the bcl-2 family member BID. We have found that expression of Fas was up-regulated >10-fold in MCF-7 breast cancer cells in response to treatment with IC60 doses of the fluoropyrimidine 5-Fluorouracil (5-FU) and the antifolates raltitrexed (RTX) and Alimta (MTA). MTT assays demonstrated that combined treatment with the agonistic Fas antibody CH-11 and either 5-FU, RTX or MTA in MCF-7 cells resulted in highly synergistic decreases in cell viability. Furthermore, cell cycle analyses indicated that apoptosis of 5-FU- and antifolate-treated MCF-7 cells was dramatically enhanced by co-treatment with CH-11. Procaspase 8 and BID were both activated following co-treatment with 5-FU and CH-11, but were unaffected by single treatment with either drug. In addition, activation of procaspase 8 coincided with PARP ((polyADP)-ribose polymerase) cleavage in 5-FU and

CH-11 co-treated cells, while inhibition of caspase 8 activity blocked PARP cleavage in these cells. Overexpression of thymidylate synthase (TS) in MCF-7 cells abrogated the synergy between both antifolates and CH-11, but had no effect on the synergistic interaction between 5-FU and CH-11. Inactivation of p53 in MCF-7 cells blocked 5-FU- and antifolate-mediated up-regulation of Fas and attenuated the synergistic activation of apoptosis in response to combined treatments of each drug with CH-11. These findings suggest that the Fas signalling pathway is an important regulator of 5-FU- and antifolate-induced cell death.

B.12
IDENTIFICATION OF A TELOMERASE
REPRESSOR ON CHROMOSOME 6Q14-22
INVOLVED IN HUMAN
PAPILLOMAVIRUS-MEDIATED CERVICAL
CARCINOGENESIS

J. de Wilde, P.J.F. Snijders, C.J.L.M. Meijer, B. Ylstra, R.D.M. Steenbergen
VUMC, Amsterdam, The Netherlands

High-risk human papillomavirus (HPV) types play a major role in cervical carcinogenesis *in vivo* and can induce immortalization of human keratinocytes *in vitro*. However, additive oncogenic events that, next to HPV functions, are involved in cervical carcinogenesis are currently unknown. A potential key event is the activation of telomerase resulting from a deregulated expression of its catalytic subunit hTERT.

Microcell-mediated chromosome transfer studies revealed that introduction of human chromosome 6 in the HPV16 immortalized cell line FK16A and in the cervical cancer cell line SiHa resulted in restoration of senescence, which was associated with a strong reduction of hTERT mRNA expression and telomerase activity. Ectopic expression of hTERT could prevent the telomeric shortening based growth arrest induced by chromosome 6. These data indicate that chromosome 6 harbours an hTERT repressor, the loss of which is involved in HPV-mediated immortalization (Steenbergen et al., *JNCI* (2001)).

Subsequent loss of heterozygosity (LOH) analysis on telomerase positive and telomerase negative cervical cancer precursor lesions (CIN III lesions) indicated that allelic imbalance at 6q14-22 is associated with telomerase activity (Van Duin et al., *Int. J. Cancer* (2003)). These findings suggest that the hTERT repressor gene is located at 6q14-22.

To identify the hTERT repressor on chromosome 6q14-22 and downstream pathways involved, differential expression analysis has been performed on 10 hTERT-negative and 10 hTERT-positive FK16A-chromosome 6 hybrids. The 10 FK16A-chromosome 6 hybrids, in which hTERT is endogenously repressed, express ectopic hTERT to maintain the immortal phenotype and prevent interference with irrelevant senescence events. The 10 hTERT-positive FK16A-chromosome 6 hybrids have regained their immortal phenotype due to inactivation of the repressor on chromosome 6.

Microarray expression analysis for 19,200 genes revealed approximately 1,000 genes that showed differential expression between the hTERT-negative and the hTERT-positive cells. A small subset of these genes is located on 6q and may therefore represent candidate hTERT repressor genes. The identified candidate genes are presently being analysed for their role in HPV-mediated immortalization.

B.13 UNRAVELING GENETIC PREDISPOSITION TO ENVIRONMENTALLY RELATED CANCER

B.J.M. Braakhuis, J. Cloos, C.R. Leemans,
S. Kasanmoentalib, W.P.H. de Boer, R.H. Brakenhoff
VUMC, Amsterdam, The Netherlands

Convincing evidence has been provided that the development of environmentally-related cancers such as head and neck squamous cell carcinoma are not only related to environmental factors such as smoking and alcohol abuse, but also to an intrinsic susceptibility. This susceptibility is strongly related to an impaired DNA damage processing capability that can be measured as the mean number of chromatid breaks in peripheral blood lymphocytes after challenging the cells with DNA damaging agents such as bleomycin or radiation. This assay is commonly referred to as the mutagen sensitivity test. When studying twin pairs we have shown that mutagen sensitivity is a heritable factor (heritability estimate of 75%). Identifying persons at highest risk for cancer development will be further improved by recognizing the genes responsible for cancer susceptibility. Aim of our study is to identify the molecular pathways and underlying genes that are involved in the susceptibility to environmentally related cancer using genetic screens and genomics approaches including expression profiling. We have compared lymphoblastoid cell lines of seven hypersensitive persons (=cases) with seven non-sensitive individu-

als (=controls). Changes of RNA expression were determined after 4 hr exposure to bleomycin on 20 K oligonucleotide arrays using a standard RNA reference. Normalization was performed using the reference and changes in expression were analyzed with a novel ANOVA-based method and another analysis method that was based on the calculation of the type 1 error rate of each gene. Both methods identified genes that were found to show a significantly different change in expression between cases and controls and these could be functionally classified in relation to the cellular processes of apoptosis, signal transduction and metabolism. Currently, the identified pathways are functionally characterized to provide evidence for a causal relationship with the cancer susceptibility phenotype.

B.14 FUNCTIONAL RESCUE SCREEN FOR THE IDENTIFICATION OF PROTEINS ACTING UPSTREAM OF THE HIF-1 PATHWAY

A. Shvarts, I. Heijnen, A.E. Greijer, A.J. Groot,
E.H. Gort, P. van der Groep, H.M. Pinedo,
P.J. van Diest, E. van der Wal
VUMC, Amsterdam, The Netherlands

To unravel the yet largely unknown molecular pathways leading to hypoxia inducible factor alpha (HIF-1 alpha) activation and stabilization, a functional rescue screen, based on the transcriptional activation property of HIF-1 alpha is used to identify novel upstream activators of HIF-1 alpha.

We have generated stable cell lines expressing the puromycin resistance gene under the control of a promoter with a hypoxia responsive element (HRE). In this system, activation of HIF-1 alpha, by upstream factors introduced by retroviral expression libraries, results in cell survival in the presence of puromycin. Puromycin resistance is thereby the functional readout for HIF-1 alpha activity. We indeed obtained puromycin resistant clones in primary screening rounds. Clear enrichment in number of colonies was achieved with the purified virus, containing the cDNA responsible for the rescue. Puromycin resistance requires the wild type HRE sequence and the activation of the promoter is not dependent on the integration site of the HRE containing construct in the original cell lines. Individual second round HIF-1 alpha immunofluorescence positive clones were analysed for the presence of the retroviral inserts and those inserts have been sequenced and cloned into the original retroviral vectors. We obtained specific cDNAs with HIF-1 mod-

ulating activity and at the moment we are in the process of the elucidating the precise mechanism by which the new isolated clones effect the activation of HIF-1 alpha. We will present data on, and the identity of cDNAs with positive HIF-1 alpha inducing activity.

This study strongly contributes to the identification of all possible regulators of HIF-1 alpha pathway. This knowledge will not only help us to unravel the precise mechanism of HIF-1 alpha action, but also will make it possible to design targeted cancer drugs that may interfere with the activation of HIF-1 alpha in defined areas of regulation, e.g., stabilization, translocation to the nucleus and transcription activation.

B.15

CYCLIN E DYSREGULATION AND CHROMOSOMAL INSTABILITY IN ENDOMETRIAL CANCER

A. Gschwendtner¹, M. Hubalek², E. Möller-Holzner², M. Widschwendter²

¹University of Innsbruck, Innsbruck, Austria, ²Gynecology, Innsbruck, Austria

Deregulation of cyclin E, an activator of cyclin-dependent kinase 2 (Cdk2), has been associated with a broad spectrum of human malignancies. Yet the mechanism linking abnormal expression of cyclin E to carcinogenesis is largely unknown. The gene encoding the F-box protein hCdc4, a key component of the molecular machinery that targets cyclin E for degradation, is frequently mutated in endometrial cancer, leading to the deregulation of cyclin E expression. Both hCDC4 gene mutation and hyperphosphorylation of cyclin E, a parameter that usually correlates with hCDC4 mutation, were found to have a strong statistically significant association with polyploidy and aneuploidy in endometrial cancer. On the contrary, elevated expression of cyclin E by itself was not significantly correlated with polyploidy or aneuploidy. These data suggest that impairment of cell cycle regulated proteolysis of cyclin E may be linked to carcinogenesis by promoting genomic instability.

B.16

P53-REGULATED TRANSCRIPTIONAL PROGRAM IMPLICATED IN GENOTOXIC STRESS-INDUCED APOPTOSIS

G.Y. Yu, P. Kho, Z. Wang, L. Zhuang, E.T. Liu
Genome Institute of Singapore, Singapore, Singapore

Using a genome-wide approach, we explored the transcriptional response of colorectal cancer HCT116

cells during apoptosis induced by anticancer drug 5-fluorouracil (5-FU) as a function of p53 status. 230 genes responded to 5-FU in a p53-dependent manner with the majority down regulated ($n = 189$). We noted that apoptotic response best correlated with the kinetics of p53-dependent gene repression. Selective blockade of p53-dependent gene repression significantly diminished apoptosis, suggesting that gene repression induced by p53 contributed to p53-dependent apoptosis. We further show that PLK, PTTG1 and CHEK1 are direct targets of p53-dependent transcriptional repression. Moreover, the targeted degradation of PLK and PTTG1 using siRNA directly induces apoptosis. However, we noted that the induction of p53 transcriptional response alone was insufficient to trigger apoptosis since a similar p53 response induced by doxorubicin resulted in G2/M arrest instead of apoptosis. Of the very few genes that differed in expression after doxorubicin as compared to 5-FU, cyclin E2 was induced in 5-FU treated cells independent of p53 status, concomitant with the absence of G2/M arrest. By contrast, doxorubicin which induced a G2/M arrest did not alter cyclin E2 expression. Thus, combination p53 response with additional molecular events such as cyclin E2 induction appears to be required for the 5-FU apoptotic response in HCT116 cells.

C.01

HAPLOTYPE ANALYSIS OF RELATED ATM MARKERS FACILITATE PRENATAL DIAGNOSIS IN IRANIAN ATAXIA TELANGIECTASIA PATIENTS

Bayat

National Research Center of Genetic, Tehran, Iran

Ataxia Telangiectasia is an autosomal recessive disorder in 1/40000 to 1/100000 in reported populations. There is 25% possibility for having an affected child when parents are carrier for ATM gene mutation. There is no cure available for this disease and prenatal testing is strongly recommended in prevention of this disease.

Although preference method is the direct mutation analysis of ATM gene, but large size of the ATM gene with 63 exons and the large number of possible mutation in patients considerably limit the facility of mutations analysis as a choice in diagnosis. Indirect method is a better tool when parent are not carrier of founder mutation and pass different mutations to their children. Indirect molecular diagnosis using ATM related molecular markers facilitate prenatal di-

agnosis of AT children. In this study four molecular markers: D11S2179, D11S1787, D11S535, D11S1343 are genotype in 18 unrelated families from different region of IRAN. Those markers are amplified using extracted sequence primers from Gene Bank with their described PCR conditions. The amplified products were separated using denaturing PAGE gels, and the data were analyzed to detect their pattern of inheritance in each family. In all families segregation of alleles were recording to mandelian inheritance and affected chromosomes were distinguishable from unaffected ones. All carriers and affected patients were diagnosed accurately. Thus this method is effectively usable in prenatal diagnosis of ataxia telangiectasia.

C.02

XRCC1 (ARG399GLN) AND XPD (LYS751GLN) POLYMORPHISMS AND RISK OF OVARIAN CANCER

E. Cecchin, G. Corona, F. Sartor, G. Biscontin, P. Bion, S. Muzzin, M. Boiocchi, G. Toffoli
CRO-National Cancer Institute, Aviano (PN), Italy

Genes involved in DNA repair pathways and maintenance of genome integrity have a well-demonstrated role in protecting cells from aberrations possibly leading to cancer. They could as well influence chemotherapeutic outcome of DNA-damaging drugs as platinum derivatives. We focused our investigation on polymorphisms altering the activity of two important DNA-repairing enzymes encoded by the base excision repair gene XRCC1 (Arg399Gln) and the nucleotide excision repair gene XPD (Lys751Gln). Aim of the study was to describe the frequency of these polymorphisms in 190 ovarian cancer patients and to compare it with a control group composed by 392 healthy blood donors. Investigations were performed on genomic DNA from peripheral blood mononuclear cells using restriction fragment length polymorphism method for single nucleotide polymorphisms. Among controls we found that 182 individuals (46.4%) were wild type (Arg/Arg), 176 (44.9%) heterozygous (Gln/Arg) and 34 (8.7%) homozygous mutated (Gln/Gln) for XRCC1 (Arg399Gln) whereas for XPD (Lys751Gln) 129 individuals (32.9%) were wild type (Lys/Lys), 210 (53.6%) heterozygous (Gln/Lys) and 53 (13.5%) homozygous mutated (Gln/Gln). Among cases the frequencies of XRCC1 (Arg399Gln) were: 77 (40.3%) wild type, 78 (41.1%) heterozygous, 35 (18.6%) homozygous mutated; for XPD (Lys751Gln): 64 (33.7%) wild type, 90 (47.4%) heterozygous, 36 (18.9%) homozygous

mutated. Case-control approach highlighted significantly higher frequency of the homozygous mutated XRCC1 genotype among cases (35/190, 18.6%) than among controls (34/392, 8.7%). Individuals with homozygous mutated genotype had a relative risk to develop an ovarian cancer 2.5 fold higher than individuals with at least one wild type allele (OR = 2.4, 95% CI 1.43–3.95, $p = 0.001$) (two-sided Fisher's Exact Test). No relationship has been evidenced between ovarian cancer and the XPD polymorphism considered (OR = 1.5, 95% CI 0.94–2.38, $p = 0.110$). We demonstrated an increased frequency of the XRCC1 Arg399Gln polymorphism in ovarian cancer patients compared to controls highlighting its role in the development of this kind of tumour. The prevalence of the mutated genotype in ovarian cancer patients could influence their chemosensitivity to platinum derivatives.

C.03

RAPID PRENATAL DIAGNOSIS OF DOWN SYNDROME USING QUANTITATIVE FLUORESCENCE *IN SITU* HYBRIDIZATION ON INTERPHASE NUCLEI

K.T. Truong¹, F. Soussaline¹, A. Gibaud², J.M. Dupont³, M.N. Guilly⁴, B. Dutrillaux², B. Malfoy²
¹IMSTAR SA, Paris, France, ²Institut Curie, Paris, France, ³Hopital Cochin, Paris, France, ⁴CEA DRR DSV, Fontenay-aux-Roses, France

Conventional analysis of metaphase chromosomes remains the reference approach in prenatal diagnosis. However, this method remains labor-intensive and time-consuming. The first step towards rapid identification of aneuploidies is achieved by interphase fluorescence *in situ* hybridization (FISH) with centromeric or locus-specific probes. Spot counting using this type of probes is a reliable approach, but remains very time-consuming with technical and biological limitations. In this study, we present a new FISH method using image cytometry for the detection of trisomy 21 within interphase nuclei. The approach is based on comparative quantitation of the fluorescence signals emitted by whole chromosome 21 and 22 painting probes co-hybridized on interphase nuclei. The chromosomal imbalance was determined with an automated image cytometer by detecting an abnormal ratio of both fluorescence emissions when compared with the ratio obtained in normal cells. Ten blood samples and twenty amniotic fluids were analyzed. Results from FISH and standard cytogenetics were compared and 100% correlation was achieved. Our method, which enables easy

detection of chromosomal imbalances without need for metaphase preparations, can be applied to the diagnosis of trisomy 21 and extended to other disorders with chromosomal imbalances avoiding any difficulties related to spot counting.

C.04

CDK9/CYCLIN T1 EXPRESSION IN REACTIVE LYMPH NODES AND MALIGNANT LYMPHOMAS

C. Bellan¹, G. De Falco¹, S. Lazzi¹, S. Vicidomini¹, R. Lio¹, K. Schurfeld¹, S. Bartolommei¹, M. Hummel², S. Pileri³, P. Tosi¹, L. Leoncini¹, A. Giordano⁴

¹Siena, Italy, ²Institut für Pathologie, Berlin, Germany, ³Hematopathology Unit, Bologna, Italy, ⁴College of Science & Technology, Philadelphia, USA

CDK9 is a member of the CDC2-like family of kinases. Its cyclin partners are members of the family of CYCLIN T (T1, T2a and T2b) and cyclin K. CDK9/CYCLIN T1 complex is very important in the differentiation program of several cell types, controlling specific differentiative pathways. Limited data are available regarding the expression of CDK9/CYCLIN T1 in haematopoietic and lymphoid tissues. The aim of this paper is to analyze the expression of CDK9/CYCLIN T1 complex in lymphoid tissue, in order to assess its role in B and T cell differentiation and lymphomagenesis.

In reactive lymph nodes CDK9/CYCLIN T1 expression was found by immunohistochemistry in germinal center and in B and T cell blasts in interfollicular areas, suggesting a role for CDK9 in the activation and differentiation of B and T cells. The staining of CDK9 and cyclinT1 complex in malignant lymphomas seems to reflect their normal counterpart, as it is highly expressed in lymphomas derived from germinal center cells, such as follicular lymphomas and classical Hodgkin's lymphomas and from activated T cells (i.e. anaplastic large cell lymphomas). Diffuse large B-cell and Burkitt's lymphomas showed a wide range of values. No expression of CDK9 and CYCLIN T1 was detected in marginal zone and mantle cells lymphomas.

However, at RNA level we found an imbalance in CDK9/CYCLIN T1 ratio in follicular lymphoma and diffuse large B cell lymphomas with germinal center phenotype, and in the cell lines of classical Hodgkin's lymphomas, Burkitt's lymphomas and anaplastic large cell lymphoma, in comparison with reactive lymph nodes.

Here we report CDK9/CYCLIN T1 complex deregulation in neoplastic conditions for the first time, representing a step towards better understanding of through which molecular mechanism CDK9/CYCLIN T1 complex acts on the activation and differentiation program of lymphoid cells and the role of its deregulation in malignant transformation.

C.05

THE INTERACTION BETWEEN HIV-1 TAT AND PRB2/P130: A POSSIBLE MECHANISM IN THE PATHOGENESIS OF AIDS-RELATED NEOPLASMS

G. De Falco¹, C. Bellan¹, S. Lazzi¹, P. Claudio², A. Giordano², L. Leoncini¹

¹Siena, Italy, ²College of Science & Technology, Philadelphia, USA

HIV-1 has long been recognized as the etiological agent of acquired immunodeficiency syndrome (AIDS). Although many neoplasms arise in HIV-1 infected patients more frequently than in other forms of immunosuppression, the role of HIV-1 as an oncogenic virus has not yet been clarified. The HIV-1 gene product Tat is a likely candidate to contribute to tumor pathogenesis in HIV-1 infected patients because of its growth promoting activity, angiogenic function and antiapoptotic effect. The oncogenic role of Tat is further supported by the development of non Hodgkin's lymphomas in Tat-transgenic mice. Furthermore, a virus-linked mechanism of lymphomagenesis, in AIDS-related lymphomas has recently been proposed, involving the RB2/p130 pathway. The absence of mutation in the RB2/p130 gene and the unusually high percentage of cells expressing pRb2/p130 in tumors with a high proliferative activity such as AIDS-related lymphomas, may in fact suggest a physical interaction of pRb2/p130 with viral oncoproteins. However, little is known about the mechanism by which HIV-1 gene products interact with RB family and other cell cycle regulatory proteins. The aim of our study was to investigate whether Tat could bind to pRb2/p130, thus impairing its tumor suppressor activity. Our results show that the two proteins interact both *in vitro* and *in vivo*, through the pocket region of pRb2/p130. Tat seems to inactivate the tumor suppressor activity of pRb2/p130, as demonstrated by a colony assay. In addition, we observed that Tat does not compete with E2F-4 in binding to pRb2/p130. Due to the overexpression of pRb2/p130 observed in AIDS-related lymphomas, we investigated whether Tat could influ-

ence either the phosphorylation status of pRb2/p130 or its expression at mRNA level. Our results show that Tat does not alter the phosphorylation status of pRb2/p130, but increases its expression at mRNA level. The interaction between Tat and pRb2/p130 may lead to a deregulation of cell growth control by Rb-related proteins. The understanding of basic information may be of significance for prognosis and implementing therapeutic regimens.

C.06 COMPUTERISED EXPERT SYSTEM FOR THE CLASSIFICATION OF CERVICAL INTRAEPITHELIAL NEOPLASIA

P.W. Hamilton¹, G.J. Price², W.G. McCluggage³,
M.L. Morrison², G. McClean³, L. Venkatraman³,
J. Diamond², H. Bharucha², R. Montironi⁴,
P.H. Bartels⁵

¹Queen's University, Belfast, Northern Ireland,
²Queen's University of Belfast, Belfast, UK, ³Royal
Group of Hospitals, Belfast, UK, ⁴University of An-
cona, Ancona, Italy, ⁵University of Arizona, Arizona,
USA

Considerable inter and intra-observer variation exists in the histological classification of preinvasive cervical squamous lesions. The aim of this study was to develop a computer-based expert system for the histological interpretation of these lesions. Knowledge was represented as a Bayesian belief network comprising eight diagnostic features, each independently linked to the diagnosis. An onscreen interface prompted the entry of diagnostic knowledge using images, collecting evidence in a cumulative manner which provided a final probability for the diagnostic outcomes. A cumulative probability graph (decision graph) was generated to map the diagnostic process. The network was tested on 50 cervical colposcopic biopsy specimens, comprising 10 cases each of normal, koilocytosis, CIN I, CIN II and CIN III. The cases were classified using conventional assessment on two occasions by two consultant and two junior pathologists. The cases were also classified using the expert system on two occasions by the four pathologists. Inter and intra-observer agreement using conventional assessment and using the expert system was explored using kappa statistics. Intra-observer reproducibility using conventional un-aided diagnosis had reasonable kappa values (0.69–0.86) but inter-observer agreement was often poor (0.35–0.75). The expert system improved overall reproducibility between individuals. An additional advantage of the sys-

tem is that a decision graph allows the assessment of feature reproducibility and how this contributes to diagnostic disagreement between individuals. Diagnostic features such as nuclear pleomorphism were shown to be particularly problematic in the grading of CIN. Comparison of decision graphs between individuals allows the assessment of disagreement at the level of individual diagnostic features level as well as for the final diagnosis. If a decision graph can be defined by an expert for a given slide then individuals can test their performance against that graph. This has enormous potential in training environments as well as for objective assessment of performance in QA/QC programmes in pathology.

C.07 DNA METHYLATION AS A PREDICTIVE MARKER AND A THERAPEUTIC TARGET IN OVARIAN CANCER

G.R. Strathdee¹, B.R. Davies², J.K. Vass³,
N. Siddiqui⁴, R. Brown¹

¹Glasgow University, Glasgow, Scotland, ²University
of Newcastle, Newcastle, UK, ³Beatson Institute, Glas-
gow, Scotland, ⁴Stobhill Hospital, Glasgow, Scotland

DNA methylation of CpG islands, located in the 5' region of genes, has been demonstrated to result in transcriptional inactivation of the associated gene. While CpG islands are almost invariably unmethylated in normal tissue, cells derived from tumours exhibit widespread methylation of CpG islands and many genes known to be important in tumour development and progression are frequently inactivated by this mechanism. We studied the methylation pattern of the Methylation Controlled DNAJ protein (MCJ) gene, both in normal and ovarian cancer tissue. Surprisingly, we identified cell type specific methylation and inactivation of MCJ expression in cells of epithelial origin, but not haematopoietic or mesenchymal origin. High levels of MCJ methylation were detected in ovarian surface epithelial cells (OSE), from which the vast majority of ovarian cancers are derived. However, the levels of methylation of MCJ varied widely in ovarian tumours, from high levels similar to those detected in OSE cells to low or undetectable, suggesting that MCJ can frequently become demethylated during ovarian tumourigenesis. In addition, high levels of MCJ methylation was significantly correlated with poor response to chemotherapy, an observation compatible with previous *in vitro* data linking absence of MCJ expression to increased resistance to several chemother-

apeutic agents. This suggests that methylation of MCJ may be a useful predictive marker in ovarian cancer. The ovarian carcinoma cell line A2780 expresses MCJ, however, we found loss of MCJ expression in 80% of cisplatin resistant derivatives of A2780, and loss of expression correlated with methylation of the MCJ CpG island. Treatment of the resistant cell lines with a DNA methylation inhibitor (Decitabine) results in demethylation of the MCJ CpG island and re-expression of MCJ, as well as at least one other protein known to be involved in drug resistance, the mismatch repair gene MLH1. In addition the Decitabine-treated cells exhibit re-sensitisation to a number of important chemotherapeutic agents. The potential utility of Decitabine as a modulator of chemosensitivity is currently being tested in clinical trials.

C.08

CRONKHITE-CANADA SYNDROME-LIKE HABITUS AND APC GENE CODON 1309 GERM-LINE MUTATION

S. Kapitanovic¹, K. Husnjak¹, S. Cukovic-Cavka², K. Pavelic¹

¹Rudjer Boskovic Institute, Zagreb, Croatia, ²KBC Zagreb, Zagreb, Croatia

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease, affecting 1 in 2000 people. Patients with FAP develop hundreds to thousands of adenomatous polyps in the colon and rectum during their second or third decades and one or more of them progress to cancer if left without surgical treatment. FAP is caused by mutations in the tumour suppressor gene APC. Cronkhite-Canada syndrome is not inherited disease and with the juvenile-type of polyposis have no malignant potential. Here we report one patient (18-year-old female) affected by FAP with Cronkhite-Canada syndrome features (alopecia, cutaneous hyperpigmentations, arachnodactyilia, dystrophic changes in the fingernails). We also examined the molecular genetic changes of APC, K-ras and p53 genes in the epithelium of patient's tubular adenoma.

DNAs were isolated from peripheral blood of patient and from the microdissections of deparaffinized tissue sections. Each microdissection was first preamplified by using primer extension preamplification (PEP) method. PCR was performed using specific pairs of primers. PCR products were analysed by heteroduplex, RFLP, LOH analysis and sequencing.

The genetic analysis confirmed the APC gene codon 1309 germ-line mutation and the diagnosis of FAP

in this patient as a first case in her family. By using microdissection and PEP-PCR we were able to prove germline mutation and LOH of the APC gene. K-ras gene codon 12 and p53 gene exon 7 mutation were detected as well.

This results show the importance of genetic analysis in the differentiation of polyposis syndromes and evaluation of the risk for development of colon cancer.

C.09

A SPECIFIC SOMATIC GENETIC PROFILE FOR BRCA1 AND BRCA2 BREAST CARCINOMAS REVEALED BY MOLECULAR CLASSIFICATION USING CGH

P.M. Nederlof¹, T. van Welsem¹, E. van Beers¹, S. Verhoef¹, F.B.L. Hogervorst¹, L.F.A. Wessels², P. Devilee³, J. Veltman⁴, R. Oldenburg³, A. Geurts van Kessel⁴, L.J. van 't Veer¹

¹The Netherlands Cancer Institute, Amsterdam, The Netherlands, ²Delft University of Technology, Delft, The Netherlands, ³LUMC, Leiden, The Netherlands, ⁴University Nijmegen, Nijmegen, The Netherlands

We developed a method to identify possible BRCA1 and BRCA2 mutation carriers based on the genetic characteristics of their breast carcinomas. We build a Bayesian classifier, which assigns a given tumor to the BRCA1, BRCA2 or sporadic group based on comparative genomic hybridization (CGH) profiles.

We analyzed proven BRCA1 germline mutation carriers ($N = 28$) and sporadic breast tumors ($N = 42$, BRCA1 or BRCA2 status unknown). BRCA1 breast carcinomas exhibit specific somatic genetic aberrations and can be distinguished from sporadic tumors with an accuracy of 84% as determined by Leave-One-Out-Cross-Validation (LOOCV) [1]. The classifier includes regions on chromosomes 3p, 3q and 5q. Only one BRCA1 tumor (5622del62) was miss-assigned by the classifier. Interestingly, also the microarray gene expression profile classified this tumor as a non-BRCA1 tumor [2].

BRCA2 tumors show some of the characteristic BRCA1 genomic changes (such as the characteristic gain on chromosome 3q) but also BRCA2 specific changes. In general, the number of alterations is considerably lower than that of the BRCA1 tumors. BRCA2 tumors can be distinguished from BRCA1 tumors with a performance of 82% (LOOCV), but the separation from the sporadic group is more difficult (69%).

We now use genomic arrays with 3700 BAC clones at 1 Mb interval to identify potential BRCA1/2 mutation carriers in a group of non-BRCA1/2 high-risk families from our family cancer clinic.

References

- [1] L.F. Wessels et al., Molecular classification of breast carcinomas by comparative genomic hybridization: a specific somatic genetic profile for BRCA1 tumors, *Cancer Res.* **62** (2002), 7110–7117.
- [2] L.J. 't Veer et al., Gene expression profiling predicts clinical outcome of breast cancer, *Nature* **415** (2002), 530–536.

C.10

ANTINEOPLASIC MANIFESTATIONS OF ANTIMETABOLITE DERIVATIVE IN PRE AND PHARMACEUTICAL PREPERATIONS WITH N-BROMOSUCCINIMIDE

P. Jaiswal

University of Allahabad, Allahabad, India

Chemical substances which take part in cellular metabolic reactions are called as metabolites and an antimetabolite is a chemical agent which by virtue of its close structural similarity to the metabolite, block its action. The antimetabolites check the production of carcinogenic compound produced by the abnormal metabolism of some organic compounds: (a) folic acid antagonists, e.g., Methotrexate, etc., (b) purine antagonists, e.g., 6-mercato-purine Azathioprine, (c) pyrimidine antagonists, e.g., fluoro-uracil, cytosine-arabino-5, etc.

Antifolates occupy a special place in antineoplastic chemotherapy in that they produced the first striking, although temporary, remissions in leukemia and the first cure of a solid tumor, choriocarcinoma and the first cure of a solid tumor (1). These compounds have the potential to be developed into new antibacterial, antiviral or cancer chemotherapeutic agents (2). Imidazole derivatives such as midaglizole, deriglidole and efaroxan are highly active antioxidant, antitumor, and anticancer activities (3).

In modern antiviral therapy an important role is played by modified nucleosides and their analogues in which the glycoside linkage at the 1-position of a pyrimidine on 9-position of a purine nucleosides is replaced by a carbon chain (4,5). Bromination is a very important reaction in organic synthesis (6). Particularly for the preparation of intermediate bromo compounds for different b-blockers (7). In the present paper we describe an accurate method for determination

of Antimetabolite in pure form and in pharmaceutical preparation with N-bromosuccinimide in glacial acetic acid medium using an indicator. For testing the quantitative validity of reaction aliquots containing 1 to 10 mg of sample were taken for different time interval 1–10-minute reaction condition was developed for pure sample and pharmaceuticals preparation. A blank experiment was also run under identical condition using all the reagent except the sample. The stoichiometry of the reaction was established and the percentage recovery of the pure sample was calculated with the accuracy of $\pm 1\%$.

C.11

ANALYSIS OF P53 GENE MUTATIONS IN MUSCLE-INVASIVE BLADDER CARCINOMA USING YEAST FUNCTIONAL ASSAY

B. Schlichtholz, M. Presler, M. Matuszewski
Medical University of Gdansk, Gdansk, Poland

Transitional cell carcinoma of the bladder is the most common urinary malignancy with an annual incidence of approximately 7000 in Poland, accounting for 8.6% of new cases in men (the fourth cancer in men) and 2% in women, and was responsible for about 3% of all cancer-related deaths. Voided urine cytology is the most accepted technique of noninvasive detection of urinary system neoplasms. Recently many bladder tumor markers have been described allowing for noninvasive detection of bladder carcinomas. The purpose of the present study was to test the feasibility and sensitivity of the p53 gene functional analysis to detect bladder cancer cells in urine. We determined the p53 status in tumor tissue and paired voided urine by functional assay in yeast followed by sequencing. The p53 mutations were found in 13 out of 29 (44.8%) stage T2–4 tumor tissues and in 6 out of 18 (33.3%) available urine specimens. The p53 mutation pattern found in urine in all but one case was always identical to that identified in the matched tumor tissue. P53 mutations correlated positively with stage ($P = 0.017$) but not with the grade or lymph node involvement. There was no relationship between the p53 mutations and overall survival (OS) ($P = 0.27$). In Cox univariate analysis only stage ($P = 0.018$) was significant prognostic variable, associated with a decreased survival. These results indicate that yeast functional assay is a useful method for p53 gene mutation analysis in urine sediment, however, in our study the p53 status had no significant predictive value concerning OS.

C.12 PROGNOSIS FOR IRINOTECAN EFFICIENCY IN ADVANCED COLORECTAL CANCER

R. Harisi¹, J. Dudas², T. Winternitz³, B. Jaray⁴,
L. Flautner³, G. Bodoky⁵

¹Teaching Hospital St. Laszlo, Budapest, Hungary,

²Department of Gastroenterology, Gottingen, Hungary,

³1st Department of Surgery, Budapest, Hungary,

⁴2nd Department of Pathology, Budapest, Hungary,

⁵Department of Oncology, Budapest, Hungary

Purpose: In spite of encouraging clinical results of topoisomerase I inhibitors they are often inefficient to achieve the required therapeutic response. Our aim was to establish correlation between clinical responses of irinotecan (Campoto) treated advanced colorectal cancer patients and topoisomerase I activity, in order to set up the bases for individualized treatments.

Patients and methods: 16 advanced colorectal cancer patients were involved in our investigation. Irinotecan was used as first line mono-chemotherapy for 6, and second line mono-chemotherapy for 10 patients. Before chemotherapy, cell nuclear protein extracts were isolated from samples taken from advanced colon and rectum cancer patients. PCNA and Ki-67 protein expression and topoisomerase I activity was determined in the cell nuclear extracts.

Results: Drug efficiency is being predicted based on topoisomerase I activity measurements as effective, transitive and ineffective. Drug efficiency for 6 of 16 patients was predicted as effective, for 4 patients as transitive, while for 6 patients as ineffective. Complete remission was not, 3 partial remissions (PR), 6 stable diseases (SD) were observed. PR was observed in case of patients whose drug efficiency was predicted as effective, SD was found for those, whose efficiency was predicted as effective or transitive. 7 patients progressed (PD), whose topo I activity based efficiency was predicted as ineffective or transitive. Chemotherapy caused death was not observed. Severe vomiting and diarrhea was observed by one patient, in his case high topo I activity was detected in the peritumoral tissue as well.

Conclusions: Our preliminary examinations demonstrate that topoisomerase I activity measurements may have predictive role for Irinotecan efficiency in colorectal cancer. Proliferation marker determination may help for prediction of drug efficiency in case of patients with transitive activity. We are planning to extend our study to involve larger patient numbers.

C.13 METHODOLOGICAL POSSIBILITIES FOR THE DETERMINATION OF THE NUMBER OF CTG/CAG REPEATS IN TRIPLET REPEAT UNITS OF THE HUMAN GENOME

M. Falk¹, M. Vojtísková¹, U. Froster²

¹Institute of Biophysics, ASCR Brno, Czech Republic,

²University of Leipzig, Leipzig, Germany

Human genome dynamic mutations are a new class of gene mutations represented by an unstable number of trinucleotide repeats and causing severe human hereditary neuromuscular and neurodegenerative diseases. The identification of pathological expanded alleles on the molecular level is important for clinical diagnostics.

For the molecular diagnostics of expanded tandem repeat trinucleotide sequences we have introduced a fast and efficient TP-PCR fluorescent method according to Warner et al. (1996). We have modified this TP-PCR method for a rapid detection of expanded CTG alleles of the DMPK gene (myotonic dystrophy, MD) into a two-level protocol; first, the heterozygote sample DNAs were selected using P1/P2 primers flanking repeat tracts and, second, the TP-PCR protocol used was focused above all on the identification of a pathological allele. A fluorescent-labelled specific primer in TP-PCR was used for the exact determination of the number of CAG repeats of the gene IT-15 (Huntington's disease, HD) in the diagnostically important region of the grey zone (35 to 39 CAG). The reproducibility of the PCR results was demonstrated on control DNA samples with the known genotype and, in the case of MD, also by Southern blot analysis. We have especially shown the possibility of a cheaper PCR-P1/P2 and TP-PCR protocol with silver staining of separated PCR products on polyacrylamide gels, which can be used also for research of genetic stability of premutated and expanded alleles in large population studies.

Conclusion. Our experience with introducing the above-mentioned PCR methods into laboratory practice clearly documents the possibilities of their general applicability in the molecular diagnostics of hereditary diseases characterised by instability of the trinucleotide repeat tracts.

C.14
MOLECULAR PHARMACODYNAMIC (MPD)
EFFECTS INDUCED BY ET-743
(YONDELISM) IN HUMAN SARCOMA
CELLS (HSC)

M. Sanchez Beato
 CNIO, Madrid, Spain

ET-743 is a marine derived anticancer agent acting through a guanine selective DNA minor groove binding plus an interaction with nuclear transcription factors. ET-743 has shown consistent activity in patients with advanced refractory sarcomas; thus we are investigating the ET-743 induced changes in the gene expression profile in a panel of 11 HSC explanted from untreated sarcoma patients. A correlation between the doubling time (DT) and the ET-743 activity is being noted with IC50s ranging from 0.4 nM at DTs < 44 hours-100 nM at DTs > 72 hours. The dynamics of the ET-743 induced MPD are being assessed in this panel by using a cDNA chip (OncoChip) that includes an array of 6700 clones for cancer relevant genes. The initial data obtained in ET-743-sensitive sarcoma cells indicates an early (6 h) ET-743-induced change in a set of genes related with apoptosis, cell cycle, transcription, cell growth and DNA-binding, with 132 genes induced (2%, 95% CL 1.7–2.3) and 13 inhibited (0.19%, 95% CL 0.10–0.33) at this time point in the sensitive cell line. The correlative data in the resistant cell line is as follows: 52 (0.8%, 95% CL 0.6–1) genes inhibited and 35 (0.5%, 95% CL 0.4–0.7) induced at the same time point. The drug-induced gene expression signature in cells harbouring ET-743 IC50s = 100 nM shows a marked delayed response involving a limited number of genes regulating critical pathways. A potentially predictive set of genes correlated with ET-743 induced sensitivity/resistance, independently of the sarcoma sub-type, is being characterized. Our initial data supports the use of these molecular tools to seek for predictors of ET-743-sensitivity/resistance, thus allowing its further validation in clinical samples.

C.15
SPONTANEOUS APOPTOSIS IN CHRONIC
LYMPHOCYTIC LEUKEMIA AND ITS
RELATION TO TREATMENT-FREE PERIOD
IN CHRONIC LYMPHOCYTIC LEUKEMIA*

K. Metze, G.B. Oliveira, F.G. Pereira,
 I.G.H. Lorand-Metze
 State University of Campinas, Campinas-SP, Brazil

Chronic lymphocytic leukemia (CLL) presents considerable variability in clinical presentation and evolu-

tion. In contrast to the inhibition of apoptosis *in vivo*, spontaneous apoptosis after short-term culture occurs. In a previous study it was shown that the total tumor mass (TTM) and the AgNOR score (percentage of cells with one AgNOR cluster) are very important independent prognostic factors for the treatment-free period (stable phase) of patients with CLL. Since apoptosis is considered to be a prognostic factor for many neoplasias, we tried to find out whether this variable could be predictive for the stable phase, comparing it with TTM and the AgNOR score of peripheral lymphoid cells. Apoptosis rate was measured by flow cytometry as the percentage of annexin V positive cells after short term culture at diagnosis. 32 patients were prospectively evaluated. There were statistically significant inverse correlations between the apoptosis rate and TTM ($r = -0.47$), the AgNOR score ($r = -0.40$) and an INDEX combining TTM and the AgNOR score ($r = -0.48$). In univariate analyses TTM, AgNOR score, and the INDEX were significant predictive variables for stable phase. Comparing all them together in a multivariate Cox regression the logarithm of the INDEX was the most important independent factor. The spontaneous apoptosis rate proved also to be of prognostic importance in an univariate Cox model. Analysing it together with the logINDEX only the latter remained as independent prognostic variable. This was confirmed in a bootstrap resampling study of 250 newly created data sets where the logINDEX was included in the final model in 91% of the cases, whereas the 'spontaneous apoptosis' in only 12.5%.

Therefore we conclude, that the previously described prognostic INDEX at diagnosis is a more important prognostic variable for the treatment free period than the spontaneous apoptosis rate in CLL patients.

*Supported by CNPq and FAPESP.

C.16
IS NUCLEAR MORPHOMETRY PREDICTIVE
OF THE RECURRENCE OF BASAL CELL
CARCINOMA?

V.B. Bedin, A.R.L. Adam, L.N.J. Leite, E. DeSouza,
 M.L. Cintra, K. Metze
 State University of Campinas, Sao Paulo, Brazil

Basal cell carcinomas (BBCs) usually follow a rather benign clinical course. A part of them, however, recurs after treatment causing local tissue destruction. Therefore, morphological parameters that could help to distinguish between recurrent and non recurrent

BBCs would be interesting. The aim of our study was to investigate whether morphometric variables of the nuclei in routinely stained histologic sections could be helpful to identify patients with a high risk for recurrence.

Our study was based on patients with surgically excised BBCs and a follow-up of at least 60 months. In all cases ($n = 67$) the primary tumor was excised without compromised resection margins. Specimen and tumor diameters, as well as the minimal distance between tumour and the resection margins were measured. Digitalized images of 100 nuclei captured at the invasion front of routinely HE-stained paraffin sections of the primary tumors were analyzed according to area, diameters, perimeters and form factors. In order to get a predictive model for tumor recurrence we calculated univariate and multivariate Cox-regressions regarding event-free survival until tumor recurrence. In univariate analysis the Manhattan formfactor proved to be of prognostic significance with lower values (equivalent to more irregular outlines) indicating a higher risk of tumor recurrence. Furthermore, the lowest specimen diameter, the maximum tumour diameter and the minimal distance between tumour and the resection margin (measured on histologic slides) were significantly predictive factors in univariate Cox analysis, but not tumor type and topography of the excision site. In a multivariate Cox-regression, however, an index created of lowest specimen diameter, maximum tumor diameter and the minimal distance between tumour and margin was more important for the prediction of event-free survival than the form factor of the nuclei. In summary simple measurements done on the resection specimens may predict better tumor recurrence than morphometric analysis of nuclei in histologic sections.

C.17

TSLC1 IS A TUMOR SUPPRESSOR AND MARKER FOR INVASION IN CERVICAL NEOPLASIA

R.D.M. Steenbergen¹, D. Kramer¹, B.J.M. Braakhuis¹, P.L. Stern², R.H.M. Verheijen¹, C.J.L.M. Meijer¹, P.J.F. Snijders¹

¹VUMC, Amsterdam, The Netherlands, ²Paterson Institute Cancer Res., Manchester, UK

Cervical cancer development is characterized by a cascade of premalignant lesions, so called cervical intraepithelial neoplasia (CIN), graded I to III, the formation of which is initiated by an infection with high-risk HPV. A persistent HPV infection, although necessary, is however not sufficient for the development of cervical cancer. Progression from a high-grade CIN le-

sion to an invasive carcinoma is driven by genetic alterations triggering invasion. A putative tumor suppressor gene (TSG) is located on chromosome 11 as chromosome 11 suppresses tumorigenicity of the HPV16 positive cervical cancer cell line SiHa and LOH at 11q23 frequently occurs in invasive cervical carcinoma. A candidate TSG is the TSLC1 (tumor suppressor in lung cancer 1) gene, which is located at 11q23.2.

TSLC1 is a member of the immunoglobulin superfamily of cell adhesion molecules (IgCAMs) and has been shown to be involved in intracellular adhesion through homophilic trans-interaction.

We showed that TSLC1 mRNA expression is undetectable or severely downregulated in 91% (10/11) of cervical cancer cell lines, compared to non-tumorigenic HPV pre-immortal and immortalized cells and primary keratinocytes. Promoter hypermethylation appeared the major mode of TSLC1 silencing and TSLC1 expression was restored upon culturing in the presence of the demethylating agent 5-azacytidine. A mechanistic involvement of TSLC1 downregulation was supported by TSLC1's ability to suppress both anchorage independent growth and tumor growth of SiHa cells. Furthermore, TSLC1 promoter hypermethylation was detected in 58% of cervical carcinomas and 35% of high-grade CIN lesions, but not in low-grade CIN lesions and normal cervix. Interestingly, TSLC1 promoter hypermethylation could be detected in archival cervical smears of women with cervical cancer taken up to 7 years before cancer diagnosis.

In conclusion, these data show that TSLC1 silencing is an important and highly frequent event in the transition of hr-HPV containing high-grade CIN lesions to invasive cervical cancer. Hence, testing for TSLC1 silencing in cervical smears may provide a powerful tool to identify women having CIN lesions with invasive potential.

C.18

RAF/RAS MUTATION IN SEROUS BORDERLINE TUMORS AND MICRO-ARRAY BASED EXPRESSION PROFILES CLEARLY DISTINCT THIS GROUP FROM SEROUS CARCINOMA; IT IS TIME FOR A REAPPRAISAL

N.L.G. Sieben¹, P. Macropoulos², G.M.J.M. Roemen¹, S.M. Kolkman-Uljee³, G.J. Fleuren³, A.M. Flanagan²
¹University Hospital Maastricht, Maastricht, The Netherlands, ²University College London, London, UK, ³University Hospital Leiden, Leiden, The Netherlands

Genes of the RAF family, which mediate cellular responses to growth signals, encode kinases

that are regulated by RAS and participate in the RAS/RAF/MEK/ERK/MAP-kinase pathway. Activating mutations in BRAF recently have been found to occur in melanomas, colorectal cancers, thyroid and ovarian tumors. Here, we perform an extensive characterization of BRAF and KRAS mutations in 264 epithelial and non-epithelial ovarian neoplasms. The epithelial tumors ranged from adenomas and borderline neoplasms to invasive carcinomas including serous, mucinous, clear cell and endometrioid lesions. We show that BRAF mutations in ovarian tumors exclusively occur in low-grade serous neoplasms (33 of 91, 36%); these included serous borderline tumors (SBT), typical and micro papillary variants, an invasive micro-papillary carcinoma and a psammocarcinoma. KRAS mutations were identified in 26 of 91 low-grade serous tumors, 7 of 49 high-grade serous carcinomas (SCA), 2 of 6 mucinous adenomas, 22 of 28 MBT and 10 of 18 mucinous carcinomas. Of note, we found 2 SBT harbored both BRAF and KRAS mutations.

Based on these findings it's tempting to assume that SBT's and SCA's are separate genetic entities. To confirm this we assessed a micro-array based expression profiling on 43 samples (SBT's, SCA grade I and III) in order to identify signalling pathways regulated by key genes implicated in tumorigenesis. Hierarchical clustering analysis showed a clear genetic separation of the SBT's and the SCA's. At present, statistical analysis of the main genetic changes is performed using Spotfire software.

C.19 DETERMINATION OF HER2 GENE AMPLIFICATION STATUS IN BREAST TUMORS: COMPARISON OF FISH AND REAL-TIME QUANTITATIVE PCR

V. Ghislain, V. Vermeeren, C. Bourgain, M. Marichal, P. in 't Veld
AZ-VUB, Brussels, Belgium

Gene amplification/overexpression of the human epidermal growth factor receptor 2 (HER2, neu) is observed in 20–30% of primary breast carcinomas. Gene amplification/overexpression is a predictive marker of both overall patient survival and of susceptibility to treatment with monoclonal antibodies (herceptin) against the HER2 molecule. Recent data indicate that the determination of HER2 gene amplification by fluorescent *in situ* hybridisation (FISH) is a more reproducible expression. In view of the increased demand for molecular HER2 testing we investigated whether

real-time quantitative PCR (Q-PCR) is a reliable alternative molecular test for the determination of HER2 gene amplification in routine breast tumor biopsies as compared to FISH.

DNA samples from 25 frozen breast tumor biopsies were analysed with Q-PCR, using TaqMan technology. Both the HER2 gene and reference genes on chromosome 21 (APP) and chromosome 17 (ATPAF2) were quantified and their mutual ratios calculated. The ratios were quantitatively compared to the results obtained with FISH (Vysis-Pathvysion) and immunohistochemistry (CB-11) on paraffin-embedded material from the same biopsy.

A statistically significant ($p < 0.001$) correlation was found between the HER2/CEP17 ratio as determined by FISH and the HER2/APP and HER2/ATPAF2 ratios as determined by Q-PCR. Comparison on a per patient basis showed that Q-PCR resulted in a concordant result in 24/25 cases (96%) with a false negative result in 1/12 cases and in a false positive result in 0/13 cases when compared to the results obtained by FISH. It was observed that the Q-PCR results are affected by the choice of reference marker, with both the FISH and the Q-PCR results indicative of multiple chromosome abnormalities in the tumor cells.

It is concluded that real-time quantitative PCR leads to highly concordant results with FISH in determining the HER2 gene amplification status.

C.20 THE MOLECULAR DIAGNOSIS OF MAMMARY CANCER

M. Ardelean, A.S. Damian
Vasile Goldis Western University Arad, Arad, Romania

The method used within the genetic laboratories of the Faculty of Medicine is based on identifying the genetic mutations at BRCA1 and BRCA2 genes that are codifying proteins containing a tumour-suppressor function.

The DGGE and TGE systems are used in investigations on DNA by electrophoresis and kit method. Regarding the mammary cancer, the frequency of each mutation and the risk that each mutation implies are established using kit specially created for these tests by PCR reaction and also using primers that allow the amplification of the two genes involved in mammary cancer.

C.21 QUALITY ASSURANCE PROGRAMMES – A NEW MODALITY

R.A. Williams¹, J.M. Skinner², P. Hamilton³
¹St. Vincent Hospital, Melbourne, Australia, ²Flinders University, Adelaide, Australia, ³Queens University, Belfast, Northern Ireland

Participation in quality assurance programmes is mandatory to maintain laboratory accreditation. It is also possible through these programmes to provide an educational component.

Traditionally, quality assurance consisted of slides of cases with a purportedly definitive diagnosis and a summary of the collective results assessed against the 'right' answer. This requires cases from which multiple sections are cut showing the same features. The process assesses laboratory performance but de-emphasises individual's performance.

In an ideal educational programme there should be a focus aimed at the maintenance of standards. On the quality assurance side there is need for a well structured and effective testing program for individual pathologists.

The RCPA and the IPD from the Queens University, Belfast, are developing a quality assurance programme based on digitization of a tissue section on a single diagnostic slide circulated on DVD. In addition to the ad hoc examination of slides using a variety of criteria we have introduced a standardized approach to subjective slide examination using a system based on a Bayesian belief network (BBN). This enables pathologists to learn a new approach to tissue analysis and standardize approach to diagnosis. It allows individuals to test repeatability of approach to diagnosis and compare their diagnostic opinion with that of peers. The background has been reliably tested in the form of the CytoInform breast cytology module.

The combination of the QAP approach with the use of an educational tool reduces the pressure on the practitioner. The variability in output is a quantum leap forward in the production of a combined QA and continuing professional development. Advantages include efficient delivery of the programmes, maintenance of QA, education for the individual and, risk minimization. It minimises the psychological stress aspect of compliance with requirements of external QA, being seen as an opportunity for continuing professional development rather than as a punitive and competitive exercise.

C.22 CLASSIFICATION OF HEREDITARY AND SPORADIC BREAST CANCERS BASED ON CLINICOPATHOLOGICAL DATA

P. van der Groep¹, A. Bouter¹, R. van der Zanden¹,
 I. Siccama², F.H. Menko¹, H.J.P. Gille¹,
 R.J.A.M. Michalides³, C. van Kalken⁴,
 E. van der Wall¹, R.H.M. Verheijen¹, P.J. van Diest¹
¹VUmc, Amsterdam, The Netherlands, ²KiQ, Amsterdam, The Netherlands, ³Netherlands Cancer Institute, Amsterdam, The Netherlands, ⁴NDDO Research Foundation, Amsterdam, The Netherlands

Introduction: About 5% of all the breast cancer cases are due to an inherited predisposition by germ line mutations in the BRCA1/2 genes. Screening for BRCA mutation in suspected carriers is however difficult and mutations may well be missed, which hampers genetic counselling. Additional features pointing to hereditary breast cancer are therefore useful.

Methods: Age of presentation, tumour size, mitotic index (MAI) and the expression of Ki67, p53, p21, p27, cyclin D1, cyclin A, estrogen (ER) progesterone (PR), Epidermal Growth Factor (EGFR), and HER-2/neu receptors by immunohistochemistry were compared between 27 proven BRCA1/2 mutation carriers and over 500 unselected controls. In addition, test cases at intermediate risk of hereditary disease based on familial history were evaluated.

Results: Hereditary breast cancers showed higher MAI, and Ki67/p53/cyclin A indices, more frequent EGFR overexpression, and lower ER/PR compared to the controls. Based on a discriminant function including age, Ki67 and EGFR, 96% of the hereditary cases and 92% of the control cases were correctly classified. Most cases at intermediate risk of hereditary disease based on familial history could be classified with high probability as either hereditary or sporadic with this classification function.

Conclusion: Breast carcinomas can be classified as sporadic or as part of a hereditary syndrome with a high level of certainty using a discriminant function based on age, Ki67 and EGFR. This could be clinically useful to guide mutation analysis in families with borderline risk of hereditary disease.

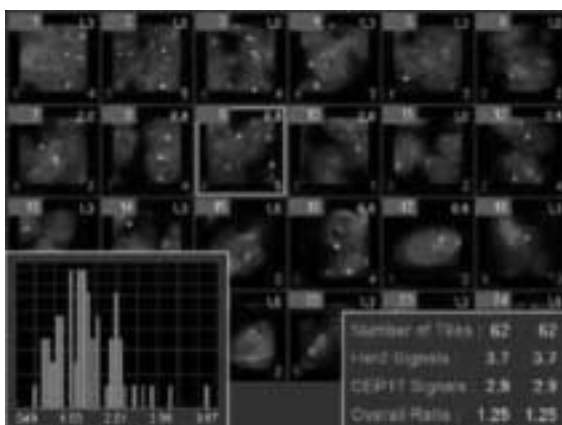
D.01 AUTOMATIC ANALYSIS OF FISH SIGNALS IN TISSUE SECTIONS

A. Plesch, Th. Lörch
MetaSystems GmbH, Altlußheim, Germany

Automation of microscopic analysis is highly desirable in numerous diagnostic applications. Thus the automatic scoring of FISH signals in interphase nuclei is of increasing importance for cancer prognosis and therapy selection. We developed an automatic slide scanning system named MetaCyte, which is based on the multipurpose scanning platform Metafer and has the capability to analyse FISH signals in up to 6 different colour channels. Its flexible architecture allows the adaptation to various assays like automatic FISH spot counting, gene amplification assays in tissue preparations, tissue microarray analyses or high content screening (HCS).

One of the most demanding tasks for the automatic analysis of slides in cancer diagnostics is the scoring of Her-2 amplification in paraffin-embedded tissue sections. Nevertheless, the certain conditions on samples prepared for this analysis bear difficulties for the automatic detection of cells and signals, namely:

- the difficult cell morphology, which makes reliable segmentation of individual cells impossible,
- highly variable signal quality and background, requiring sophisticated image treatment algorithms as well as automatic rejection if the quality is not suitable for automatic scoring,
- the occurrence of homogeneously stained regions (HSR), requiring adapted strategies of quantification beyond spot counting.



Result of gene amplification analysis in tissues.

Therefore strategies had to be developed, which cope with the specific problems of this assay. We will introduce a new method called 'Tile Sampling', which allows fast and reliable segmentation and detection of cells even in tissue sections. We will also present data about the achievable agreement between human and automatic scoring results for Her-2 amplification assays, proving the feasibility of automatic FISH signal analysis in tissue sections.

D.02 THE USE OF EXPRESSION MAPS IN THE ANALYSIS AND REPRESENTATION OF CHROMATIN PHENOTYPE BY TEXTURE ANALYSIS

C. Toland¹, P. Bartels², R. Montironi³, J. Diamond¹,
P. Hamilton¹

¹Queens University of Belfast, Belfast, Northern Ireland, ²The Optical Sciences Centre, University of Arizona, USA, ³Institute of Pathology, University of Ancona, Italy

Chromatin texture analysis for the study of chromatin phenotype in diagnostic and prognostic studies results in large multivariate, multidimensional datasets which are difficult to interpret. Most studies use multivariate statistics to reduce dimensionality. While this can be an effective approach for multivariate data interpretation, one can lose diagnostically valuable information. Methods such as signature analysis and expression maps can provide an alternative approach for the visualisation of complex data such as that seen in texture analysis.

A series of studies were carried out measuring chromatin phenotype in prostate neoplasia using digital texture analysis. A total of sixty texture features were calculated and classed into different groups based on their computational statistics. For each texture feature, a median for a population of nuclei may be calculated. This median value was used as a threshold to define whether a given lesion/diagnostic group median was positive (above) or negative (below) for a given feature. This was presented in the form of a binary expression map for each group where an array with features on the y axis and cells/cases/diagnostic groups on the x. White boxes represented positive values and black boxes, negative values. This allowed visualization of patterns in the data associated with changes across the entire feature set quantifying higher order chromatin remodelling. Alternatively, range expression maps may be used to illustrate a measure of disruption from a predefined normal or base range. Here distinct patterns associated with texture abnormalities (black boxes) can

again be visualised indicating deviation from the normal range.

Expression profiles applied to a series of studies in chromatin texture analyses made it possible to visualise the changes in the texture features simultaneously. Binary expression maps are more amenable to pattern recognition techniques such as artificial neural networks. This may allow a better approach to the classification of complex textural patterns associated with chromatin reorganisation in neoplasia.

D.03

QUANTITATIVE ASSESSMENT OF H&E STAINED BIOPSIES OF PRE-INVASIVE AND EARLY INVASIVE LUNG NEOPLASMS

A. Doudkine¹, M. Guillaud², J. LeRiche², Z. Chen¹, S. Granleese¹, B. Palcic², C. MacAulay²

¹*Cancer Prevention Centers Inc., Vancouver, Canada,*

²*British Columbia Cancer Agency, Vancouver, Canada*

Repeatable and objective grading of pre-cancerous and early cancerous lesions of the lung remains a serious challenge even for the most experienced pathologists. The root of the problem traditionally has been the lack of diagnostic material of this type and subsequent lack of experience and common knowledge associated with this task. In order to provide pathologists with instrument that can be used for more objective and repeatable grading, we developed an interactive computer system that employs image processing methods for quantitative assessment of H&E stained biopsies of pre-invasive and early invasive lung neoplasms. The system consists of a microscope, 3.3 mega-pixel color CCD camera, computer and a high resolution LCD tablet with a light pen interface. The system can be operated in clinical setting as it is designed to work with conventionally stained (H&E or Pap stain) biopsy samples. The pathologist examines the biopsy visually and then defines the region of interest (ROI) using light pen on the display. The system performs auto-focusing on every cell found within the ROI and calculates cell morphological features, tissue architecture information and textural features. Those features are combined to provide a single tissue score as well as cell morphology and tissue architecture scores. The results are then displayed in a form of scatter plots and histograms. The interface enables the operator to easily access a large set of reference images in a database linked to the results, to provide images and diagnosis of lung tissue in the database which are the most similar to the tissue imaged for the operator's consideration. Data analysis performed on more than 500 consensus-graded lung biopsies included in the database provided 70% to 95%

correct classification when discriminating between hyperplasia and low grades of dysplasia on one hand and high grades of dysplasia and CIS on another.

D.04

CELLULAR SOCIOLOGY FOR THE ANALYSIS OF MIB1 IMMUNOHISTOCHEMICAL STAINING OF BRONCHIAL BIOPSIES

M. Guillaud, N. Lam, S. Lam, C. MacAulay
BC Cancer Agency, Vancouver, Canada

A variety of molecular markers are under investigation as possible surrogate endpoint biomarkers as part of ongoing lung cancer chemoprevention trials. There are a number of qualitative scoring schemes used to evaluate immunohistochemical staining. We have evaluated a number of different quantitative methods (% positive cells, % positive cell in the basal layer, % positive cells in the supra basal layer, % positive cells in layer X as defined by voronoi tessellation, etc.) on a set of 329 MIB1 stained bronchial biopsies. Some of these are existing methods, others are novel which make use of quantitative tissue architectural tools. Currently, consensus histopathological interpretation of H & E stained sectioned biopsied material is the gold standard lung chemoprevention surrogate endpoint biomarker. Thus we have evaluated the different quantitative methods of MIB1 as a biomarker against the gold standard. A detailed description of the methods evaluated, their correlation with the gold standard and their strength and weakness will be presented.

D.05

A MORPHOMETRIC STUDY OF BONE MARROW ANGIOGENESIS IN MULTIPLE MYELOMA

N. Kavantzias¹, P. Korkolopoulou¹, K. Anargyrou², M. Kirtsonis², I. Thymara¹, E. Terpos², M. Dimopoulou², D. Boutsis², N. Viniou², E. Plata², E. Patsoutis¹, X. Yataganas², G. Pangalis²

¹*University of Athens Medical School, Piraeus, Greece,*

²*1st Dept. Internal Medicine, Athens, Greece*

Indisputable evidence has accumulated implicating bone marrow angiogenesis in the pathophysiology and course of various hematologic malignancies. In multiple myeloma (MM) angiogenesis has been associated with features of active disease and unfavorable prognosis. These studies, however, have focused on microvessel density (MVD) as the only factor reflecting angiogenesis, overlooking parameters related to the morphology of the microvessels that might be signif-

icant. In this study, we evaluated multiple morphometric characteristics of microvessels highlighted by means of F-VIII immunohistochemistry in the bone marrow of 68 patients with MM and 7 patients with MGUS, in relation to several clinicopathologic parameters and prognosis. Quantitation of microvascular parameters was performed in the region of most intense vascularization ('hot spot') by computerized image analysis. The mean follow-up period was 44.1 months. MVD was higher in MM than in MGUS ($p = 0.008$). Total vascular area (TVA) increased with paraprotein levels ($p = 0.031$), whereas low shape factor values (evidenced as flattened vessel sections) inversely correlated with $\beta 2$ -microglobulin ($p = 0.003$) and the degree of plasmacytosis ($p = 0.046$). Advanced age and stage, increased paraprotein and CRP levels, increased ESR, pronounced plasmacytosis, increased MVD ($p = 0.0036$), TVA ($p = 0.00314$) or branching values ($p = 0.0435$) and low shape factor values ($p = 0.0314$) adversely affected survival. Multivariate analysis (after factor analysis of morphometric variables) selected the degree of plasmacytosis and the combination of MVD and TVA as the independent predictors of survival. It is concluded that besides MVD, qualitative aspects of the microvessels are associated with unfavorable characteristics and the prognosis of patients with MM.

D.06 TOPOGRAPHY OF TELOMERES AND STRUCTURE OF CHROMOSOME TERRITORIES IN HUMAN LYMPHOCYTES

J. Amrichova¹, S. Kozubek², E. Lukasova²,
M. Kozubek¹

¹Faculty of Informatics, Masaryk University, Brno, Czech Republic, ²Institute of Biophysics, Brno, Czech Republic

Nuclear and territorial positioning of p- and q-telomeres and centromeres of chromosomes 3, 8, 9, 13 and 19 were studied by repeated fluorescence *in situ* hybridization, high-resolution image cytometry and three-dimensional image analysis in human blood lymphocytes before and after stimulation.

Our results suggest that in both G0- and stimulated lymphocytes, centromeres of HSA 3, 8, 9, and 13 are predominantly localized at the periphery of the cell nucleus; only centromere of HSA 19 in G0-lymphocytes is localized more centrally. Both p- and q-telomeres mostly adopt positions in the nuclear interior; q-telomeres are usually nearer to the center of the cell nucleus as compared to p-telomeres. Chromosome territories occupy different mean radial positions

depending on the average level of gene expression or gene density.

We found that chromosome territories are polar, with the centromere localized on one side of the territory and both telomeres on the other side. Chromosomes are polar independently of their positions inside cell nuclei. In addition, we found that the majority of the polar chromosome territories are oriented with the centromere localized near the nuclear periphery and both telomeres placed in the interior of the cell nucleus. Mutual distances between telomeres of submetacentric chromosomes were very short, usually shorter than centromere-to-telomere distances, which means that the chromosome territory is non-randomly folded.

Using repeated hybridization, tethering between telomeres of heterologous chromosomes 8, 9, and 19 was investigated. An attempt was made to find which particular telomeres are involved in telomere associations and whether any associations can be found also between homologous telomeres. We found no tethering of heterologous telomeres of chromosomes 8, 9, and 19. In contrary, both pairs of homologous telomeres of chromosome 19 (but not in other chromosomes) are tethered (associated) very frequently.

D.07 CHANGES IN GENE EXPRESSION INDUCED BY PML-RARA FUSION PROTEIN CORRELATE WITH THE DEGREE OF CHROMATIN COMPACTION*

M. Falk¹, E. Lukasova¹, S. Kozubek¹, G.I. Pellici²,
M. Faretta², M. Kozubek³, M. Rocchi⁴

¹Institute of Biophysics, ASCR, Brno, Czech Republic, ²European Institute of Oncology, Milano, Italy, ³Faculty of Informatics, MU Brno, Brno, Czech Republic, ⁴University of Bari, Bari, Italy

The reciprocal translocation between PML and RARa genes, associated with acute promyelocytic leukemia, results in disintegration of PML bodies and expression of the fusion protein PML/RARa in transformed cells. We have examined the relationship between transcription and large-scale chromatin structure using the gene expression microchip analysis and 3D-FISH in combination with high-resolution cytometry. Chromatin structure of three chromosomal regions Xq28-Xqter (up-regulated by PML/RARa), cen17-17q11.2 (small clusters of down-regulated genes) and 19p13.3 (down-regulated) were studied and compared between Zn-treated and untreated U937 PIR9 cells, stably transfected by the expression vector carry-

ing the PML/RAR α fusion gene under the control of Zn-inducible promoter. The degree of chromatin compaction was evaluated according to mutual 3D-distances between pairs of DNA probes separated by short molecular distances (1.5–4 Mbp); changes in chromatin compaction were determined after Zn-treatment of U937 PIR9 cells. The distances between probes in the up-regulated chromosomal region Xq28-Xqter were significantly elongated after the PML/RAR α induction. In contrast, the mutual distances between two probes of the down-regulated region 19p13.3 were shortened. The degree of chromatin condensation of the slightly down-regulated region 17q11.2 was very similar for control and Zn-treated cells. These results show the dynamic condensation/decondensation of chromatin in relation to the transcription level, for the first time determined in natural DNA sequences and directly measured as the distances between DNA probes. Our results also reveal the dependence of the changes of chromatin condensation on the radial position in the cell nucleus after induction of PML/RAR α chimera. The changes of chromatin condensation were significantly more pronounced in the nuclear interior contrary to the periphery. On the other hand, centre-to-locus nuclear distances of the above-described loci remained uninfluenced by Zn-treatment of PIR9 cells.

*This work was supported by the grants: GACR 202/01/0197, GAAS S5004010, IGA MH NC6987-3.

D.08 LARGE-SCALE ORGANIZATION OF THE HUMAN GENOME AND CHROMOSOMAL TERRITORIES IN THE NUCLEUS OF NORMAL AND CANCER CELLS AND ITS FUNCTIONAL IMPLICATIONS

S. Kozubek¹, E. Bartová¹, E. Lukasova¹,
M. Kozubek², J. Amrichova², R. Taslerova², M. Falk¹,
V. Ondrej¹

¹*Institute of Biophysics, ASCR, Brno, Czech Republic,*

²*Masaryk University, Brno, Czech Republic*

Knowledge of the large-scale chromatin arrangement and its effect on the genome function is still very limited. We have found that genetic elements (GE) such as genes, centromeres or chromosome territories (CTs) are localized at similar radial distances in both normal and cancer cells (chromosoma 111,321–331,2002). It demonstrates that the global structure of cell nuclei does not change dramatically during the cell transformation. Radial arrangements of GEs in cancer

cells are element-specific with central localization of the elements pertaining to highly expressed chromosomal regions.

Chimeric chromosomes that are typical of some leukaemia were found approximately midway between the nuclear positions of both original CTs (*Human Genet.* **112** (2003), 143–155). For example, in Ewing sarcoma cells, the radial positions of both of the fusion genes are shifted compared with the radial positions of the non-aberrant EWSR1 (CT 22) and FLI1 (CT 11) genes. The chromosome 11 fusion gene is shifted more centrally, whereas the chromosome 22 fusion gene lies towards the periphery.

The potential influence of heterochromatin on RB1 gene silencing was investigated in differentiated human retinoblastoma tumour cells with an (X;13) translocation (*J. Cancer. Res. Clin. Oncol.* **129** (2003), 89–99). Spreading or proximity of the heterochromatic and methylated chromosome X to the q arm of chromosome 13 was suspected to induce functional monosomy of the RB1 gene. It seems that the regulation of gene activity during carcinogenesis may be realized in some cases through heterochromatin-mediated gene silencing.

To find the influence of increased gene expression and amplification in colorectal carcinoma on the chromatin structure, the nuclear distances between two BAC clones with short genomic separation were measured and compared between the tumour and parallel epithelial cells. Larger nuclear distances were found for tumour as compared with epithelial cells for the same genomic separation. The ratios of the mentioned nuclear distances decreased with the degree of the amplification of genetic loci. Changes of the large-scale chromatin arrangement in cancer cells and their possible functional implications are discussed.

D.09 OBSERVATION OF COILED BODY – MICROCELL TRANSITION IN TUMOUR CELLS USING FLUORESCENCE RESONANCE ENERGY TRANSFER TECHNIQUE

T. Freivalds, I. Buikis, L. Harju

Latvian Inst. of Experimental Medicine, Riga, Latvia

Recently we named by term sporosis an ultrastructurally observed path of microcell formation from coiled bodies of damaged tumour cells (*Anal. Cell. Pathol.* **18** (1999), 73–85). The aim of this study was to demonstrate in fluorescence microscope the coiled body – microcell transition of damaged tumour cells.

Development of microcells were observed in human sarcoma line HT-1080 cells or in Jungarian hamster fibroblastoma cells induced with medium containing thiophosphamidum (10–20 mkg/ml) or vincristinum (2–5 ng/ml) for 24 h. Imprints and paraffin slides of Guerin carcinoma of rats, treated with cyclophosphamidum, human squamos carcinoma, irradiated with 28 Grey, and human breast cancer of patients, received neoadjuvant chemotherapy were also investigated. The cells were stained with protein stain – 8-anilino naphthalene-1-sulfonic acid NH₄-salt (ANS, donor chromophore, 5 mkg/ml) and nucleic acid stain – ethidium bromide (EB, acceptor chromophore, 3 μ g/ml). With both chromophore stained cells were excited with UV light 365 nm. In the case of closely spaced ANS and EB in cell structures fluorescence resonance energy transfer between ANS and EB occur expressing EB red fluorescence.

In such conditions the coiled bodies as proteinaceous structures expressed bright blue fluorescence, but intact and damaged macrocells showed dull red fluorescence. Coiled bodies as a rounded bright blue fluorescent objects were observed not only over macrocells, but also in necrotic debris and intercellular space. The coiled bodies progressively accumulate pink fluorescent material at first in a form of granules or globules, usually in one of the poles of coiled body. This process finished with confluence of separate granules and globules and transformation of blue fluorescent coiled body into pink fluorescent micronucleus. Micronuclei formed around themselves a bright blue cytoplasm. The fluorescent features of microcells may be a good distinctive mark of them. Coiled body – microcell transition of damaged tumour cells *in vitro* and *in vivo* occur in the same way. Fluorescence resonance energy transfer technique may be a useful method for observation of sporosis in tumour cells.

D.10 AN ASSESMENT OF CHROMATIN PHENOTYPE AND H3K9 ACETYLATION IN COLORECTAL CARCINOMA USING DIGITAL TEXTURE ANALYSIS

M.I. Hegab¹, J. Diamond¹, P. Hamilton¹, C. Toland¹, P. Maxwell²

¹*Institute of Pathology, QUB, Belfast, UK*, ²*Royal Group of Hospitals Trust, Belfast, UK*

Histone modifications are important in chromatin remodelling. The assessment of the acetylation status of lysine9 of core histone3 (AcH3K9) in normal and colonic cancer tissues may reflect alterations in histone

acetylation and consequently, chromatin remodelling. Our aim was to assess AcH3K9 immunocytochemistry of normal colon and colon cancer cases and to correlate these results with chromatin phenotype as measured using texture analysis.

An Ambion colon tissue microarray was used to study 52 adenocarcinoma cores and 6 normal colorectal mucosa cores. Colonic tumours were classified as well, moderately and poorly differentiated. Duke's stage was also recorded. Serial microarray sections were stained with H&E and with anti-AcH3K9 by immunocytochemistry. Visual and digital texture analysis was used to quantify chromatin phenotype and measure nuclear density and distribution of AcH3K9. Statistical analysis was carried out to determine differences in chromatin phenotype and H3K9 expression between normal and malignant colorectal epithelium.

Quantitative analysis showed hyperacetylation in adenocarcinoma. Higher grade and stage of cancer was associated with increasing acetylation although poorly differentiated adenocarcinoma exhibited hypacetylation. Chromatin density followed a similar increase with stage and grade where increased acetylation within the nuclei was associated with increased DNA content, however, direct correlations in distribution of chromatin and distribution of AcH3K9 were seldom observed. A discriminant function based on four texture features was able to correctly classify 98% of cores. Quantitative AcH3K9 immunoexpression and distribution was more distinct between normal and malignant colorectal cells. A discriminant function based on three texture features could distinguish 100% of tissue cores into benign/malignant groups. As AcH3K9 expression and chromatin pattern were not strongly associated AcH3K9 immunoexpression may act as an independent marker of malignancy in the colon.

The evaluation of acetylation immunoexpression and chromatin using texture analysis illustrates the role of histone modifications and chromatin remodelling in colorectal neoplasia. This may have implications in the study of histone deacetylases inhibitors in the treatment of colorectal cancer.

D.11 APPROXIMATE ENTROPY AS A TOOL FOR TEXTURE ANALYSIS OF NUCLEI*

K. Metzke, R.I. Adam, P.V.V.T. Silva, N.I. Leite
State University of Campinas, Campinas-SP, Brazil

Approximate entropy (ApE) is usually determined in time series where it provides information on the complexity of both deterministic and random processes. ApE measures the probability that runs of patterns

that are close to each other will remain close in the next incremental comparisons. The aim of our study was to investigate whether ApE could be used for texture analysis of chromatin. The 2D images of the segmented cells were transformed into 1D signals by peel-off-scanning. The pixel row was divided into blocks of 512, 1024 or 2048 pixels. The input variables were defined between 1 to 5 and the tolerance varied between 10 to 30 % (in steps of 5) of the standard deviation of the data sets. For each of these 75 combinations the ApE was calculated as the average value of the blocks per nucleus. We tested the ApEs in two different biological models:

1. ApEs were calculated from hematoxylin stained cytologic preparations of nuclei of cardiomyocytes of normal rats of 7 different ages (between 19 days of fetal age and 60 days post partum; 100 nuclei per rat; total of 90 rats). In all ApE combinations the Kruskal Wallis test indicated significant differences at least in one age group thus indicating the detection of differences of the chromatin pattern.
2. In a pilot study we calculated the ApEs of nuclear images of bronchial brush cytology, comparing 7 cases without neoplasia with 16 cases with neoplastic cells. Significant differences of ApEs between the groups could be detected and they were more pronounced when blocks of 2048 pixels or the whole pixel row were used.

Our investigation indicates that ApE may be useful for texture analysis of chromatin.

*Supported by CNPq, FAPESP.

D.12 NUCLEAR TEXTURE ANALYSIS BY GRANULOMETRIC RESIDUES*

K. Metze, R.I. Adam, P.V.V.T. Silva, N.I. Leite
State University of Campinas, Campinas-SP, Brazil

Granulometry describes quantitatively the coarseness of an image in a hierarchical way by means of a decomposition of its components. It obtains an ordered and complete description of an image from morphologic residues, which are defined as the differences between two granulometric levels. Granulometry has rarely been used for image analysis in medicine. The aim of our study was to investigate its application for the texture analysis of chromatin. From each granulometric level of gray scale images the residues were

extracted by progressive filtering (openings and geodesic reconstruction), using the gray level height of the basins as filter parameter. The number of residues and their mean area was registered for each level (between 1 to 128 gray levels). Two different biological models were investigated:

1. We analysed cytologic preparations of hematoxylin stained nuclei of cardiomyocytes of normal rats of 7 different ages (between 19 days of fetal age and 60 days post partum; 100 nuclei per rat; total of 90 rats). Comparing different age groups by the Kruskal–Wallis-test, we found that mean areas of residues of the levels 9 to 40 described differences according to the age. With increasing age larger residues could be found. The number of residues (levels between 12 to 57) was also varying with a greater number of residues in the younger ages.
2. In a pilot study we compared nuclear images of HE-stained bronchial brush cytology: 7 patients without neoplasia and 16 patients with neoplasias. Significant differences were found for the residues with levels <12 and for the mean areas with levels >9.

Therefore we conclude that the extraction of granulometric features may be useful for the texture analysis of chromatin.

*Supported by CNPq, FAPESP.

D.13 TEXTURE ANALYSIS OF ERYTHROBLASTS IN MYELODYSPLASTIC SYNDROMES*

K. Metze, E. Ribeiro, R.I. Adam, N.I. Leite,
I.G.H. Lorand-Metze
State University of Campinas, Campinas-SP, Brazil

Dyserythropoiesis is an important criterion for the diagnosis of myelodysplastic syndromes (MDS). Its evaluation is routinely done by subjective examination of May-Grünwald-Giemsa-stained bone marrow (BM) smears. In order to objectivate this diagnosis we studied the utility of several morphometric and granulometric parameters. Bone marrow smears of 25 MDS patients and 20 controls (patients from lymphoma staging) entered the study. Diagnosis of MDS was performed by two independent observers and was based on BM cytology as well as on cytogenetics and peripheral blood counts applying FAB criteria. Gray-scale transformed digitalized images of erythroblast nuclei

were analysed and the nuclear area, perimeter, longest chord and optical density determined. Granulometry was applied with the gray level height of the basins as filter parameter. The number of residues and their mean area was registered for each gray level. Area, longest chord, mean transversal diameter, as well as the mean optical density and its standard deviation discriminated well between erythroblasts of MDS and normal BM. Looking at the subtypes of MDS, we could not distinguish erythroblasts of patients with refractory anemia (RA) from erythroblasts of patients with refractory anemia with ring sideroblasts (RARS), but both were different from refractory anemia with excess of blasts (RAEB). Applying granulometric features similar results were obtained. Significant differences between the groups could be seen for nearly all mean area levels and for the lower levels of the number of residues. Granulometry was also not able to differentiate between erythroblasts of RA and RARS patients. In conclusion usual morphometric parameters are useful in order to distinguish between normal and MDS erythroblasts and between RA/RARS and RAEB. Granulometry does not provide additional information for this differential diagnosis.

*Supported by FAPESP and CNPq.

D.14 CELLULAR SOCIOLOGY OF CYCLING CELLS DURING COLON TUMOUR PROGRESSION: A VIRTUAL TMA (TISSUE-MICRO-ARRAY) APPROACH

F. Giroud¹, M. Julien¹, J. Simony-Lafontaine²,
M.-P. Montmasson¹, N. Lequeux²

¹Laboratoire TIMC-CNRS/UJF, La Tronche, Cedex,
France, ²CRLCC Val d'Aurelle, Montpellier, France

Objective: A virtual TMA approach is used to demonstrate the influence of sampling on the study of cellular sociology of cycling cells combined to AgNORs quantification during colon tumour progression.

Tissue data basis: 120 colon cancer cases were collected at the CRLCC (Montpellier, France) and documented by patient, clinical, biological and anatomopathological data. For each tissue-block 2 consecutive sections were prepared. The first one was HES (Hemalun–Eosine–Safranine) stained, scanned at low magnification, images were stored in data basis and annotated according to the colon cancer pathology ontology defined in the knowledge basis included in the TMA-Explorer project promoted in our group. One to

5 areas of special interest were delineated for each case and specifically annotated.

Image analysis: The second section was Ki67-AgNORs double-stained. Areas of interest were scanned at high magnification using the AcCell™ system (SAMBA technologies): 40 to 450 fields were acquired for each area. AgNORs were estimated by their relative nuclear area and proliferating cells were detected from Ki67 immuno-labelling. A total of 25 parameters was calculated.

Virtual TMAs: sampling simulations were made to build virtual TMAs in order to test the influence of core size and/or location on the various parameters and the corresponding interpretation.

Results: Among the 120 colon cases actually introduced in our TMA-Explorer data basis only 28 cases are selected for this preliminary study. The preliminary results show that only 6 major parameters are sufficient to explain 86% of the information to separate the different patient cases using principal component analysis. The first component (57%) is mainly represented by parameters from cellular sociology and the second one (21%) by parameters related to AgNORs. That demonstrates the major importance of cellular sociology of cycling cells to explain tumour cases differences and the complementary information bring by AgNORs quantification. Finally we illustrate the variations of parameters depending on the annotation type of the areas of interest, and the importance of core sampling (size and location).

D.15 ANALYSIS OF DNA METHYLATION STATUS OF CELL NUCLEI USING HIGH-RESOLUTION CYTOMETRY*

M. Skalnikova¹, E. Bártová², S. Kozubek²,
M. Kozubek²

¹FI, Masaryk University, Brno, Czech Republic, ²Institute of Biophysics, AS, Brno, Czech Republic

Epigenetic silencing in eukaryotes is associated with the formation of a transcriptional repressive higher-order chromatin structure. Differential methylation of CpG islands has been inversely correlated with gene activity: transcriptionally active genes were found to be hypomethylated, whereas transcriptionally silent genes were hypermethylated [4].

In our experiments, the detection of sub-nuclear localization of DNA methylated regions was performed. Using indirect immunofluorescence technique combined with FISH. A high-resolution cytometer was

used as a powerful tool for image acquisition of fluorescence images [2]. FISH was used to determine selected metaphase chromosomes HSA 1, 6, 10, 11, 18, 19, 20 and 22. Sequential 5-methylcytosin-rich regions were visualized on the selected chromosomes using immunofluorescent labelling in different cell types. We have found, for example, that the HSA 19, 20 and 22 involve heavy methylated regions. On the other hand, HSA 18, 10, 11 and 6 were found to be unmethylated in CpG islands in our experiments, which is in accordance with Barbin [1].

Another modification linked to silencing is methylation of histone H3 on lysine 9 (H3-K9). Domains of heterochromatin are characterised by methylation of H3-K9 and euchromatin is characterised by methylation of H3 on lysine 4 (H3-K4) [3].

Therefore, *in situ* immunofluorescence, was performed using antibody against H3-K4. We observed orientation of methyl groups of H3-K4 into the interchromatin space in the interphase nuclei of K-562 cells.

This work shows that immuno-FISH method provides a powerful tool for studying DNA methylation states at the chromosomal level in human cell types.

References

- [1] A. Barbin et al., *Human Genet.* **94** (1994), 684–692.
- [2] M. Kozubek et al., *Cytometry* **45** (2001), 1–12.
- [3] M.G. Mattei et al., *European Cytogenetics Association, Newsletter* **11** (2003), 3–13.
- [4] R. Singal and G.D. Ginder, *Blood* **93** (1999), 4059–4070.

*This work was supported by the Ministry of Education of the Czech Republic (Grant No. MSM-143300002) and partly also by the Academy of Sciences of the Czech Republic (Grant No. B 5004102).

D.16

“CHIPS AND CHEAP”: LOW RESOLUTION TISSUE ARRAY IMAGE ANALYSIS

N. Signolle¹, R. Françoise¹, N. Elie¹, J. Bosq², P. Herlin¹

¹Greccan, Caen, France, ²Dept. Pathologie Institute G. Roussy, Villejuif, France

Recently introduced, tissue array technique offers pathologists a promising tool for screening potentially prognostic significant molecules in oncology [1]. Nevertheless, slide lecture done visually remains time consuming. We developed a simple, fast and inexpensive method for acquiring a single image of the whole slide, taking advantage of a 4000 dpi slide scanner. Two fully automatic image processing strategies are proposed, allowing low resolution identification and analysis of in-

dividual chips. The two labelling methods were evaluated and compared with respect to rapidity and reliability.

Reference

- [1] O.P. Kallioniemi, U. Wagner, J. Kononen and G. Sauter, *Hum. Mol. Genet.* **10**(7) (2001), 657–662.

D.17

NUCLEAR TEXTURE ANALYSIS OF TWO DIFFERENT PROGNOSTIC CLASSES OF EARLY OVARIAN CANCER BASED ON GRAY LEVEL ENTROPY MATRICES

B. Nielsen¹, F. Albrechtsen¹, H.E. Danielsen²

¹University of Oslo, Oslo, Norway, ²Norwegian Radium Hospital, Oslo, Norway

The main aim of the present study has been to develop textural features with a potential prognostic value in tumour pathology. Features based on local entropy values were extracted from nucleus images from two different prognostic classes of early ovarian cancer. 134 cases of ovarian cancer classified as FIGO stage 1 were included in the analysis. 94 cases had a good prognosis, which means that they survived the follow-up period without a relapse. The 40 cases included in the bad prognosis group died of cancer-related disease or relapsed during the follow-up.

Gray Level Entropy Matrices (GLEM) were computed from each nucleus image. The GLEM element $P(i, j|W)$ contains the probability of having an entropy value j within a window of size $W \times W$ centered around a pixel having gray level value i . Based on the training set images, we computed a class distance and a class difference matrix. The class difference matrix contains the difference between the average GLEM of each of the classes. The class distance matrix contains information on which GLEM elements that will provide high class discrimination. The GLEM matrices were extracted separately from the peripheral and central parts of the nucleus images, as well as from the complete nucleus.

The class distance and class difference matrices between the two classes contained one to three distinct areas of consistently high values. There was a marked difference between the matrices computed from the peripheral and central parts of the nuclei. These matrices clearly illustrated the difference in chromatin texture between the two different prognostic classes. High local entropy values were more probable in nucleus images from the good prognosis class, while lower local entropy values were more probable in the bad prognosis class.

The classification performance of two new adaptive GLEM features was compared with nine earlier defined non-adaptive GLEM features. We found that a single adaptive feature contained most of the discriminatory power of the GLEM texture analysis method.

D.18 LOCAL BINARY PATTERN MATRIX – A NEW APPROACH TO NUCLEAR TEXTURE ANALYSIS

B. Nielsen¹, F. Albrechtsen¹, H.E. Danielsen²
¹*University of Oslo, Oslo, Norway*, ²*Norwegian Radium Hospital, Oslo, Norway*

In 1996 Ojala et al. proposed a binary version of the texture spectrum method of Wang and He. Each 3×3 pixels neighborhood in the gray level image is thresholded by the center pixel value. Putting binomial position weights on the eight binary pixel values, the weighted summation gives a unique local binary pattern (LBP) index to each such binary pattern. There are 256 possible LBP values within a 3×3 neighborhood.

We now introduce a local binary pattern matrix (LBPM). The contents of a sliding 3×3 window is thresholded by the center pixel value, and the eight binary values are mapped into an 8-bit pattern. A mapping of all 256 possible 8-bit patterns to the 30 possible rotation and mirroring invariant patterns is stored as a look-up-table. Finding the local binary pattern index over the whole image, we accumulate a normalized LBPM(i, j), giving the probability of finding a window having center pixel value i and LBP index j .

We have applied the method to about 37 700 nucleus images from two different prognostic classes of early ovarian cancer. 134 cases of ovarian cancer classified as FIGO stage 1 were included in the analysis. 94 cases had a good prognosis, which means that they survived the follow-up period without a relapse. The 40 cases included in the bad prognosis group died of cancer-related disease or relapsed during the follow-up. The LBP and LBPM histograms were extracted separately from the peripheral and central parts of the nucleus images, as well from the complete nucleus.

The nine most uniform LBP's contributed 89% of all observed patterns. The LBP histograms of the two classes was not sufficient to discriminate them. However, partitioning each nucleus into a peripheral and a central part, and utilizing the gray level in the LBPM representation, increased the discriminative information. Uniform patterns with high center pixel gray level values turned out to carry almost all of the discriminative information.

D.19 COMPLEXITY GRAYLEVEL MATRIX, A NEW TEXTURE ANALYSIS METHOD

S. Baheerathan¹, F. Albrechtsen¹, B. Nielsen¹,
H.E. Danielsen²
¹*University of Oslo, Oslo, Norway*, ²*Norwegian Radium Hospital, Oslo, Norway*

The complexity of a binary image has been defined in different ways, related to entropy, the number of connected components, and the total length of black-to-white boundaries. We have earlier proposed five texture features based on the complexity curve, i.e. the global number of black-to-white transitions for all possible graylevel threshold values.

The new Complexity Graylevel Matrix (CGM) describes the local binary complexity of an image as a function of the graylevel. Using a sliding 3×3 window, the number of black-to-white transitions within the window is computed, when the center pixel value is used as a threshold. The complexity value will vary from 0 for a homogeneous binary to 12 for a checkered pattern. Accumulating a normalized 2D CGM(i, j) over the whole graylevel image, we obtain the probability of finding a window having center pixel graylevel value i and local complexity value j within a given image.

Two adaptive features based on a Class Difference and a Class Distance Matrix are extracted from the Complexity Graylevel Matrix.

The prognostic value of the method is studied by applying the method to a pilot data set of 10 000 cell nuclei images from 40 early ovarian cancer patients with good and bad prognosis.

Segmenting the cell nuclei into peripheral and central parts, large class distances correspond to darker pixel values in the periphery than in the central segment. In the peripheral segments the good prognosis class will have a higher probability at high gray levels and the bad prognosis class dominates at slightly lower gray levels, while the opposite is true for the central segments. So the two segments should not be mixed.

The classification performance of the adaptive CGM features is compared with some other texture analysis methods like GLCM, GLRLM, Cooccurrence of graylevel runlength matrix and Complexity curve. The classification results, using the leave-one-out method, showed 80% correct classification rate. It is an increase of 15% compared to other methods.

D.20 DNA CONTENT MEASUREMENTS AFTER FISH TREATMENT

D.M. Chassoux¹, L.G. Linares-Cruz²
¹INSERM, Paris, France, ²AIPC, IFR St-Louis, Paris,
 France

We have carried out DNA content measurements on cultured human cells submitted to Mock-FISH treatments. Measurements were performed by quantitative fluorescence imaging as described in *Anal. Quant. Cytol. Histol.* **21** (1999), 489. FISH protocols were as in Visser et al., *Exp. Cell. Res.* **243** (1998), 398 or as in Solovei et al., *Exp. Cell. Res.* **276** (2002), 10 but DNA probes were not applied. Briefly, '3D preserved' fixed cells were denaturated at 72 or 75 degrees for 2 minutes in 50% Formamide, then incubated at 37 degrees for 48 hours, washed in 0.05 SSC at 37 degrees and stained with Hoechst. We observed that Hoechst fluorescence intensity was not altered after Mock-FISH treatments. Incorporated BrdU was revealed, indicating that nuclear DNA was indeed partially denaturated.

These observations imply that a direct assessment of ploidy level or cell cycle position (given by DNA content) and of chromosome territories organisation (given by FISH) on the same cell should be possible. A variety of cell types from normal or pathological samples have been adequately analyzed for cell cycle *in situ* by fluorescence imaging. Data regarding the arrangement of chromosome territories under these biological situations are being gathered.

D.21 CONFOCAL 3D DNA CYTOMETRY: IMPROVEMENT OF STAINING AND MEASUREMENT PROCEDURE

J.A.M. Beliën, L.S. Ploeger, A.Y. Abbaker,
 D. Cremers, M.A.M. Broeckaert, N.M. Poulin,
 P.J. van Diest
 VUMC, Amsterdam, The Netherlands

Background: Confocal Laser Scanning Microscopy (CLSM) presents the opportunity to perform 3D DNA content measurements on intact cells in thick histological sections and biopsies. So far, these measurements have been performed in a semi-automated fashion. Due to the manual inspection this is still quite time consuming task.

Methods: In this paper an improvement of staining and measurement procedure for automatic 3D CLSM image cytometry of nuclei in thick histological sec-

tions is presented. The suggested improvement will be used as set-up for a quality assurance and quality control procedure. To evaluate the improvements, we have measured DNA content and volume of human liver- and prostate cancer nuclei in 3D CLSM images.

Results: The throughput of the fully automated measurement procedure has been improved to approximately 200 nuclei per 5 minutes. However, manual inspection and correction is still required but is less time consuming due to improved initial automated segmentation of nuclei.

Preliminary analysis of DNA-histograms of human liver indicate that a coefficient of variation less than 10% is feasible.

Conclusions: The improved automated 3D CLSM image cytometry procedure enables measurement of volume and DNA content of large numbers of nuclei in thick histological sections within an acceptable time. This makes large scale studies feasible, whereby the advantages of CLSM can be fully exploited.

D.22 STUDY ON THE MORPHOLOGICAL ATYPIA IN THE THIRD STRUCTURE OF DNA OF COLORECTAL CARCINOMA *

H. Shen¹, Q. Chen²
¹The First Military Medical University, Guangzhou,
 China, ²Department of Epidemiology, Guangzhou,
 China

Aim: to discover the morphological features of DNA third structure of colorectal carcinoma cell.

Methods: Nuclei DNA was extracted from the cultured cell line (HR8348) first; Second, The extracted DNA was floated and unfolded on the surface of the solution of 0.05 M NH₄Ac (pH 7.5); Third, the unfolded DNA samples was stick to copper grids with Formvar membrane; Finally, observed DNA structure under the transmission electron microscope (TEM).

Results: (1) A lot of hairpin like structures could be found in some of DNA strain. Some of the hairpin like structures are longer, some are shorter; and, base loops could be seen in DNA hairpin like structures, some are obvious larger, some are smaller; (2) bulb like structures could be seen in DNA strains, which may be possibly formed from the dissolving of DNA double strain from each other; (3) there are a lot of ring like structure in DNA strains; (4) some of DNA structures looked like flower with petal like structure, and we called it as flower like structure, which consisted of ring like structures; (5) the phenomenon that DNA strains branched

could be seen under TEM, and this kind of structure of DNA strains was called as branch DNA in the paper.

Conclusions: The atypia of nuclei DNA third structure in morphology is existent in the colorectal carcinoma cells from cells line HR8348. The atypia of DNA third structure could be valuable to differentiate malignant tumors from benign injuries.

*This study was supported by National Natural Science Foundation of China NSFC (30271462).

D.23 NUCLEAR CHROMATIN PHENOTYPE IN PROSTATE CANCER IS ASSOCIATED WITH EPIGENETIC MODIFICATIONS IN ACETYLATION AND METHYLATION

J.A. Orr, P. Maxwell, J. Diamond, O. El Sharaf,
P. Hamilton

Queen's University Belfast, Belfast, Northern Ireland

Chromatin reorganisation associated with reversible epigenetic modifications is considered responsible for changes in gene expression observed during prostate cancer progression. The aim of this study was to identify alterations in chromatin distribution and global epigenetic status in prostate cancer.

A 76 core prostate cancer microarray (Ambion Europe UK) was used, consisting of adenocarcinoma samples at varying stages and grades and matched benign tissue cores. Sequential sections from the prostate microarray were stained with H&E and immunostained for acetylated histone lysine 9 (AcH3K9) and 5'-methylcytosine (5'Mec). Digital texture analysis was used to measure chromatin phenotype, and 5'Mec and AcH3K9 density and distribution within each of the tissue microarray cores.

Statistical analysis showed an increase in the density of chromatin in prostatic adenocarcinoma ($P < 0.001$). This was associated with global hypomethylation and global hyperacetylation in prostate cancer as measured using quantitative immunocytochemistry ($P < 0.001$). Numerous features measuring the distribution of nuclear chromatin/AcH3K9/methylation patterns were also significantly different in prostatic adenocarcinoma when compared to normal and showed monotonic progression with increasing Gleason grade. Discriminant analysis of normal and high Gleason grade prostate cancer allowed the definition of a classification function based on overall chromatin texture which provided 78% correct classification of nuclei and could be used to illustrate increasing disruption in chromatin phenotype with increasing grades

of prostate cancer. Comparison of chromatin texture, methylation and H3K9 acetylation status showed chromatin phenotype to be the strongest biomarker of malignancy. Chromatin phenotype, methylation and acetylation patterns showed subtle correlations indicating a relationship between global chromatin organisation and epigenetic profile.

Immunocytochemical reactions can be accurately quantified using digital texture analysis. A strong relationship exists between chromatin texture and epigenetic markers in prostate neoplasia. It is possible that epigenetic markers may provide additional biomarkers of diagnosis and prognosis and help to untangle the underlying mechanisms responsible for higher order chromatin phenotype alterations in malignancy.

D.24 BRCA1 INDUCED CHANGES IN CHROMATIN PHENOTYPE AS MEASURED USING TEXTURE ANALYSIS

C. Toland¹, P. Bartels², R. Montironi³, P. Harkin⁴,
J. Quinn⁴, P. Hamilton¹

¹*Queens University of Belfast, Belfast, Northern Ireland*, ²*The Optical Sciences Centre, University of Arizona, USA*, ³*Institute of Pathology, University of Ancona, Italy*, ⁴*Cancer Research Centre, Belfast, Northern Ireland*

Background: Changes in nuclear chromatin phenotype is likely to be related to specific alterations in the genotype. BRCA1 has been implicated in important cellular pathways including DNA repair and regulation of transcription and co-localises with Rad51 in nuclear foci in the S/G2 phases of the somatic cell cycle, indicating a shared interaction with a specific nuclear structure. The aim of this study was to examine if the induction of BRCA1 leads to conformational changes in chromatin phenotype as measured using texture analysis.

Materials and methods: The Tet-off gene expression system (BD Biosciences) was used to sequentially transfect and regulate a breast cancer cell line (MDA435) with a transactivator (MDA47) and BRCA1 (MBR62-BCL2). Tetracycline is used to block the transactivator, regulating the expression of the exogenous BRCA1 gene. Cell lines were grown in T75 flasks. MBR62-BCL2 was grown with and without tet and northern blotting was carried out to detect expression of exogenous BRCA1. Cells were fixed, paraffin embedded, sectioned, stained with H&E and images of cells lines were digitally recorded. Nuclei were seg-

mented and a series of 60 texture features calculated for each nucleus.

Results: Significant differences in chromatin pattern were seen for the MBR62 BCL2-tet cell line compared to the other cell lines, suggesting that BRCA1 induction causes specific chromatin pattern changes. BRCA1 induction caused a smoothing effect on the nucleus, and the chromatin was more spatially organised, with reduced chromatin clumping. A range expression map was set at the mean for the MDA435 nuclei plus and minus one standard deviation. The range expression map shows quite clearly the changes in all the texture features for the cell line due to BRCA1 induction.

Conclusions: Chromatin phenotype alterations in neoplasia can be influenced by the expression of specific genes such as BRCA1.

E.01 CHROMOSOMAL IMBALANCES IN COLORECTAL CANCER

E.I. Douglas¹, H. Fiegler¹, I.P.M. Tomlinson², N.P. Carter¹

¹The Sanger Institute, Cambridge, UK, ²Cancer Research UK, London, UK

Comparative genomic hybridisation (CGH) has been widely used for the genome-wide analysis of DNA copy number changes in tumours. However, conventional CGH has a limited resolution, of approximately 3 Mb, which can be improved by replacing the metaphase chromosomes as the hybridisation target with mapped sequences arrayed onto glass slides. The resolution is limited only by the insert size and density of the mapped sequences used.

The sequencing of the human genome has provided an ideal resource of mapped and sequenced "Golden Path" clones for the construction of arrays. We have utilised these clone resources to generate a genomic microarray consisting of large insert clones, which are spaced at approximately 1 Mb intervals across the human genome (~3500 clones). In addition, a set of human chromosome specific subtelomeric probes and 167 clones containing genes associated with cancer are included.

A collection of 49 colorectal cancer cell lines and 40 primary tumours was hybridised to our genomic microarray and recurrent regions of copy number change were identified. Gain of chromosome 20 was a widespread finding in both cell lines (50%) and primary tumours (65%). Other consistent regions of copy number gain included chromosome 13 (35% of cell lines, 40%

of tumours), chromosome 7 (25% of cell lines and tumours) and chromosome 8q (25% of cell lines, 30% of tumours). Common areas of copy number loss were found on chromosome 18q (25% of cell lines and tumours) and chromosome 8p (20% of cell lines and tumours). In addition to the broad regions of copy number change, discrete aberrations were detected. Recurrent deletions of small regions of chromosomes 4q, 5q, 9p, 10q and 20p were reported along with amplifications of sections of chromosomes 10q and 17q. Thus, consistent regions of copy number change have been identified by array CGH which are likely to contain genes important in the development and progression of cancer.

E.02 HETEROZYGOUS DELETIONS ON CHROMOSOME 7 IN SPLENIC MARGINAL ZONE LYMPHOMA MAPPED BY ARRAYCGH

K. Kok¹, K. Hillmann¹, P. van der Vlies¹, R. Boonstra², S. Poppema², A. Van den Berg², C.H.C.M. Buys³

¹University Hospital Groningen, Groningen, The Netherlands, ²Department of Pathology, Groningen, The Netherlands, ³Department of Medical Genetics, Groningen, The Netherlands

Splenic marginal zone lymphoma (SMZL) is a rare non-Hodgkin lymphoma that recently has been recognized as an entity. In general SMZL are characterized by primary involvement of the spleen and a very characteristic immunophenotype (IgM+, IgDdim, and CD5-, CD10-, CD23-). In a previous study we analysed the presence of chromosomal aberrations in this entity by cytogenetic analysis and comparative genomic hybridization (CGH). Loss of chromosome 7 was observed in 4 out of 13 cases. These findings indicate that deletions in chromosome 7q31-32 may be a characteristic feature of SMZL with primary splenic presentation and the typical IgM+, IgDdim, CD5-, CD10-, CD23- immunophenotype. In order to enhance the resolution of the deletion analysis, we are performing arrayCGH analysis on 13 cases of SMZL. An array was constructed consisting of DOP-PCR-amplified material from 340 chromosome-7-specific BACs with an average spacing of about 0.5 Mb, each spotted in quadruplicate. Three out of the ten cases that have been analysed thus far had large heterozygous deletions at 7q31-7q33. The overlap between the three deletions of 26 Mb, 17 Mb and 23 Mb, respectively, is 17 Mb.

E.03**GAIN OF CHROMOSOME 8Q23-24 IS A PREDICTIVE MARKER FOR LYMPH NODE POSITIVITY IN COLORECTAL CANCER**

M.G. Grade, B.M. Ghadimi, T. Liersch, C. Langer, A. Siemer, L. Füzesi, H. Becker

University Medical Center, Goettingen, Germany

The prognosis of patients with colorectal cancer is largely determined by the tumor stage, and especially lymph node metastasis indicate a bad prognosis. At the time of diagnosis, approximately 60% of colorectal cancers have already spread into the lymph nodes. In this respect, rectal cancers might benefit from a pre-operative radiochemotherapy which is widely used in Germany and France. Accordingly, there is considerable clinical interest to find specific chromosomal regions that can be used as predictive biomarkers for a pretherapeutic molecular staging and an individual risk estimation.

To investigate the genomic differences in colorectal cancer progression, we used comparative genomic hybridization (CGH) to screen the DNA of 50 patients with different stages of colorectal cancers: small tumors without lymph node metastasis (group 1: T2, N0; $n = 15$), large non-metastasizing tumors (group 2: T3/4, NO; $n = 15$) and large tumors with lymph node metastasis (group 3: T3/4, N1/2; $n = 20$). The statistical analysis was done by pairwise comparisons and by using the Fisher's exact test. Differences with a P -value < 0.05 were considered statistically significant.

The lymph node positive tumors showed the highest degree of chromosomal instability, which is reflected by an ANCA of 5.7 in group 1, 7.5 in group 2 and 9.8 in group 3. Commonly described markers of advanced colorectal cancers such as gains of 20q and losses of 18q21 were not statistically different. The major finding was the frequent gain of chromosome 8q23-24 in the vast majority of lymph node positive cancers, whereas it was rather rare in lymph node negative tumors with the same depth of infiltration ($p = 0.0016$).

This study reveals that the 8q23-24 locus might possibly pinpoint to relevant target genes. It could be speculated whether the detection of gains of 8q23-24 by using interphase DNA-probes or DNA-chip technology might predict lymph node positivity. Such molecular approaches could improve the pretherapeutic staging which is mandatory for an individual cancer treatment such as neoadjuvant radiochemotherapy.

E.04**COMPREHENSIVE CHARACTERIZATION OF CELL LINES AND LARYNGEAL CARCINOMAS CONTAINING GENOMIC AMPLIFICATIONS IN THE CHROMOSOME 11Q13 REGION BY USE OF HIGH DENSITY ARRAY CGH**

J.H. Gibcus¹, J.E. van der Wal¹, M. Hermsen², E.F. Schoenmakers³, J.A. Veltman³, R.P. Takes⁴, K. Kok¹, E. Schuurin¹

¹Groningen University MC, Groningen, The Netherlands, ²Free University MC, Amsterdam, The Netherlands, ³Nijmegen University MC, Nijmegen, The Netherlands, ⁴LUMC, Leiden, The Netherlands

Laryngeal cancer is preferably treated with radiotherapy in order to preserve the larynx and vocal cords. Although highly curable when diagnosed at early stage, radiotherapy of advanced (T3-T4) laryngeal carcinomas fails in local control in up to 50% of patients, making salvage laryngectomy necessary. At present no appropriate predictive tumor marker is available. Amplification of the 11q13 region is found in several malignancies, with the highest frequency in head/neck carcinomas (36%). Amplification in head/neck cancer correlates with decreased disease-free-survival and is associated with poor prognosis. Several overexpressed genes situated within the amplified 11q13 region including CCND1 (cyclin D1), myeov, EMS1 (cortactin) and TAOS1. Therefore, these genes are likely candidates to serve as a prognosticator for laryngeal carcinoma.

We have developed a CGH-matrix microarray containing ± 600 overlapping BACs PACs and cosmids covering the whole 11q13 and neighboring regions in an overlapping fashion to determine the relative copy number of all markers/genes. Furthermore, we have added ± 100 control BACs to confirm hybridization quality. Due to the high density design of this array we are able to generate a map of the 11q13 region identifying copy number changes as small as duplications within the smallest overlapping region (± 100 kb).

Using this array we identified 3 different amplification patterns existing in ovarian, breast and head and neck cell lines. An amplicon of approximately 3 Mb covering the region near the CCND1 gene could be identified in head and neck cell lines as well as in patient material. Furthermore, we have identified an amplicon at the telomeric side of 11q13 and a previously identified amplicon harboring the cIAP gene at 11q22. The latter two amplicons were both present in head and neck cell lines.

E.05 CHROMOSOMAL CHANGES IN SECONDARY MANIFESTATIONS OF BREAST CANCER

K. Friedrich¹, T. Weber¹, J. Scheithauer¹, W. Meyer¹,
Z. Kirsnerova¹, G. Haroske², K.D. Kunze¹,
G. Baretton¹

¹University Hospital TU Dresden, Dresden, Germany,

²General Hospital, Dresden-Friedrichstadt, Germany

The capability to metastasize or recur is a crucial feature of malignant tumors. The purpose of this study was to determine genetic differences between primary breast cancers with and without progression and between primary tumors and their corresponding metastases or local recurrences by CGH.

Eighty primary tumors with and without secondary manifestations, twenty lymph node metastases, ten systemic metastases and ten local recurrences were examined by CGH.

The primary tumors with lymph node metastases showed more frequent losses at 9p21–p23 than the node negative tumors. The lymph node metastases displayed more frequent chromosomal losses, whereas the primary tumors exhibited more frequent chromosomal gains. The lymph node metastases exhibited more frequent losses at 7q32, 14q24–q31 and gains at 8q23 than their corresponding primary tumors, whereas primary tumors showed more frequent losses at 2q13, 2q31–q32, 6p25, 9pter–p23 and 18p than the lymph node metastases. In the cases with systemic metastases losses at 5q23 were more frequent than in the cases without metastases, whereas in the cases without metastases gains at 16p and losses at Xp21 were more frequent than in cases with metastases. The metastases displayed more frequent losses at 5p, 8p21–p23, 11p14–p15, 12q24, 17p13 and 17q22–q24 than the corresponding primary tumors. The primary tumors with local recurrences showed more frequent losses at 18q12–q21 than tumors without local recurrence. However, the comparison of primary tumors and the local recurrences revealed no additional imbalances in the local recurrences.

The comparison of primary tumors showed that losses at 9p and 5q are associated with the development of metastases, whereas losses at 18q were associated with a higher risk for local recurrences. A lot of additional changes were detected in the genome of metastases, it remains unclear in all CGH studies whether they are features of a small 'metastazing' cell clone in the primary tumor or whether the imbalances in the metastases are newly acquired. This question may be answered by interphase cytogenetics.

E.06 GENOME-WIDE ARRAY COMPARATIVE GENOMIC HYBRIDISATION ANALYSIS OF PREMALIGNANT LESIONS OF THE STOMACH

M.M. Weiss¹, E.J. Kuipers², C. Postma³, A. Snijders⁴,
M. Stolte⁵, M. Vieth⁶, D. Pinkel⁴, S.G.M. Meuwissen³,
D.G. Albertson⁴, G.A. Meijer³

¹Molecular Genetics Laboratory, Leiden, The Netherlands, ²Erasmus University MC, Rotterdam, The Netherlands, ³VUMC, Amsterdam, The Netherlands, ⁴UCSF Cancer Center, San Francisco, USA, ⁵Klinikum Bayreuth, Bayreuth, Germany, ⁶University Magdeburg, Magdeburg, Germany

Background: Gastric cancer is one of the most frequent malignancies in the world, ranking fifth in The Netherlands as cause of cancer death. Surgery is the only possible curative treatment for advanced cases, but results of gastrectomy largely depend on the stage of the disease. A better understanding of the mechanisms leading to the progression from a pre-neoplastic condition through intraepithelial neoplasia to invasive cancer may provide new insights that are relevant for designing focused prevention strategies.

Methods: Since knowledge of the pattern of chromosomal aberrations in precursors of gastric cancer is limited, eleven gastric polyps with intraepithelial neoplasia (three hyperplastic polyps, and 8 adenomatous polyps) were analysed by microarray CGH to study the presence of chromosomal instability in precursors of gastric cancer.

Results: Chromosomal aberrations were detected in all three gastric hyperplastic polyps and eight adenomas. Adenomas did not show more chromosomal aberrations than the hyperplastic polyps with intraepithelial neoplasia. The most frequent aberrations in the adenomas were gain of 7q36 and 20q12, and losses of 5q14–q21. In the hyperplastic polyps the most frequent chromosomal changes were loss of 15q11–14, 1p21–31, and 21q11–21.2. The most frequent chromosomal aberration that both types of polyps had in common was loss of 9p21.3.

Discussion: Hyperplastic polyps in this study showed many chromosomal aberrations underlining the fact that neoplastic transformation can occur in these lesions. The observations in this study are consistent with the existence of two morphologically and genetically different pathways to gastric cancer, i.e. the hyperplastic polyp pathway and the (intestinal type) adenoma pathway. The relative contribution of each

of these pathways to gastric carcinogenesis in general, and how they compare to patterns of chromosomal aberrations in the more prevalent flat foci of intraepithelial neoplasia remains to be determined.

E.07

A FULL-COVERAGE, HIGH-RESOLUTION, HUMAN CHROMOSOME 8Q GENOMIC MICROARRAY FOR COMPARATIVE GENOMIC HYBRIDIZATION OF PROSTATE CANCER

M. van Duin¹, R. van Marion¹, J.E.V. Watson², A. Lapuk², N. Brown², D.G. Albertson², D. Pinkel², P.J. de Jong³, C. Collins², H. van Dekken⁴

¹Erasmus MC, Rotterdam, The Netherlands, ²Comprehensive Cancer Center, San Francisco, USA, ³Children's Hospital (CHORI), Oakland, USA, ⁴Erasmus Medical Center, Rotterdam, The Netherlands

Prostate cancer represents a very commonly diagnosed malignancy in European and in North-American males. The behaviour of prostate cancer is variable and it is important to discriminate between indolent and progressive disease. Therefore, markers capable of assessing the malignant potential of prostate cancer are required. Genetic alterations have been identified which are associated with aggressive behaviour of prostate cancer. These alterations include increased copy number of the long arm of chromosome 8 (8q). Therefore, a high-resolution contig array was developed specific for array comparative genomic hybridization analysis of chromosome 8q. A minimal tiling path contig of 702 8q-specific BAC clones was generated using a novel computational tool (BAC Contig Assembler). BAC clones were amplified by DOP-PCR and subsequently printed onto glass slides. For validation of the array DNA samples of various cancer specimens were used, which were previously characterized by multicolor FISH and conventional CGH. Both single and double copy gains were confidently demonstrated using the 8q array. Single copy loss and high-level amplifications were accurately detected. In both fresh and archival samples additional alterations were disclosed. In comparison with conventional CGH the resolution of the detected changes has much improved, which is illustrated by an amplicon of 718 kb and a deletion of 554 kb, both spanned by only 6 BAC clones. In a series of prostatic adenocarcinomas including fresh-frozen and archival specimens ($n = 17$), xenografts ($n = 9$) and cell lines ($n = 3$) 5 regions on 8q were found to be frequently amplified, including the c-myc region. Subsequently, we will perform

FISH analysis using the BAC clones identified by array CGH on a tissue microarray containing both progressive and non-progressive prostate adenocarcinomas with a long-term followup.

E.08

ANALYSIS OF CRITICAL REGIONS ON CHROMOSOME 1, 3 AND 8, ASSOCIATED WITH CLINICAL ENDPOINTS IN UVEAL MELANOMA, USING DNA-CHIP TECHNOLOGY

W. van Gils, M.M.P.J. Verbiest, E. Kilit, G.P.M. Luyten, H.T. Brüggewirth, A. de Klein
Erasmus MC, Rotterdam, The Netherlands

Cytogenetic studies in Uveal Melanomas (UM) revealed several non-random chromosomal aberrations, such as loss of chromosome 1 and 3 or gain of 8q or extra copies of chromosome 8. Mutual loss of chromosomes 1p and 3 is a strong predictor of poor prognosis. Amplification of chromosome 8q is also correlated with poor prognosis. By using micro array technology we want to identify genes located on these chromosomes which can be used to distinguish between high or low risk patients. DNA and RNA isolated from UM will be analyzed on genomic Bacterial Artificial Chromosome (BAC) arrays and cDNA expression arrays respectively to identify certain tumor-related genes. In this way, we can make a correlation between gains and losses on chromosomes and changes in gene expression and obtain more precise information on partial deletions of chromosome 1 and 3 and partial amplifications of chromosome 8q, in relation with prognosis.

E.09

CHROMOSOMAL CHANGES IN RELATION WITH CLINICAL OUTCOME IN LARYNX AND PHARYNX SQUAMOUS CELL CARCINOMA

M.A.J.A. Hermsen¹, M.M. Alonso Guervas¹, G.A. Meijer², P.J. Van Diest², C. Alvarez Marcos³, A. Sampedro¹

¹University of Oviedo, Oviedo, Spain, ²VUMC, Amsterdam, The Netherlands, ³Hospital Valle del Nalón, Oviedo, Spain

Chromosomal gains and losses in 34 larynx and 22 pharynx primary untreated carcinomas were investigated with comparative genomic hybridization (CGH) and results were related to a series of clinical parameters and follow-up information. Tumours

varied from stage I–IV. Twenty-six cases were lymph node positive. Eighteen out of 50 cases measured by flow cytometry were DNA diploid and 32 were DNA aneuploid. The average follow-up time was 25.5 months.

CGH analysis on average detected 11.1 gains and 8.5 losses. The most frequent gain were at chromosome arms 3q, 7q, 8q, 5p, 11q13, 17q and 18p, and losses at 3p, 11qter, 4p, 18q, and 5q. The segments most frequently showing high level amplification were 3q26-qter, 11q13, 11q22, 3q12-13, 18p11.3, and 18q11.2, and occurred more frequently in aneuploid cases than in diploid cases. No differences in chromosome abnormalities were found between the larynx and pharynx cases.

No specific chromosomal abnormality correlated with clinical outcome. Amplification at 11q13 occurred only in lymph node positive cases, all of which were stage IV tumors. However, although tumor stage and lymph node status are strongly correlated with poor clinical outcome, amplification 11q13 did not, possibly due to the low number of cases. These results seem to indicate that in advanced larynx and pharynx squamous cell carcinomas, chromosomal aberrations may not be useful as prognosticators for clinical outcome.

E.10

A CHARACTERISTIC PATTERN OF CHROMOSOMAL CHANGES IN FLAT ADENOMAS AND CARCINOMAS OF THE LARGE INTESTINE RESEMBLES THAT OF A SUBGROUP OF POLYPOID COLORECTAL TUMOURS

C. Postma¹, M. Hermsen¹, J. Coffa¹, J.P.A. Baak¹, J.D. Mueller², E. Mueller², B. Bethke³, J. Schouten⁴, M. Stolte³, G.A. Meijer⁵

¹VUMC, Amsterdam, The Netherlands, ²Baystate Medical Center, Springfield, USA, ³Klinikum Bayreuth, Bayreuth, Germany, ⁴MRC Holland, Amsterdam, The Netherlands, ⁵VUMC, Amsterdam, The Netherlands

Controversy exists on the pathogenesis of colorectal carcinomas. While according to the classic adenoma-carcinoma sequence all carcinomas arise from adenomatous polyps, recent observations have demonstrated the existence of a flat adenoma to carcinoma pathway. These flat adenomas are flat or slightly elevated lesions, mostly with tubular architecture. Compared with polypoid adenomas of similar size, flat adenomas show

more frequently high-grade dysplasia and rapid submucosal invasion.

The aim of this study was to study whether flat colorectal lesions differ in their pattern of chromosomal aberrations from their polypoid counterparts.

Formalin-fixed paraffin embedded tissue samples of 6 flat adenomas and 12 flat carcinomas were analysed by comparative genomic hybridisation (CGH). Their pattern of chromosomal aberrations was compared to those earlier obtained in a series of 112 polypoid adenomas and 82 polypoid carcinomas. In addition, multiplex ligation-dependent probe amplification (MLPA), for a series of genes on 20q, was done on the flat tumours.

With CGH, flat adenomas showed on average 1.8 gains (range 1–4) and 3.2 losses (range 0–4), and the flat carcinomas 4.5 gains (range 0–8) and 3.5 losses (range 1–6). In both adenomas and carcinomas a high frequency of 20q gain (83% and 92%, respectively) and 18q loss (83% and 92%, respectively) were found, which resemble the pattern of chromosomal aberrations as was earlier observed in a subgroup of polypoid colorectal tumours. In further analysis of chromosome 20q with MLPA, the following genes had a ratio higher than 2.5: BCL2L1, HCK, TOP1, MYBL2, STK6, EEF1A2, TNFRSF6B and FLJ20517, which are spread over the whole 20q arm.

We conclude from the CGH results that the pattern of chromosomal instability in flat colorectal adenomas and carcinomas is marked by a high prevalence of 18q loss and 20q gain, resembling one of the distinct patterns of chromosomal changes found in polypoid colorectal adenomas and carcinomas.

E.11

IDENTIFICATION OF MULTIPLE GENETIC ALTERATIONS ON CHROMOSOME 3P IN ORAL TUMORS BY HIGH RESOLUTION ARRAY CGH

C.A. Garnis¹, C. Baldwin¹, B. Coe¹, L. Zhang², M. Rosin¹, W. Lam¹

¹BC Cancer Agency, Vancouver, Canada, ²University of British Columbia, Vancouver, Canada

Each year, there are approximately 300,000 cases of oral squamous cell carcinoma (SCC) world-wide. Most cases are not diagnosed or treated until the advanced stages of disease. As a result, patients have a poor prognosis with a five-year survival rate of just 50%. However, if diagnosed and treated in the early stages, prognosis and survival rates will increase. Identification of

gene alterations in oral cancer will provide novel targets for diagnosis and treatment.

Chromosome 3p is implicated to harbor multiple tumor suppressor genes in a variety of cancers including renal, breast, uterine cervical, endometrial, ovarian, testicular, lung, and in particular, oral SCC. Although loss of heterozygosity (LOH) studies have shown regions with high frequency of alteration on 3p in oral SCC, this approach is not designed to distinguish amplifications from deletions. Furthermore, LOH analysis is limited to the availability of microsatellite markers. Here, we present a new approach to fine-map these alterations to identify novel genes.

Objective: The objective of this study is to develop high-resolution array CGH technology for chromosome 3p-copy number profiles for oral tumors.

Methods: In this study we constructed a BAC CGH array comprised of 539 BAC clones spanning the 3p arm from 3p12.3 to 3p26.3. Tumor and normal DNA were extracted from oral tumor specimens and surrounding connective tissue, labelled with cyanine-dyes, then co-hybridized onto 3p BAC array, and scanned for each dye to determine the hybridization signal intensity ratios for each loci. The data was plotted to reveal amplifications and deletions across the entire 3p arm.

Results: 20 oral tumors were profiled using BAC array CGH of the 3p arm. Four regions of frequent alterations were identified: amplifications at 3p14.2–21.2 and 3p21.31–21.33, and deletions at 3p14.1–14.2 and 3p22.3–23.

Conclusion: The high resolution BAC array CGH technology detects alterations on 3p. Alignment of alterations from multiple patients defines minimal regions, which potentially harbor tumor suppressor genes and oncogenes.

E.12

HIGH RESOLUTION ARRAY CGH DETECTS RECURRENT REGIONS OF AMPLIFICATION ON CHROMOSOME ARM 1P IN SMALL CELL LUNG CANCER

L.J. Henderson¹, B.P. Coe¹, J.D. Minna², S. Lam¹, C. MacAulay¹, W.I. Lam¹

¹BC Cancer Research Centre, Vancouver, BC, Canada, ²U of T Southwestern Med. Ctr., Dallas, USA

Introduction: Many previous studies have indicated the presence of one or more cancer-related genes on chromosome arm 1p. Current methods for mapping genetic alterations in cancer, including loss of heterozygosity, comparative genomic hybridization (CGH), and fluorescence *in situ* hybridization, lack the resolution

to identify these genes. The recent advance of array CGH (aCGH) technology has improved the resolution and accuracy of alteration mapping. The objective of this project was to construct a high resolution array of BAC clones from chromosome arm 1p, and to apply this tool to identification of candidate genes on 1p in lung cancer through the use of array CGH.

Methods: A physical map of chromosome 1p was determined by combining data from publicly available sequenced-based and BAC fingerprint-based human genome maps. Human BAC clones representing 1p were selected from this map. The DNA was isolated, and clone identity was verified. The DNA was amplified using a linker-mediated PCR protocol, and amplified DNA was spotted onto amine-coated glass slides. For aCGH experiments, DNA isolated from 13 small cell lung cancer (SCLC) cell lines was labelled with cyanine-5 dCTP, and was co-hybridized to the array with normal male reference DNA labelled with cyanine-3 dCTP. Arrays were imaged using a Arraywrx CCD-based system, and analyzed using Softwrx software (Applied Precision).

Results: 666 clones were selected to represent 1p, resulting in a resolution of approximately 5 BACs per megabase. Array hybridization detected two distinct regions that were recurrently amplified in at least six of the cell lines.

Conclusion: Using aCGH, we have detected two regions of amplification in 13 SCLC cell lines. This indicates the presence of multiple cancer-related genes on this chromosome arm. Further investigation using clinical samples and of expression levels of candidate genes will be necessary to identify the genes important to SCLC.

E.13

SUB-MEGABASE RESOLUTION ARRAY CGH PROFILING OF CHROMOSOME ARMS 3P AND 5P IN LUNG CANCER

B.P. Coe¹, C. Garnis¹, A. Ishkanian¹, J. Minna², S. Lam¹, M. Tsao³, C. MacAulay¹, W. Lam¹

¹BC Cancer Research Centre, Vancouver, Canada,

²UT Southwestern Medical Center, Dallas, Canada,

³Ontario Cancer Institute, Toronto, Canada

Background: Several regions on 3p have been reported to contain tumour suppressor genes and oncogenes. Additionally amplification of regions on chromosome arm 5p have been observed in all histological subtypes of lung cancer. Array-based comparative genomic hybridization (aCGH), which permits the

screening of DNA samples for deletions and amplifications of chromosomal regions at a resolution 10–100 times greater than that of conventional CGH methodologies, will allow the rapid identification of the genes frequently amplified on these arms.

Objectives: To construct and utilize high resolution array CGH analysis of chromosome arms 3p and 5p to fine map breakpoints and detect small deletions and amplifications that are not detectable by conventional CGH in lung cancer.

Design: A minimal overlapping tiling set of >600 fingerprint verified BAC clones spanning the 3p arm and >500 fingerprint verified BAC clones spanning the 5p arm were selected from FPC contig maps and the UCSC golden path in order to create a minimal overlapping tiling set of PCR amplified BAC clones at approximately 0.1 megabase resolution across these arms. These tiling sets have allowed us to produce a BAC CGH Array for copy number analysis of DNA from lung cancer cell lines and tumours from patients. Sample DNA was labeled with cyanine 5 through a random priming reaction and co-hybridized with a cyanine 3 labeled reference DNA to the arrays. Post-hybridization the arrays were scanned using a CCD based imaging system from Applied Precision. Signal ratios were then determined using the Softworx array analysis program.

Results: We have delineated breakpoints in lung cancer cells lines to within one BAC clone in single experiments. Additionally we have identified micro-amplifications that have not been detected by conventional methodologies. This has allowed us to rapidly identify candidate genes in lung cancer tumorigenesis.

Conclusions: The p arms of chromosomes 3 and 5 contain multiple tumour suppressors and oncogenes relevant to lung cancer. Array CGH has facilitated fine mapping of the regions containing these genes.

E.14

HIGH RESOLUTION MAPPING OF COPY NUMBER CHANGES INVOLVING THE LONG ARM OF CHROMOSOME 6 IN GASTRIC CANCER

B.P.M. Carvalho¹, A. Koning², P. van der Vlies², F. Carneiro³, C.H.C.M. Buys², R. Seruca³, K. Kok²
¹VUMC, Amsterdam, The Netherlands, ²University Hospital Groningen, Groningen, The Netherlands, ³IPATIMUP, Porto, Portugal

Copy number changes of the long arm of chromosome 6 occur frequently in gastric carcinoma. We

have constructed a chromosome 6-specific BAC-array to analyse these copy number changes in more detail. The array consists of 336 BACs, all sequence-linked to the human genome. Of these, 200 BACs map to the long arm of chromosome 6. Taking into account the total length of all BACs, 23% of chromosome 6 is covered with an average distance between the BACs of 400 kb. All BACs have been spotted in quadruplicate. The array is complemented with a collection of 600 BACs, representing genomic regions for 200 cancer-related genes. With this array we have thus far analysed 3 gastric cancer derived cell lines, 5 gastric cancer derived xenografts, and 10 primary tumours. High-level amplifications were detected in 2 cell lines. In each case the amplification could also be detected in the corresponding xenograft. The 50-fold amplification of the EGFR gene region that was detected in one of the cell lines, has been confirmed by Southern analysis, and was shown by immuno-histochemistry to correlate with a high level of EGFR expression. Copy number gains and losses of parts of the long arm of chromosome 6 were detected in all gastric cancer-derived samples. The data clearly demonstrate the power of these array-CGH analyses, but more tumour samples need to be analysed to define the hotspot(s) of the chromosome-6 specific changes.

E.15

HIGH RESOLUTION MICROARRAY-CGH ANALYSIS USING SPOTTED OLIGONUCLEOTIDES

B.P.M. Carvalho, E. Ouwerkerk, G.A. Meijer, B. Ylstra
 VUMC, Amsterdam, The Netherlands

Array-CGH is nowadays the method of choice for studying DNA copy number changes. So far, either amplified representations of BACs/PACs or cDNAs have been spotted as probes. Here, we propose to use spotted oligonucleotides, recently used in expression profiling, as probes for array-CGH. We show that oligo-CGH is able to detect amplifications with high accuracy and greater spatial resolution, compared to other current array-CGH platforms. In addition, single copy-number changes can be detected. Oligos are easy to handle and flexible, since they can be designed for any part of the genome without the need for laborious amplification procedures. The availability of a full-genome array (around 30,000 clones) would represent a big step forward in the analysis of DNA copy number changes. Moreover, this technique can be easily applied to any organism for which the full-genome sequence is known.

E.16
GENOME-WIDE-ARRAY-BASED
COMPARATIVE GENOMIC HYBRIDIZATION
REVEALS GENETIC HOMOGENEITY AND
FREQUENT COPY NUMBER INCREASES
ENCOMPASSING CCNE1 IN FALLOPIAN
TUBE CARCINOMA

J.C. Dorsman¹, A.M. Snijders², M.E. Nowee³,
 J. Fridlyand², J.M. Piek³, A.N. Jain², D. Pinkel⁴,
 P.J. van Diest⁵, R.H. Verheijen³, D.G. Albertson²
¹VUMC, Amsterdam, The Netherlands, ²Cancer Re-
 search Institute UCSF, San Francisco, USA, ³Gynaeco-
 logical Oncology, VUMC, Amsterdam, The Nether-
 lands, ⁴Laboratory Medicine UCSF, San Francisco,
 USA, ⁵Pathology, VUMC, Amsterdam, The Nether-
 lands

Fallopian tube carcinoma (FTC) is a rare, poorly studied and aggressive cancer, associated with poor survival. Tumorigenesis is related to the acquisition of genetic changes and corresponding changes in gene expression. In order to obtain a better understanding of FTC carcinogenesis and to identify potential targets for therapy, we used genome-wide array comparative genomic hybridization to analyse DNA copy number aberrations in FTC. We used arrays of 2464 genomic clones, providing approximately 1.4 Mb resolution across the genome, to map genomic DNA copy number aberrations quantitatively from 14 FTCs onto the human genome sequence. All tumors showed a high frequency of copy number aberrations with recurrent gains on 3q, 6p, 7q, 8q, 12p, 17q, 19 and 20q, and losses involving chromosomes 4, 5q, 8p, 16q, 17p, 18q and X. Recurrent regions of amplification included 1p34, 8p11-q11, 8q24, 12p, 17p13, 17q12-q21, 19p13, 19q12-q13 and 19q13. Candidate, known oncogenes mapping to these amplicons included CMYC (8q24), CCNE1 (19q12-q21) and AKT2 (19q13). The FTCs were remarkably homogeneous. Some aberrations (8p, 19q and X) occurred in more than 70% of samples, suggesting a stereotyped pattern of tumor evolution. A comparison study of DNA copy number profiles between FTCs and matched ovarian carcinomas is in progress.

E.17
DNA COPY NUMBER CHANGES AT 8Q23-24
IN METASTASIZED COLORECTAL CANCER

T.E. Buffart¹, J. Coffa¹, M.A.J.A. Hermsen¹,
 J.P. Schouten², G.A. Meijer¹
¹VUMC, Amsterdam, The Netherlands, ²MRC-Holland,
 Amsterdam, The Netherlands

Background: C-myc, a well known oncogene located on 8q24.1, is often amplified and overexpressed in both primary and metastasizing colorectal cancer. In addition, PRL-3 (also known as PTP4A3), a tyrosine phosphatase located on 8q24.3, is amplified in colorectal cancer metastasis. Beside PRL-3 and c-myc, other oncogenes located on the 8q23-24 region might be involved in this process. Therefore, the present study aims to analyse DNA copy number changes at 8q23-24 in colorectal cancer at high resolution in correlation to metastatic disease.

Materials and methods: 35 cases of colorectal cancer (12 of which with liver metastasis) were included in this study. DNA from the primary colorectal tumor and the corresponding liver metastasis (when present) was isolated. A chromosome 8 specific MLPA probe mixture was used to analyze the presence of DNA copy number changes. The probe mixture contained 30 probes covering a number of genes on chromosome 8, as well as 10 control probes on other chromosomes.

Results and discussion: No differences in DNA copy number between Dukes B1 and B2 cancers were observed. When comparing these with Dukes D tumors, several genes differed significantly (GATA4 ($p = 0.033$) and CTSB ($p = 0.002$) on 8p, MOS ($p = 0.032$), TPD52 ($p = 0.002$), EIF3S6 ($p = 0.002$), MYC ($p = 0.004$), SLA ($p = 0.000$) and PTP4A3 ($p = 0.004$) on 8q). When comparing primary tumors and their corresponding liver metastases, approximately the same pattern of gains and losses was observed. However, most metastases showed higher levels of amplifications than primary tumors.

Conclusion: In addition to c-myc, several other amplified genes on 8q are associated with liver metastasis in colorectal cancer.

E.18
SCREENING FOR LARGE GENOMIC
DELETIONS OR DUPLICATIONS IN GENES
INVOLVED IN HEREDITARY CANCER BY
MLPA

G. Pals¹, J. Schouten², F.B.L. Hogervorst³,
 P.N. Nederlof³, J.J.P. Gille¹, F.H. Menko¹,
 N. Ameziane¹, A. Errani²
¹VUMC, Amsterdam, The Netherlands, ²MRC-Holland,
 Amsterdam, The Netherlands, ³NKI, Amsterdam, The
 Netherlands

The majority of the families seeking advice at a family cancer clinic are those with familial breast/ovarian cancer and colon cancer. Often they are offered muta-

tion analysis of the genes involved, which is usually restricted to the analysis of PCR fragments from genomic DNA. However, a wide variety of different exon deletions and duplications in the BRCA1, MLH1 and MSH2 genes have been described. Here, we present a simple and reliable method to detect all possible single or multiple exon deletions and duplications in these genes. The method, called multiplex ligation-dependent probe amplification (MLPA), is PCR based and allows the relative quantification of many different sequences in a single tube. In a collaborative study, we have tested more than eight hundred individuals in which routine DNA-diagnostic analysis did not reveal a mutation in either BRCA1 or BRCA2. This study identified 5 different mutations in this gene among which are a deletion of exon 8 and duplications of exon 21–23 and 17–19 that are not reported before. Furthermore, all identified mutations were found only once, suggesting that there is no additional high frequency founder mutation like the genomic deletions of exons 13 or 22. Also in the MLH1 and MSH2 genes single and multi exon deletions were found with MLPA, including a deletion of the entire MLH1 gene that had been missed previously by Southern blotting. The MLPA test proved to be a very efficient and reliable test, which can easily be implemented in the DNA-diagnostic laboratory. Furthermore, it provides a means to screen all counselees for large genomic deletions/duplications and thus improves the diagnostic mutation screening.

E.19

SMALL UNIT OF HUMAN DNA POLYMERASE EPSILON AND BREAST CANCER

Y.U.I. Collan¹, Q. Zhou¹, R. Effati¹, K. Talvinen¹, H. Pospiech², J.E. Syväoja²

¹University of Turku, Turku, Finland, ²BioCenter Oulu, Oulu, Finland

24 breast cancer DNAs and 4 normal tissue DNAs (lymph node, placenta) were studied for genomic DNA changes in the gene of the small subunit of human DNA polymerase epsilon. The technique included PCR amplification with 19 primer pairs covering all coding regions, single strand conformation polymorphism electrophoresis with a Pharmacia ALF Sequencing Machine, and sequencing with the same instrument. All abnormal samples in SSCP were sequenced. The sequencing results were compared with the sequencing data from chromosomal BAC clones and POLE2 sequencing data from HeLa cells. When

compared with the BAC clone data there were clearly fewer sequence abnormalities than when comparison was made with HeLa cell DNA data. The following conclusions are based on comparisons with BAC clone sequence database. SSCP showed abnormalities with 11/19 primer pairs. In two breast cancer cases a TTAA deletion was found in intron 18, similar deletions were not found in control DNAs. In addition, single base transitions were found in intron 14, but the same changes could be found in control DNA, suggesting polymorphism. The results suggest that specific changes can occur in the small unit gene of DNA polymerase epsilon in breast cancer. This mutation will not affect the protein code, but can influence splicing efficiency and expression levels, possibly impairing function of pol epsilon.

E.20

THE EVENTS OF MITOTIC CATASTROPHE ARE CAUSED AFTER GENOTOXIC STRESS IN LYMPHOID TUMOUR CELLS BY ACTIVATION OF MOS/MAPK PATHWAY

J. Erenpreisa¹, A. Ivanov^{1,2}, M.S. Cragg³, T.M. Illidge²
¹Latvian University Biomedical Research and Study Center, Riga, Latvia, ²Cancer Research UK Wessex Oncology Unit, UK, ³Tenovus Research Laboratory, Southampton, UK

In radioresistant p53 mutated tumours, cells following genotoxic stress enter the post-G2-arrest mitosis, which fails and is transiently by-passed by endoreduplication accompanied by delayed apoptosis. We studied this process, termed mitotic catastrophe, on three lymphoid tumour cell lines, with different resistance to gamma-irradiation and different TP53 status. Quantification of the arrest in a spindle checkpoint, the main event of mitotic catastrophe, revealed that it was paradoxically the largest in the most resistant cell-line and did not correlate with the amount of chromosome breaks in the cell-lines. Therefore, we decided to study the Mos/MAPK signalling pathway which may be responsible for this arrest. It turned out that translational upregulation of mos, phosphorylation of MAPK1/2, and prevention of degradation and nuclear export of cyclin B1 (all characteristic of the cytostatic action of mos), coincided in time and amount with the arrest in a spindle checkpoint. From that point, mitotic cycle is transiently abrogated and transformed into endocycle. Although also transcribed in the radio-sensitive wild-type p53 cell line, mos was not translationally activated and the events of mitotic catastrophe did not oc-

cur in it. Inhibition of the MAPK kinase by UO126 did not impact the non-irradiated cells, however retained most of the irradiated cells in the G2 arrest, abolishing endoreduplication and delayed apoptosis. Our findings show that mitotic catastrophe in lymphoid tumours is caused after genotoxic damage by upregulation of Mos/MAPK pathway, which is licensed by deficiency of p53 function and induced from the G2/M DNA damage checkpoint.

F.01

STUDY OF C-MYC ONCOGENE IN HEPATOCELLULAR CARCINOMA

S.M. Abdel Salam², N.A. Moustafa¹, A.F. Amer², A.F. Amira¹, M.F. El Moufty², A. Aziz³, S.A. Zaki⁴
¹Departments of Pathology, ²Microbiology & Immunology, ³Clinical Oncology, ⁴Internal Medicine, Faculty of Medicine, Alexandria University, Alexandria, Egypt

Structural alterations in c-myc oncogene, copy number and expression have been implicated in the pathogenesis and progression of several human neoplastic diseases. However, the biologic significance of c-myc gene in human hepatocellular carcinoma (HCC) is unconfirmed. In the present study we correlated c-myc gene amplification and protein expression with the clinicopathologic features in 20 HCC cases c-myc amplification in tumour tissue was determined using a differential PCR, a procedure for the evaluation of gene amplification in comparison with a dopamine D2 receptor gene. The c-myc gene was amplified in 6 out of 20 tumour specimens (30%). Amplification of c-myc was more frequent in younger male patients with HBV infection and in less differentiated tumours. All cases demonstrated positive staining using anti-c-myc monoclonal antibody with increasing percentage of immunoreactive cells in less differentiated tumours. However, the high protein expression was not statistically correlated with c-myc amplification.

F.02

DETECTION OF FURIN OVEREXPRESSION IN OVARIAN TUMOR TISSUE ARRAYS

A.J.P. Klein-Szanto, R. Lopez De Cicco, R.E. Page, D.E. Bassi
 Fox Chase Cancer Center, Philadelphia, USA

Furin is a serine protease member of the family of proprotein-convertases (PC's) that catalyzes the hydrolytic cleavage at the C-terminus of the motif RXR/KR. Many inactive proteins, such as metallopro-

teinases, growth factors and growth factor receptors, contain this motif and become activated after furin-dependent proteolysis. The immunohistochemical and molecular detection of this proprotein convertase has been described previously (e.g., Bassi et al., *Am. J. Pathol.* **162** (2003), 439–447). Furin overexpression has been demonstrated in human lung, gastrointestinal and head and neck tumors.

We investigated furin expression in human primary ovarian tumors and in 14 cell lines (nine ovarian carcinoma cell lines and five derived from normal ovarian surface epithelium or OSE). Tissue micro-arrays were constructed with 80 surgical specimens of primary ovarian carcinomas using two 1 mm diameter cores per tumor sample, together with 10 normal ovarian samples containing OSE and several other normal human tissues designed to occupy the peripheral rows and columns. Furin was detected immunohistochemically with the furin-specific monoclonal antibody MON-152 using an ABC kit. Semi-quantitative analysis of furin expression in ovarian tissues demonstrated cytoplasmic staining in normal ovarian OSE, borderline lesions and low-grade carcinomas. The highest levels of furin expression were observed in the high-grade carcinomas. To confirm these results, proteins were extracted from representative frozen primary tumors and furin levels were evaluated by Western analysis. These levels correlated well with those found by immunohistochemistry. Four colon carcinomas that expressed high levels of furin were used as positive control.

All tumor cell lines studied showed expression of furin. Interestingly, the cell lines derived from normal OSE showed negligible levels of this protease. The aggressive tumor cell lines showed increased processing of furin substrates such as insulin-like growth factor receptor, IGF-1R and membrane-type metalloproteinases. This enhanced processing resulted in increased proliferation and invasive ability. Taken together, these results suggest that furin may be a suitable tumor marker as well as a possible target for therapeutics.

F.03

BONE MARROW LYMPHOCYTE SUBSETS IN MYELODYSPLASTIC SYNDROMES*

K. Metze, E. Ribeiro, C.S.P. Lima, I.G.H. Lorand-Metze
 State University of Campinas, Campinas-SP, Brazil

In myelodysplastic syndromes (MDS) quantitatively and functionally abnormal lymphocyte subpopulations

can be found, which are believed to modify both proliferation and apoptosis of hemopoietic cells. We analysed the bone marrow (BM) lymphoid subsets in 42 newly diagnosed MDS patients by flow cytometry, paying attention to their relation to the CD34+ precursors, the expression of CD95 (Fas) and the peripheral blood counts. Normal BM from 11 allogeneic BM transplantation donors was taken as control. There was no correlation between BM lymphocyte and peripheral blood counts. In MDS and normal BM the total number of BM lymphocytes was similar. In MDS, B lymphocytes (CD19+), however, were decreased and T (CD3+) lymphocytes increased, with an inverse correlation between them ($r = -0.75$). The number of NK cells (CD16+) was elevated. There was no correlation between the number of CD 34/CD95 cells and the total number of lymphocytes. A direct correlation between the ratio of CD4/CD8 cells ($r = 0.64$) and total number of lymphocytes as well as the B lymphocytes ($r = 0.44$) could be detected. NK cells showed an inverse correlation with total lymphocytes ($r = -0.74$) and with CD8+ cells ($r = -0.56$). An inverse correlation was also found between the total number of lymphocytes and that of CD34+ cells ($r = -0.37$) and between the percentage of BM blasts and CD95/CD34 positive cells. In a linear stepwise regression we achieved the following equation: $\text{Log}(\text{number of blasts}) = -0.59 \log \text{BM lymphocytes} - 0.90 \log \% \text{CD34/CD95} + 5.8$ ($R^2 = 0.36$). Our study suggests that an increase of the number of blasts in MDS is accompanied by a decrease of lymphocytes and the apoptosis rate of CD34 cells. Thus, immunologic mechanisms may be important for the increased apoptosis and the inhibition of blast proliferation in MDS.

*Supported by FAPESP and CNPq.

F.04

TOWARDS AN UNDERSTANDING OF SMALL INTESTINAL ADENOCARCINOMAS – A COMPARISON OF ITS MOLECULAR PATHOLOGY TO AMPULLARY AND PANCREATIC CANCER

K. Maude¹, C.S. Verbeke², P. Quirke², P. Howdle³, M.I. McMahon⁴

¹Leeds Teaching Hospitals, Leeds, UK, ²Histopathology Department, Leeds, UK, ³Department of Gastroenterology, Leeds, UK, ⁴Academic Unit of Surgery, Leeds, UK

Introduction: Small bowel adenocarcinoma is rare and little is known about the carcinogenesis process

involved. Studies have suggested that the molecular profile of ampullary tumours is divided into 2 groups, those more similar to intestinal tumours and those of a 'pancreaticobiliary' type. Are pancreatic, ampullary and small intestinal adenocarcinomas distinct biological entities with different molecular pathology dependent on site of origin? An extensive molecular study on a larger series is required to improve our understanding.

Method: Suitable cases of primary small bowel adenocarcinoma were identified from the BSG (British Society of Gastroenterologists) National Survey (June 1998–May 2000). The histopathology records of Leeds NHS Trust (1980–2002) identified suitable cases of pancreatic, ampullary and primary small bowel adenocarcinoma. Clinical information and survival data was provided by case note review and questionnaires retrieved from the BSG Survey. Based on this information and stringent histological review only primary small bowel adenocarcinomas were included. All specimens were graded and staged according to the UICC (Union Internationale Contre le Cancer) TNM Classification (1997). TMA (tissue microarray) technology was employed to allow high-throughput immunohistochemistry. Formalin fixed paraffin embedded tumour and control tissues were collected and each case was represented by 3 tissue cores. Monoclonal antibodies for tumour suppressor genes p53, p16, and SMAD4, mismatch repair genes hMLH-1, hMSH-2 and the APC/ β -Catenin pathway were used.

Results: 64 cases of pancreatic, 32 ampullary, and 64 small bowel adenocarcinoma identified from histopathology records and 100 cases of primary small bowel adenocarcinoma from the BSG survey were collected. Tumour anatomic distribution comprised 10 periampullary, 47 duodenal, 33 jejunal, 38 ileum and 36 indeterminate small intestine. The profile of each cancer will be examined in terms of frequency and type of molecular changes. All findings will be correlated with clinicopathological parameters.

Conclusion: This unique large series will allow analysis and identification of the molecular relationships between pancreatic, ampullary and small bowel adenocarcinomas. Comparisons between tumours will elucidate any shared common molecular mechanisms of carcinogenesis.

F.05
STUDY OF THE AMPLIFICATION
MECHANISM LEADING TO NON-SYNTENIC
CO-AMPLIFICATION OF ATBF1 AND MYC IN
AN EARLY NEURAL CREST DERIVED CELL
LINE SJNB-12

N. van Roy¹, J. Vandesompele¹, E. de Smet¹,
 K. Swerts¹, J. Philippé¹, A. de Paepé¹, S. Pahlman²,
 F. Speleman¹

¹Ghent University Hospital, Gent, Belgium, ²University of Lund, Malmö, Sweden

One of the possible consequences of genomic instability in human tumors is oncogene amplification. Oncogene amplification usually leads to overexpression of one or more target genes located within the amplicons and is typically associated with an aggressive tumor phenotype. In many instances, extra-chromosomally double minutes or intrachromosomally homogeneously staining regions are the cytogenetic hallmark of this amplification process. Despite the fact that oncogene amplification is a typical characteristic of many tumor types, the mechanisms controlling amplicon formation have remained largely unresolved. In this study, we present the finding of non-syntenic co-amplification of MYC and ATBF1 in neuroblastoma cell line SJNB-12 that coincides with a translocation t(8;16) with breakpoints close to the known chromosomal position of both co-amplified genes, i.e. 8q24 and 16q22.3, respectively. In order to study the role of this particular translocation as a trigger for the amplification process, we studied the genomic content of the amplicon and the position of the translocation breakpoints. Our results are in keeping with the occurrence of excision and deletion of breakpoint flanking sequences and subsequent amplicon formation leading to non-syntenic co-amplification of MYC and ATBF1. As both MYC and ATBF1 amplification have not been previously reported in neuroblastoma we also investigated the possible origin of this cell line in further detail. Combination of immunocytochemical, flow cytometric and gene expression profiling data provided evidence for an early neural crest/sympathetic progenitor derived origin for cell line SJNB-12.

F.06
ASSESSMENT OF CELL CYCLE
CHECKPOINTS IN PLEURAL MALIGNANT
MESOTHELIOMA (PMM): CORRELATION TO
SV40 AND PROGNOSIS

A.-R.M. Zekri, A.A. Bahnassy, R.M. Gaafar,
 F.M. Aboul-Kasim, S. El-Gerzawi, A. Rafeat
 National Cancer Institute, Cairo, Egypt

Pleural malignant mesothelioma (PMM) is a highly aggressive tumor without a satisfactory curative modality. Recently, an increased incidence of PMM has been reported in many countries. Knowing the role of the cell cycle checkpoints in PMM may help in predicting patients' prognosis and developing effective treatment strategies.

We investigated 20 cases of morphologically and immunophenotypically confirmed PMM for the presence of SV40-DNA sequences (by PCR), DNA content and S phase fraction (by flowcytometry) and for p53, Rb, p27kip1, p21waf, p16INK4 and NF2 gene status (by IHC and molecular techniques).

SV40-DNA was detected in 70% of the cases, 4/20 cases (20%) were aneuploid and 50% had high SPF (?10%). Loss of p27kip1, p21waf and Rb expression was reported in 65%, 55% and 50% of the cases respectively. p53 overexpression was present in 65%, SSCP revealed aberrant conformers in 4 cases however, sequence analysis showed no p53 mutation. Abnormal gene status of p16INK4 and NF2 genes was reported in 50% and 75 % of cases respectively. Statistical analysis revealed a significant correlation between p27kip1, p21waf expression and the overall survival (OS). The expression of both proteins accurately identified patients with prolonged OS (? the median; 12m).

Conclusion: Although p53, Rb, p27kip1, p21waf, p16INK4 and NF2 genes are involved in the pathogenetic cascade of PMM, only p27kip1, p21waf and high SPF represent reliable predictive factors and useful prognostic markers to identify patients with relatively favorable prognosis.

F.07
MULTI TISSUE ARRAYS – A TOOL IN
PROGNOSTICATION OF BREAST CANCER

K. Friedrich, K. Stief, A. Kochan, W. Meyer,
 M. Kotzsch, T. Luther, K.D. Kunze, G. Baretton
 University Hospital TU Dresden, Dresden, Germany

The search for markers with prognostic value for the clinical outcome in breast cancer requires an expensive and time consuming analysis of a high number of cases. Multi tissue arrays are a tool for high throughput examination of up to 500 tumor samples in one paraffin block, increasing the efficacy and decreasing the cost of analyses.

The aim of this study was the evaluation of the prognostic relevance of cell cycle and proliferation associated markers in breast cancer by multi tissue arrays.

Cores of 390 breast cancers with known clinical outcome were arrayed into five multi-tissue arrays and were studied for Ki-67, p53, Cyclin A and HER-2/neu expression by immunohistochemistry and by FISH for HER-2/neu gene copy number changes. The statistical analyses were performed by Cox regression and Kaplan Meier statistics.

The analysis of all cases revealed the polysomy of chromosome 17, the lymph node stage and the Ki-67 expression as independent markers for clinical outcome. The tumors were stratified into subgroups by clinico-pathological features. In pT1 tumors, HER-2/neu aberrations were a significant marker for outcome. In well differentiated tumors, aneusomy of chromosome 17 and/or HER-2/neu gene and p53 expression showed prognostic relevance. In the groups of pT2-pT4 tumors, node-negative tumors, node-positive tumor and tumors with histological grade 2 or 3, Ki-67 expression and/or polysomy of chromosome 17 were significant markers for outcome. In HER-2/neu negative and moderately positive tumors, Ki-67 expression, lymph node stage, patient age, histological grade and polysomy for HER-2/neu and chromosome 17 were independent markers for clinical outcome. In cases with strongly positive HER-2/neu expression, however, none of the markers studied showed a significant correlation with outcome.

Multi tissue arrays are an effective tool for high through-put analysis of immunohistochemical and FISH markers. This study identifies proliferation associated markers and aneusomy of chromosome 17 as potential prognostic markers in breast cancer. However, in subgroups stratified by clinico-pathological features different markers may be of prognostic relevance.

F.08

PTEN AND EGR-1 EXPRESSION IN THYROID PROLIFERATIVE LESIONS

C. Di Loreto¹, G. Tell², M. Pestrin¹, F. Puglisi¹, M. Pandolfi¹, G. Damante¹

¹University of Udine, Udine, Italy, ²University of Trieste, Trieste, Italy

Aim: PTEN (MMAC1/TEP1) is a tumor suppressor that exerts its tumor-suppressive effect on non-medullary thyroid cancer through the inhibition of cell cycle progression and cell death. Recently Virolle et al.

found that PTEN transcription is upregulated by Early Growth Response Protein 1 (Egr-1). Therefore, Egr-1 can directly regulate PTEN triggering the initial step in the apoptotic pathways. Very little is known about the Egr-1 expression and functioning in thyroid cells and its relationship with tumor progression. Aim of the present work is to study the expression of PTEN and Egr-1 in normal thyroid tissue and in benign and malignant proliferative lesions of follicular thyroid cells.

Materials and methods: Paraffin-embedded, formalin-fixed specimens from 6 normal thyroid tissue, 10 nodular hyperplasia, 10 follicular adenoma, 10 follicular carcinoma, 10 papillary carcinoma and 6 undifferentiated carcinoma cases were selected for tissue microarray. In constructing the microarrays, we identified representative areas of the lesions and took two 1 mm cores. 5-mm-thick sections of the microarrays were cut and processed for streptavidin-biotin-peroxidase complex (ABC Elite-Vector) immunohistochemistry using rabbit polyclonal antiserum anti-Egr-1 (Santa Cruz) and anti PTEN (Zymed Lab).

Results: Positive staining for PTEN was found in the cytoplasm and occasionally in the nucleus (<5 % of cells) of the follicular cells; in the normal thyroid tissue the mean percentage of cells with positive cytoplasmic staining was 97.5% and this value was similar in the nodular hyperplasia, adenoma and papillary carcinoma. We found a slight decrease in the positive cells percentage and in staining intensity in follicular and in undifferentiated carcinoma (68 and 52.5%, respectively). The mean percentage of nuclear positivity for Egr-1 was 30.6 % in normal tissue and this value was similar in nodular hyperplasia and papillary carcinoma. We have found an important decrease of mean percentage of nuclear positivity in adenoma, follicular and undifferentiated carcinoma (4.3, 2.6, 6.75, respectively).

Conclusion: Our findings support that a loss of PTEN and Egr-1 expression in follicular and undifferentiated thyroid carcinoma can play a role in their pathogenesis. Nevertheless the relationship of PTEN and Egr-1 in thyroid follicular lesions requires further study.

F.09**CHARACTERIZATION OF OVR110 EXPRESSION, A NOVEL TYPE II MEMBRANE PROTEIN, IN SEROUS, ENDOMETRIOID, AND CLEAR CELL OVARIAN CARCINOMAS**

K.R. Shroyer¹, B. Tringler¹, R.M. Lindsay², S. Salceda², R. Cafferkey², R. Macina², A. Tobler², M. Ghosh², L. Corral², G. Pilkington², J. Papkoff², T. Enomoto³, K.R. Shroyer¹

¹University of Colorado Health Sci. Ctr., Denver, USA, ²DiaDexus Inc., San Francisco, USA, ³University School of Medicine, Osaka, Japan

Ovr110 is a novel Type II integral membrane protein that was identified by genomic database mining as an EST/gene showing up-regulation in ovarian cancer cells. Quantitative PCR (QPCR) analysis in over 200 human tissue samples verified relatively high levels of expression of Ovr110 in ovarian cancer compared to matched normal ovarian tissue and normal tissues from a wide range of other somatic tissues. The full-length gene encodes a mature protein of 282 amino acids, predicted to have a single transmembrane domain. Monoclonal antibodies to Ovr110 were prepared and screened against transfected 293T cells and mammalian recombinant protein. Biochemical experiments confirmed cell surface expression in tumor cell lines expressing Ovr110 mRNA and Western blot analysis of cell extracts showed that Ovr110 is heavily glycosylated. Ovr110 expression was evaluated in histologic sections of benign, borderline and malignant epithelial ovarian tumors by an indirect immunoperoxidase method. Intense cytoplasmic and circumferential membranous staining was detected in serous cystadenocarcinomas (23/23), endometrioid adenocarcinomas (16/17), and in clear cell carcinomas (16/16) of the ovary. Focal apical membranous staining was also observed in borderline serous tumors, serous cystadenomas, and endometriomas. By contrast, benign, borderline, and malignant mucinous tumors of the ovary were uniformly negative for Ovr110 expression. Apical membranous staining was also observed in fallopian tube columnar epithelium, bladder urothelium, and benign ductal epithelium of the breast but was not detected in most other normal tissues. The highly restricted nature of Ovr110 to the most common ovarian cancers makes this cell surface antigen an ideal target for a monoclonal antibody therapeutic strategy – *in vitro* validation and animal efficacy studies are in progress.

F.10**HIGH RESOLUTION ANALYSIS OF DNA COPY NUMBER CHANGES AT CHROMOSOME 20Q IN GASTRIC AND COLORECTAL CANCER**

J. Coffa¹, M.A.J.A. Hermsen¹, J. Schouten², G.A. Meijer¹

¹VUMC, Amsterdam, The Netherlands, ²MRC-Holland, Amsterdam, The Netherlands

Background: DNA copy number changes at chromosome 20 are frequent findings in human solid cancers. We recently proposed a 800 kb region at 20q13.2 to harbor a putative oncogene relevant for gastric cancer, and gain of 20q appeared to be one of the major chromosomal changes involved in colorectal adenoma-to-carcinoma progression. Yet, still several candidate oncogenes remain at 20q13, while also areas outside this region frequently show gains. To this end, we tested the presence of DNA copy number changes for multiple genes in a series of gastric and colorectal cancers, using multiplex ligation-dependent probe amplification (MLPA). MLPA is a quantitative multiplex PCR based approach that allows to determine the relative copy number of up to 40 genes in a single experiment, requiring only minimal amounts of DNA.

Methods: A dedicated oligonucleotide probe set for MLPA containing 28 genes on chromosome 20 and 13 reference genes spread over the genome was designed. DNA samples of 28 colorectal and 8 gastric tumors, that previously had been found to contain 20q copy number changes, were analyzed with the chromosome 20 MLPA probe mix.

Results: Almost half of the colon samples showed an amplification of 20q13.3 including the loci of TNFRSF6B and EEF1A2. In addition, 10% of all colon samples showed a clear amplification at 20q11 while more than 30% of these tumors had an amplification of BCL2L1 in this area. Other candidate genes here are ID1, C20orf1, REM and HCK. In the gastric carcinoma samples tested so far, amplifications seem to be more concentrated around 20q13.2.

Conclusion: Based on the current data, DNA amplifications at chromosome 20q in gastric and colorectal cancer seem to involve different genomic regions (20q11 and 20q13.3 versus 20q13.2), which could mean that different biological mechanisms would be involved in the pathogenesis of colorectal and gastric cancer.

F.11 APOPTOSIS AND PROLIFERATION IN COLORECTAL ADENOMAS

B.M. Visser¹, M. Hermsen¹, C. Postma¹, A. de Goeij²,
W. Giaretti³, G.A. Meijer¹

¹VUMC, Amsterdam, The Netherlands, ²University
Maastricht, Maastricht, The Netherlands, ³NICR,
Genoa, Italy

Introduction: Colorectal carcinomas arise from pre-existing benign tumours (adenomas), but only about five percent of all adenomas really do progress to carcinoma. To improve screening and treatment of colorectal cancer it is important to know what mechanisms underlie the transformation of these adenomas into carcinomas. Two important alterations that play a role in this transformation are the resistance to apoptosis and uncontrolled proliferation of tumour cells. Aim of the present study was to evaluate apoptosis and proliferation associated variables in relation to adenoma progression risk.

Materials and methods: 97 and 98 colorectal adenomas were analysed by means of immunohistochemistry for M30 Cytodeath and Mib1, respectively, and results were correlated to K-ras, APC and p53 mutation status, p53 protein expression, dysplasia, adenoma size, genomic adenoma-cluster (Hermsen et al., *Gastroenterology* **123** (2002), 1109–1119) and the most frequently occurring chromosomal aberrations. Cytoplasmic M30 positivity was scored semi-quantitatively, and Mib1 was scored by interactive quantitation of the area percentage of positive tumour nuclei using the Q-Prodit system.

Results: Fifteen tumours showed low, 33 showed moderate and 49 showed high M30 positivity. There was a trend of apoptosis correlating with a 18q loss and 20q gain Mib1 positivity ranged from 6% to 84% with a mean of 29.8%, and correlated with adenoma-cluster number ($p = 0.009$), 20q gain ($p = 0.02$), and 17p loss ($p = 0.04$). **Conclusion:** Apoptosis measured by M30 staining seemed to correlate with 18q loss and 20q gain, and proliferation, measured by Mib1 positivity correlated with genomic adenoma cluster, 20q gain and 17p loss.

F.12 P21 EXPRESSION IN COLORECTAL ADENOMAS

B.M. Visser¹, M. Hermsen¹, C. Postma¹, A. de Goeij²,
W. Giaretti³, G.A. Meijer¹

¹VUMC, Amsterdam, The Netherlands, ²University
Maastricht, Maastricht, The Netherlands, ³NICR,
Genoa, Italy

Introduction: In colorectal carcinogenesis, two distinct mechanisms for genetic instability are known: microsatellite instability (the consequence of a defective mismatch repair) and chromosomal instability. Recently, evidence was found for three independent chromosomal instability pathways of carcinogenesis. It is hypothesized that in each of these pathways similar cell biological functions are disrupted, but different proteins are affected (Hermsen et al., *Gastroenterology* **123** (2002), 1109–1119). One of the above mentioned pathways is characterized by chromosomal loss on 17p. This loss is associated with loss of function of the p53 gene, a gene involved in cell cycle arrest, DNA repair or apoptosis after DNA damage. One of the downstream genes regulated by p53 is the p21/Waf gene, which can act both p53-dependent and p53-independent. The aim of the present study was to analyse the relation between p21 expression and other variables like p53, K-ras and APC mutation status, dysplasia, size of the tumour and different kinds of chromosomal aberrations.

Materials and methods: Thirty-six colorectal adenomas from the cluster associated with the 17p loss were immunohistochemically stained for P21 with the monoclonal mouse antibody Waf1. P21 expression was scored semi quantitatively by two observers.

Results: Five of the 36 tumours showed low, 14 scored moderate and 17 scored high positivity for p21 expression. These staining scores correlated negatively with the p53 staining ($r = -0.42$, $p = 0.01$). No correlation was found between P21 staining and any of the other variables of the adenomas.

Conclusion: p21 expression showed a negative correlation with a disruption of the p53 pathway as measured by positive p53 immunohistochemistry, not with other variables.

F.13 LACK OF LYMPHANGIOGENESIS DURING BREAST CARCINOGENESIS

M.M. Vleugel, R. Bos, P. van der Groep, A.E. Greijer,
A. Shvarts, H.V. Stel, E. van der Wall, P.J. van Diest
VUMC, Amsterdam, The Netherlands

Introduction: The process of lymphangiogenesis is still poorly understood in human cancer. Overexpression of lymphangiogenic growth factors in experimentally induced tumors in mice induces intratumoral lym-

phangiogenesis and lymphatic metastases. However, recent evidence suggests that functional intratumoral lymph vessels may be absent from some human cancers. This could be due to the failure of some tumors to induce lymphangiogenesis or collapse of lymph vessels caused by high interstitial tumor pressure. This study was undertaken to differentiate between these two hypotheses by studying early and late lesions from the breast carcinogenetic spectrum.

Methods: Paraffin-embedded clinical specimens from normal breasts ($n = 13$), usual ductal hyperplasia ($n = 11$), ductal carcinoma in situ (DCIS, $n = 21$) and 40 invasive breast cancers were compared for lymphatic and blood vessel density by immunohistochemistry with antibodies to the lymphatic endothelial hyaluronan receptor LYVE-1 and CD31, respectively.

Results: Normal breast tissue showed low lymph vessel density compared to blood vessel density. Lymph vessels were absent within breast lobuli. In premalignant lesions blood microvessel density increased whereas no increase in lymph vessels could be seen intralesionally. Furthermore, in invasive cancers, lymph vessels were absent in all but a few cases where probably some pre-existent lymph vessels remained, although blood microvessel density was once again increased.

Conclusions: Although angiogenesis parallels progression of premalignant breast lesions, lymphangiogenesis appears to be lacking, resulting in complete absence of lymph vessels in the newly formed stroma of most invasive breast cancers. This points either to a lack of lymphangiogenic growth stimuli or to the presence of inhibitors of lymphangiogenic growth. Identification of such lymphangiogenic growth inhibitors might increase knowledge about mechanisms of decreased tumor lymphangiogenesis.

G.01 STUDY OF THE GENE EXPRESSION OF PATIENTS WITH COLORECTAL CARCINOMA BY CDNA MICROARRAY TECHNOLOGY*

E. Faltyskova¹, P. Gajduskova², S. Kozubek²,
M. Kozubek¹

¹Faculty of Informatics MU, Brno, Czech Republic,
²Institute of Biophysics AVCR, Brno, Czech Republic

Colorectal carcinomas represent the most frequent cause of cancer-related death. The tumors can spread by direct invasion into the surrounding tissues and also by metastasis to regional lymph nodes and internal or-

gans such as liver and lung. The progression of tumor is related to the accumulation of a series of genetic events such as mutations, methylations, and others. cDNA microarray technology appears to be a suitable method for identifying genes playing a significant role in colorectal carcinoma.

In our experiments, we used a 1700-element human cDNA microarrays for monitoring and comparison of gene expression levels of tumor tissue and normal colon tissue obtained from patients of the Masaryk Memorial Cancer Institute, Brno, Czech Republic and the Faculty Hospital, Brno, Czech Republic with the diagnosis of colorectal carcinoma. We used clustering and statistical methods for microarray data analysis, such as SAM (Significance analysis of microarrays) from software packages TIGR. On the basis of SAM, we have identified about 40 genes, which appear to have significantly different level of expression in tumor tissue than in normal tissue. Several genes seem down-regulated in tumor tissue including, for example, HMGCS2, API5, RARA, HDAC5. Genes that appear as up-regulated in tumor tissue include COL5A2, COL1A2, TIMP1, S100P, HLA-DRB3, HLA-DQA1.

We applied clustering methods to identify gene expression levels of patients with colorectal carcinoma. Using cluster analysis, genes were arranged to clusters according to similarity in pattern of gene expression. Gene expression data were used for projecting the expression maps of single chromosomes utilizing molecular distances of genes on chromosomes.

This study contributes to the identification of tumor markers for colorectal carcinoma that can be used for the determination of colorectal tumors in biomedical routine.

*This work is supported by the Grant Agency of the Ministry of Health of the Czech Republic (grant number NC6987-3/2002) and The Ministry of Education of the Czech Republic (project MSM-143300002).

G.02 SERIAL ANALYSIS OF GENE EXPRESSION IN HODGKIN LYMPHOMA

J.L. Kluiver, D. de Jong, A. van den Berg, S. Poppema
University Medical Centre Groningen, Groningen, The
Netherlands (E-mail: j.kluiver@path.azg.nl)

Hodgkin lymphoma (HL) is a common lymphoma in humans and can be divided into two different groups. One is the nodular lymphocyte predominant (NLP) type and the other, which comprises 95% of all cases, is the classical form (cHL). The classical

type can be further subdivided in the nodular sclerosis (NS) and mixed cellularity subtypes. HL is distinct from other malignant tumours because of its unique cellular composition: a minority of neoplastic cells in an abundant inflammatory background. The neoplastic cells, Reed-Sternberg cells (RS-cells), are most likely derived from germinal centre B-cells that have escaped apoptosis during negative selection in the germinal centre reaction.

We have used the serial analysis of gene expression (SAGE) technique to construct gene expression profiles of the HL derived cell lines, L428 (NS, cHL) and DEV (NLP, HL). As a control a SAGE library was constructed of an Epstein-Barr virus transformed lymphoblastoid B-cell line (Ray). In total, more than 40,000 tags were collected. By comparing the HL libraries with the control library and other previously published SAGE libraries, we aim at the identification of genes involved in the pathogenesis of HL.

In a first screening we have identified several genes known to be up regulated in the RS-cells of HL tissues, including Fascin, CCL17/TARC, BIC and Restin. These data indicate that it is feasible to use HL derived cell lines for the identification of differentially expressed genes in RS-cells of HL compared to a control cell line.

Further comparisons and analysis of differentially expressed genes will reveal other genes of interest in the pathogenesis of HL.

G.03 POSSIBLE GENE REGULATORY ROLE FOR BIC; A NONCODING GENE HIGHLY EXPRESSED IN ALL SUBTYPES OF HODGKIN LYMPHOMA

A. van den Berg, J. Kluiver, D. de Jong,
G.J. te Meerman, B.-J. Kroesen, S. Poppema
University of Groningen, Groningen, The Netherlands

In a search for genes specifically expressed in Reed-Sternberg (RS) cells of Hodgkin lymphoma (HL) we applied the serial analysis of gene expression (SAGE) technique on HL derived cell lines. One of the SAGE tags was frequently observed in HL, but not in the normal control SAGE library. RT-PCR analysis revealed PCR products only in the HL cell lines, confirming a HL specific expression. This SAGE tag corresponds to the human BIC gene, which shows a high degree of homology to the Gallus gallus Bic gene. Gallus gallus BIC was originally identified as a target of viral insertion in avian leucosis virus induced lymphomas

in chicken. Moreover, an oncogenic potential was reported for BIC in cooperation with c-myc upon infection of chicken embryo's. The human and Gallus gallus BIC genes belong to the family of noncoding mRNA-like molecules and function through their noncoding RNA transcripts. RNA *in situ* hybridization (ISH) indicated a predominant nuclear localization of BIC transcripts in the majority of RS cells in 91% of HL cases. The presence of BIC transcripts in the nuclei suggests a possible gene regulatory role for BIC, this was also observed for other noncoding genes. To gain insight in the function of BIC we studied gene expression changes in BIC transfected Burkitt's lymphoma derived cell line Ramos using Agilent micro arrays containing more than 17,000 genes. These analysis revealed a strong upregulation for 2 genes and a strong downregulation for 7 genes. These putative BIC target genes include, amongst others transcription factors (ELF-1, CBP and MSX-1) and DNA repair/replication genes (Ku-70, PCNA, RFC37 and NTH-1). In summary, our data show that expression of BIC is specific for RS cells of HL and that BIC has a gene regulatory role.

G.04 SUBSTRUCTIVE HYBRIDIZATION FOR IDENTIFYING NOVEL EXPRESSED SEQUENCES IN PROSTATE CANCER

S.N. Quayle, H. Hare, D. Hwang, S. Jones, J. Schein,
M. Marra, M.D. Sadar

British Columbia Cancer Agency, Vancouver, Canada

Prostate cancer is the most frequently diagnosed cancer as well as the second leading cause of cancer deaths among North American men. Androgen ablation therapy for patients with advanced prostate cancer inevitably fails as the disease progresses to an androgen-independent stage. At present, no effective therapy can be offered to patients with this terminal stage of the disease.

The goal of this study was to isolate and identify novel genes differentially expressed during hormonal progression using an *in vivo* model. Relative mRNA transcript levels were determined from two subtractive hybridization experiments, each comparing three points in disease progression. Each of the clones isolated in these experiments was sequenced and identified by comparing search results from multiple sequence databases. Approximately half the clones isolated in each experiment matched known or predicted cDNA molecules. Another 25% of the clones were ei-

ther of poor sequence quality, matched mitochondrial genes or showed inconsistent results between the databases searched. However, the final 25% of clones isolated in each experiment did not match known or predicted cDNAs, but did match human genomic DNA sequences. The majority of these occurred either within intronic regions or nearby known or predicted cDNAs, possibly representing novel splice variants or novel 5' or 3' untranslated regions. We have now spotted the clones from the initial subtractive hybridization experiment as a custom microarray. Preliminary data obtained from these custom microarrays, as well as from Northern blot analyses, indicate that a number of the clones matching genomic sequences are expressed. We are currently confirming these results via additional Northern blotting and microarray experiments. PCR experiments are also underway to determine if these clones represent novel splice variants of known genes or if they represent entirely novel genes. These results suggest subtractive hybridization is an effective method for identifying novel genes and splice variants. Additionally, screening of the custom microarray produced here may yield novel biomarkers or therapeutic targets for the management of androgen-independent prostate cancer.

G.05 DEVELOPMENT OF PANCREATIC PRECURSOR CELLS FROM MOUSE EMBRYONIC STEM CELLS IN VITRO

M. Popovic Hadzija¹, M. Korolija², M.H. Hadzija³
¹Rudjer Boskovic Institute, Zagreb, Croatia, ²Marina, Zagreb, Croatia, ³Mirko, Zagreb, Croatia

Stem cells are self-renewing elements that can generate the many cell types in the body. These cells are derived from an early stage of the embryo and are named embryonic stem (ES) cells. ES cells isolated from 129/sv mice can be differentiate into insulin-producing cells (Lumelsky et al., 2001). When injected into diabetic mice, these cells undergo rapid vascularization and maintain a clustered, islet-like organization. There is no data about such work on NOD (non-obesity) mice, which spontaneously developed diabetes mellitus. The aim of our work was generated structure like this from blastocysts of NOD mice. The procedure involved some steps and until today we successful generated embryoid bodies, which presents the base levels for expansion of insulin-producing cells. Blastocysts are isolated from uterus of 3.5 days pregnant NOD mice. After 48 hours of culturing, on mouse

embryonic fibroblasts feeder layer, the zona pellucida was dissolved in acid media. Inner cell mass of blastocysts was expanded on gelatin-coated tissue culture surface in medium for ES cells in the presence of leukemia inhibitory factor (LIF). Four days later, in medium for embryonic cells without LIF were generated embryoid bodies. Such embryoid bodies were pick up and total cellular RNA was isolated using RNazol B. The cDNA synthesis was carried out using Moloney murine leukemia virus. The amount of cDNA was normalized based on the signal from expressed b-actin mRNA. As markers of immature pancreatic cells we examined the expression of nestin and OCT-4 genes.

In the future work, these cells positive to both tested genes should be propagated in insulin-secreting cells by using specific condition.

This is a good way for producing of immunocompatible tissue for transplantation in diabetic recipients. It presents powerful tool for prevention of problems associated with diabetes late complications.

G.06 CONSTRUCTION OF HUMAN LIVER CANCER ENDOTHELIUM CDNA EXPRESSION LIBRARY AND SCREENING OF ITS ASSOCIATE ANTIGENS

Z. Xing¹, L. Jin-Ning², Y. Zhi-Hua¹
¹Cancer Institute, PUMC, Beijing, China, ²China-Japan Friendship Hospital, Beijing, China

Aim: To gain some tumor endothelium associated antigens by serological analyses of recombinant human liver cancer endothelial cells (HLCVECs) cDNA expression library, and offer new targets for the diagnosis and therapy of tumor.

Methods: HLCVECs isolated and purified from a hepatocellular carcinoma sample were constructed a cDNA expression library. Here we show the way of SEREX (serological analyses of recombinant cDNA expression libraries) using fixed HLCVECs as a vaccine intraperitoneally immunized BALB/c mice with high doses. Sera was obtained from the BALB/c mice cannot only have antibodies specially aim to HLCVECs but also inhibit HLCVECs proliferation and induce apoptosis in HLCVECs. By using this functional sera screen the HLCVECs cDNA expression library, positive clones were sequenced and analyzed by bio-informatics.

Results: The primary library consisted of 2×10^6 recombinants. Immunoscreening of this library led to

the identification of 36 positive clones representing 18 different transcripts. Among them fifteen were already known genes; two sequences were already identified but their functions were still unknown (hypothetical protein); one was novel. SAGE database analysis showed that these genes of ERP70, GRP58, GAPDH, SSB, S100A6, BMP-6, DVS27 and NAC alpha in fifteen known genes have been reported to be associated with endothelial cell and angiogenesis. But the relationship with liver tumor vascular endothelium is not found. Genes of GAPDH, S100A6, BMP-6 and hsp70 identified by SEREX in the others tumor cDNA expression libraries. The functions of others genes in tumor angiogenesis are under-researching.

Conclusion: The screening of HLCVECs cDNA expression library by the sera immunized with fixed HLCVECs indeed acquired the antigen gene associated with endothelium or tumor endothelium. The method of improved SEREX can offer a new way for analyzing tumor endothelium and other tumor cell antigen genes. The tumor endothelium associated antigens described in the current study should therefore provide a valuable resource for basic, clinical studies of angiogenesis and human tumor facilitated the development of vaccine-based therapies and of disease.

G.07 GENE EXPRESSION PROFILES IDENTIFY HISTOLOGICALLY DEFINED RHABDOMYOSARCOMA SUBTYPES

M. Wachtel¹, M. Dettling², E. Koscielniak³,
S. Stegmaier³, P. Buehlmann², B. Schaefer⁴
¹Zurich, Switzerland, ²Seminar for Statistics, Zurich,
Switzerland, ³Olga Hospital, Stuttgart, Germany,
⁴Kinderspital Zurich, Zurich, Switzerland

Rhabdomyosarcoma (RMS) is a pediatric tumor type with characteristics of skeletal muscle precursor cells. Based on histological criteria, tumors are classified into 2 major subgroups, namely embryonal RMS (ERMS) and alveolar RMS (ARMS). The majority, but not all, ARMS carry the specific PAX3(7)/FKHR-translocation. To gain further insight into the genetic characteristics of the different subtypes, we used oligonucleotide microarrays to measure the expression profiles of a group of 29 RMS biopsy samples (15 ERMS, 10 translocation-positive and 4 translocation-negative ARMS). Unsupervised two-way hierarchical clustering revealed an expression signature composed of several hundred genes consistently upregulated in translocation-positive samples when com-

pared to translocation-negative ones. At least some of these genes might be *in vivo* targets of the chimaeric PAX3(7)/FKHR transcription factors, participating in their oncogenic activity. Furthermore, supervised analysis allowed for clear discrimination of all three tumor subgroups ERMS, translocation-positive as well as -negative ARMS. These gene signatures are likely to influence the distinct biological behaviour of these subgroups and might enhance future molecular diagnostics.

G.08 EXPRESSION PROFILE ANALYSIS OF THE SMALL AND LARGE INTESTINE OF THE WISTAR RAT

R.M. van Elburg¹, R.M. Reis², B. Ylstra¹,
M.F. Schreuder¹, W.P. Fetter¹, G.A. Meijer¹
¹VUMC, Amsterdam, The Netherlands, ²Minho Uni-
versity, Braga, Portugal

The Wistar rat, *Rattus norvegicus*, is one of the most common animal models used in life science research and it has been preferentially helpful in physiological studies. The gastrointestinal tract is one of the largest organs in the human and rat body, composed of several types of tissues with particular functions. Among them, the ileum and colon have a paramount role in nutrient absorption. Aim of the present study was to compare gene expression patterns in the ileum and colon.

Methods. Ileum and colon tissue of six one-year-old female Wistar rats were analyzed by a lab-made expression microarray containing near 5,000 oligonucleotides (Rat oligoLibrary™, Compugen). Following microarray data normalization, ileum and colon tissue expression profiles were compared using SAM (Significant Analysis of Microarrays) software. SAM paired data analysis revealed 33 differentially expressed genes at greater than twofold levels (median false discovery rate <1). Twenty-eight genes were downregulated and five were upregulated in colon versus ileum. The identified genes have wide range of biological functions including cell adhesion (e.g., Galectin-9 and L1-like cell adhesion molecule), nutrients transport (e.g., sodium dependent sulfate transporter, BAT1, and solute carrier family) and antimicrobial defense (e.g., defensin-5 precursor), as well as some with unknown function (e.g., MARRLC9B).

The results of this study provide an overview of gene expression profiles of the ileum and colon of female Wistar rats under normal conditions, which allows us to gain insight into the molecular physiology of the gut and can serve as a reference for gene expression changes under pathological conditions.

G.09
GENE EXPRESSION PROFILING USING TWO
DIFFERENT OLIGO MICROARRAY
PLATFORMS REVEALS BOTH
CONFIRMATORY AND COMPLEMENTARY
RESULTS

F. Acquadro¹, G. Chiorino¹, S. Viscomi², M. Mello Grand¹, R. Segir¹

¹Research Lab., Edo Tempia Foundation, Biella, Italy,

²University of Milan, Milano, Italy

The degree of reliability of results obtained from different gene expression evaluation platforms in similar experimental contexts necessarily depends on the comparability level between the different array technologies utilized. In this study we focus on the comparison between two different Oligo Microarray commercial sources: Agilent Galss Array Human 1 A with 22,575 spots and Affymetrix Gene Chip U95Av2 with 12,625 represented sequences.

LNCaP and PC-3 prostate cancer cells were infected with a recombinant retrovirus expressing an activated gene of interest together with GFP, in parallel with a retrovirus expressing GFP alone.

With the Agilent system the expression of gene-infected and non infected cells was simultaneously compared on one slide (comparative hybridization), while with the Affymetrix system one chip corresponded to one determination of expression for one sample. For both systems duplicates were performed, with dye-swap in the case of comparative hybridization. A new method to separate single channel data information from Agilent glass arrays and make it comparable to single channel signals from other Agilent arrays was applied to compare PC3 vs LNCaP basal gene expression. Procedures of data filtration, normalization, reproducibility assesment and consequent extraction of modulated genes were made separately for the two platforms applying suitable methods.

Using free bioinformatic tools it was possible to obtain the list of Unigene Clusters that are simultaneously represented on the two platforms (9,654).

Differently from previous published work that considered cDNA Microarray and Affymetrix systems, we compared two oligonucleotide-based systems and obtained a best agreement of results from the two platforms. But the results obtained with the two arrays were only partially redundant, since some genes were found to be modulated only on one array, and vice versa, indicating that arrays of different sources have the potential providing both confirmatory and comple-

mentary results, which would not be obtained by use of one microarray system alone. Modulated genes with the greatest potential of significance were then confirmed by real time PCR.

G.10
MICRO-ARRAY TECHNOLOGY ON THE
SPOT: HYBRIDIZATION TECHNIQUES

P.R.L.A. van den IJssel, P. Eijk, S. Kasanmoentalib, B. Ylstra

VUMC, Amsterdam, The Netherlands

Hybridization is one of the major steps that determine the quality of a micro array experiment. Although many researchers perform their manual micro array hybridizations using a coverslip (non-moving target), we have chosen to hybridize our arrays 'in the open' (moving target) according to the classical Northern- and Southern blot hybridization procedures where the 'probe' in solution is actively moved over the 'target'. In order to perform this open array hybridization, we created a hand-made array hybridization chamber with rubber cement, according to the array-CGH procedure developed by Snijders et al. [1]. Analysis of micro array results produced using this method shows that more than 80% of non-flagged spots could be confirmed by Taqman.

Automation of micro array hybridization would have several advantages over manual hybridization methods, such as throughput, reproducibility and more standardized and thorough wash procedures. Recently, several companies have developed hybridization stations with advanced 'moving target' techniques. We have now tested two of these hybridization stations, one from Tecan and one from Perkin Elmer, and compared the results with our manual open hybridization method. Detailed analysis shows that both hybridization stations produce similar results. Compared to the manual open hybridization method, higher spot intensities were obtained and significantly less spots were flagged bad or empty in arrays hybridized by either one of the hybridization stations.

Reference

- [1] A.M. Snijders, N. Nowak, R. Segraves, S. Blackwood, N. Brown, J. Conroy, G. Hamilton, A.K. Hindle, B. Huey, K. Kimura, S. Law, K. Myambo, J. Palmer, B. Ylstra, J.P. Yue, J.W. Gray, A.N. Jain, D. Pinkel and D.G. Albertson, Assembly of microarrays for genome-wide measurement of DNA copy number, *Nat. Genet.* **29** (2001), 263–264.

G.11
SERIAL ANALYSIS OF GENE EXPRESSION
(SAGE) IN MICRODISSECTED DUCTAL
CARCINOMA *IN SITU* (DCIS) CELLS OF THE
BREAST

I. Verlinden, J. Janssens, J. Raus, L. Michiels
LUC, Diepenbeek, Belgium

Breast cancer is the most frequent female cancer and causes the highest mortality from cancer in women in the European Community. Breast carcinogenesis is a multistep process starting with benign, then atypical hyperplasia; progressing into *in situ*, then invasive carcinomas; and culminating in metastatic disease. Up to now still little is known about this multistep process leading to metastatic breast cancer and even more important, the interplay between normal surrounding tissue and the tumor tissue. This mutual control probably plays an important role in establishing tumor characteristics. Ductal carcinoma *in situ* (DCIS) is believed to be the true precursor of invasive ductal carcinoma, but the pathophysiology of this disease is still poorly understood. One of the problems to overcome is the fact that in addition to the heterogeneity of the neoplastic cell, tumor tissues are composed of a variable mixture of stromal, immune and endothelial cells and normal pre-existing tissue. The presence of multiple cell types may dilute out the significant changes that occur in specific cells and therefore we used a new microdissection method to histologically separate DCIS cells from the surrounding stroma, immunological infiltrates and endothelial cells. The system operates with a novel ultrasonic cutting tool. The tumor cells in the ducts were 'carved' out of the previously HE stained frozen tissue slides (20 μm). The isolates were collected in an organic solvent and then aspirated with an electronic aspiration pipette. We successfully applied an adapted microSAGE (Serial Analysis of Gene Expression) protocol to reveal the gene expression profile of microdissected ductal carcinoma *in situ* cells of the breast and we generated 29534 tags. Sage analysis of these 29534 tags made it possible to identify 3201 different transcripts. Of these 3201 unique tags 2826 (88.3%) matched known expressed sequences and 375 (11.7%) were novel without a genbank match. Comparison of this microdissected SAGE library with a total DCIS SAGE library revealed genes expressed only in surrounding normal stromal cells and not or significantly less in DCIS tumor cells. This work shows that microdissection provides important additional information on differential gene expression both in tumor cells and normal surrounding tissue because these differences fade away upon total tumor mass analysis.

G.12
CLASSIFICATION OF HUMAN BREAST
CANCER USING GENE EXPRESSION
PROFILING AS A COMPONENT OF THE
SURVIVAL PREDICTOR ALGORITHM

Gennadi V. Glinsky, Takuya Higashiyama,
Anna B. Glinskii
Sidney Kimmel Cancer Center, 10835 Altman Row, San Diego, CA 92121, USA

Selection of treatment options with highest likelihood of successful outcome for individual breast cancer patients is based to a large degree on accurate classification into subgroups with poor and good prognosis reflecting a different probability of disease recurrence and survival after therapy. Here we propose a breast cancer classification algorithm taking into account three main prognostic features determined at the time of diagnosis: estrogen receptor status, lymph node status, and gene expression signatures associated with distinct therapy outcome. The algorithm allowed highly accurate classification into sub-groups with dramatically distinct 5- and 10-year survival after therapy a large cohort of 295 breast cancer patients with either ER+ or ER- tumors as well as lymph node-positive or lymph node-negative disease. Since identified algorithm appears highly compatible methodologically with state of the art laboratory practice and seems to provide additional predictive values over conventional markers of the outcome, its application promises immediate significant clinical and socio-economic benefits. Our data imply that quantitative laboratory tests measuring expression profiles of identified small gene clusters may be useful in stratification of breast cancer patients into sub-groups with distinct likelihood of positive outcome after therapy and assisting in selection of optimal treatment strategies. The estimated increase in survival due to the optimization of a treatment protocol may reach many thousands breast cancer survivors every year at the 10-year follow-up check point.

G.13 CYTOKINE-INDUCED GENE EXPRESSION PROFILES AS TOOL TO IDENTIFY ASTHMA PHENOTYPES

W. Ten Hove, L. Koenderman, H.G. Leufkens,
M. Bracke

Department of Pulmonary Diseases (University Medical Center) and Department of Pharmacoepidemiology and Pharmacotherapy, Utrecht University, The Netherlands

Allergic asthma constitutes of an increased responsiveness of the tracheo-bronchial tree that can be triggered by inhalation of (rather common) allergens. A Th2-driven inflammatory process in the airways sustains the chronic process in asthma. Several studies have suggested that participation of neutrophils in this inflammatory process is associated with more severe phenotypes of the disease. Well-documented cytokines involved in the inflammatory events in asthma are $\text{TNF}\alpha$ and GM-CSF.

Using array technology, we examined the cytokine-regulated gene expression status of peripheral blood neutrophils. Individual genes are identified as monitoring and/or diagnostic markers in these gene profiles. Among those were CD83, MIP-1 β , IL-1RA and IL-1 β . By using real-time PCR, the expression of these genes is compared with the gene expression status of neutrophils of asthmatic patients before and after (6 and 24 hours) an allergen challenge. In this study we investigated the expression pattern of CD83.

With this knowledge, the gene expression status of cytokine-regulated neutrophils is used as a read-out for the inflammatory response during exacerbations of asthma. Epidemiological tools can establish links between the gene expression profiles and the response to therapy (measured by clinical parameters). Therapy should then be adjusted to the gene profiles of the different disease phenotypes.

H.01 ON THE VARIABILITY OF THE CCR IN ML CLASSIFICATION

F. Albrechtsen¹, T. Nygreen²

¹*Department of Informatics, Oslo, Norway,* ²*Schola Osloensis, Oslo, Norway*

In many medical data analysis applications, only a limited set of data is available for classifier training and classification error estimation. A simulation has been performed in order to study the reliability of cor-

rect classification rates (CCR) obtained by the leave-one-out procedure for data sets of a practically realistic size.

We assume that each of the sample (patient) feature mean values are drawn at random from normal distributions in two classes described by a set of class specific mean values and standard deviations. In order to ensure that the samples are realistically modelled, a number of observations (i.e. feature values from individual images) are made for each sample. These are drawn randomly from a normal distribution having the mean value of the sample, but possibly a different sample standard deviation. The real observed mean value and the real standard deviation for each sample is then calculated on the basis of these simulated observations.

Then, for each sample in the two classes, the sample in question is excluded from its class, new class mean values and class standard deviations are computed, and the two Mahalanobis distances between the sample and the two classes are computed.

For each distance pair computed, the sample is classified by the ML principle, and a CCR is computed. This is in essence the proper leave-one-out procedure. This is repeated many times, and the mean value and the standard deviation of the CCR is computed. The experiment is run for a set of values of the parameters, and the resulting distribution of the CCR for each parameter setting is obtained.

The aim of the simulation experiment is to provide a practically useful estimate of the uncertainty the CCR's obtained for different settings of the parameters, so that the confidence of a future CCR obtained by the "ave-one-out" procedure may be estimated from the known parameters and the implicit assumption of normal distributions.

H.02 LEAVE-ONE-OUT CLASSIFICATION ERROR ESTIMATION IN HIGH DIMENSIONAL FEATURE SPACES – THE USUAL WAY, AND THE RIGHT WAY

F. Albrechtsen¹, H. Schulerud²

¹*Department of Informatics, Oslo, Norway,* ²*SINTEF, Oslo, Norway*

We address the problems of analyzing many feature candidates when performing feature selection and error estimation on a limited data set. A Monte Carlo study on normal distributed data has been performed to illustrate the problems of selecting the correct features and estimating the performance.

The simulations demonstrate that in order to find the correct features, the number of features initially analyzed is an important factor, besides the number of samples. Moreover, the sufficient ratio of number of training samples to feature candidates is not a constant. It depends on the number of feature candidates, training samples and the distance between the classes.

Furthermore, the simulations demonstrate how the leave-one-out error estimate can be a highly biased error estimate when feature selection is performed on the same data as the error estimation. It may even indicate complete separation of the classes, while no real difference between the classes exists.

However, if feature selection and leave-one-out error estimation are performed in one process, an unbiased error estimate is achieved, but with high variance. The holdout error estimate gives a reliable estimate with low variance, depending on the size of the test set. Two feature selection methods are tested: 'Plus-1-Minus-1' and 'Sequential Forward Floating Selection'. The two feature selection methods analyzed gave the same result.

H.03 LONG RANGE CORRELATION IN DIFFERENT FUNCTIONAL GENOME REGIONS

I. Abnizova

University of Hertfordshire, Hatfield, UK

The statistical analysis of DNA sequences is of importance for understanding the structure, function and evolution of genomes. From a molecular biological point of view, long range correlations are not surprising since the complex organization of genomes involves many different scales.

We suggest to apply a set of known computational statistical methods, as well as our novel statistical algorithm, to search for difference in short and long range correlation properties between noncoding, regulatory and noncoding DNA, focusing on regulatory DNA. This analysis is based on cross genome comparison. It is known that if you compare genome sequences for properly evolutionary distant species, then you will observe different levels of conservation along the sequences. Because noncoding, regulatory and non regulatory noncoding DNA have different rates of evolutionary recombination which are likely to be reflected in their conservation levels, it is very natural to assume that corresponding regions will have different short and long range correlation type of behaviour. Numerical results which are mutually consistent, are obtained.

H.04 COMPARISON OF DIFFERENT NORMALIZATION METHODS IN THE ANALYSES OF OLIGO-NUCLEOTIDE MICROARRAY DATA

S. Kasanmoentalib, F.A.E. Kruyt, B.J.M. Braakhuis, B. Ylstra, J. Cloos, W.P.H. de Boer, J.H. van der Meulen-Muilenman
VUMC, Amsterdam, The Netherlands

Two-channelled microarray data are based on signal ratios, and the relative fluorescence intensities between the two channels must be normalized to adjust for systematic biases. Proper normalization of these data is crucial and has profound effect on further upstream analyses. The usual approach is to equate the mean intensity of one channel to that of the other by a normalization factor before log-transformation. Another approach is to bring the log-transformed intensities in both channels on the same scale by transforming each channel separately to the standard normal distribution. This transformation to z -scores is a valid statistical method in both neuro-imaging and psychological studies, and has recently also been applied on microarray data.

In this presentation we compare the effects of global mean normalization and z -score transformation on the log-ratios and the results of the t -test, the z -score test and, SAM. Because the positions of the spots on the array appear to have a persisting effect on the log-ratios, we had to adjust for spatial effects and also normalized locally by the two methods. The above will be demonstrated on basis of two data sets, recently produced by the Micro Array Core Facility of the VU University Medical Center, Amsterdam. In one dataset the effect on gene expression was investigated of a novel anticancer modality, being the conditionally replicating adenovirus vector CRAd-D24-RGD. The other dataset shows the differences in response to DNA damage between sensitive and insensitive cell lines. We will discuss the criteria that are relevant for the choice between the two methods.

H.05 TIMAN: AN OPEN SOURCE SOFTWARE FOR TISSUE MICROARRAY DATA MANAGEMENT

V. Della Mea, I. Bin, R. Spizzo, C. Di Loreto, C.A. Beltrami
University of Udine, Udine, Italy

Introduction: Tissue Microarray is a novel technique that, by collecting up to hundreds of samples on the

same specimen, allows to obtain three main advantages:

- reuse of a scarce resource (the tissue block);
- uniformity in staining;
- decreased antibody use per sample.

The preparation of a TMA involves the design of the recipient block, which should host all its samples in recognisable positions; after having prepared and stained a number of glass slides from the block, each sample should be analysed and reported in correspondence with its original patient data. This gives some difficulties, due to the large amount of data to be managed in each phase. Aim of the present paper is to describe a specifically developed software for TMA data management, able to help during the whole TMA life-cycle.

Methods: At present, just two papers describe software for supporting TMA activities. Both are based on off-the-shelf products (Excel, Photoshop), and deal with either data and images. We followed a differ-

ent approach: to develop a multiuser system, based on open source software, and preliminarily to deal with just TMA data.

Results: After requirement analysis, we developed a prototype system using the following software: MySQL, PHP, Apache. The resulting system, TIMAN (TIssue MicroArray maNagement) is a web-accessible TMA data management system able to cover most of the pathologist' needs. The technologies used make TIMAN highly platform independent, on server as well as on client side: the server application has been tested with Windows98, RedHat Linux and MacOSX, while the client has been tested with Mozilla 1.2 and Explorer 5-6.

Discussion: The developed system focuses on data management, leaving image management and processing to other software modules; it is accessible through the network by means of any recent browser. Thorough tests are ongoing inside a multicentre trial led by the University of Udine; after that, it will be released as open source.



Hindawi
Submit your manuscripts at
<http://www.hindawi.com>

