The Second International Symposium on Circulating Nucleic Acids in Plasma and Serum (CNAPS-2) held in conjunction with the 6th Annual Scientific Symposium of the Hong Kong Cancer Institute

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Invited speakers’ abstracts

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Tumor-derived genetic alterations in plasma and serum P. Anker Plant Biochemistry and Physiology, Faculty of Science, University of Geneve, Switzerland
Small amounts of free DNA circulate in both healthy and diseased human plasma/serum, and increased concentrations of DNA are present in the plasma of cancer patients. Characteristics of tumor DNA have been found in genetic material extracted from the plasma of cancer patients. These features include decreased strand stability, mutations of specific oncogenes or tumor suppressor genes, gene rearrangements and amplifications, microsatellite alterations and hypermethylation of several genes. Moreover, free viral DNA such as EBV has been detected circulating in the plasma. Finally, mDNA characteristic of tumor cells has also been found in the serum of cancer patients. Malignancies studied include breast, colorectal, pancreatic cancer, head and neck, lung, kidney, breast, ovarian, bladder, nasopharyngeal, liver cancer, non-Hodgkin’s lymphoma, acute B cell leukemia and melanoma. The results obtained, sometimes prior to clinical diagnosis, have opened a new research area indicating that plasma DNA might eventually be a suitable target for the development of non-invasive diagnostic, prognostic and follow-up tests for cancer.

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Nuclear genetic changes drive the progression of human cancers David Sidransky Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.
Since the identification of clonal genetic changes in the serum of cancer patients, new directions are emerging in the development of these diagnostic approaches. Among our most promising nuclear markers is epigenetic promotor methylation. We have demonstrated the presence of promoter methylation at various target genes in a variety of head and neck and lung cancers. We are now interested in real time quantitation of methylated genomes in patients before the development of cancer. Moreover, we have also assayed the presence of HPV DNA in the serum of patients with head and neck tumors as well as cervical cancer. Finally, we have recently demonstrated the presence of homoplasmic mitochondrial mutations in human tumors. Increased copy number of these mitochondrial mutations makes them an attractive target for various diagnostic purposes. In addition to their promise as markers in draining bodily fluids, we are also investigating their use as serum markers in various tumors.

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Viral DNA as a tumor marker in plasma and serum: application to nasopharyngeal and cervical carcinoma Wichai Pornthanakasem, Kanjana Shoteler-suk, Wichai Termrungtragoolrt, Narin Voravud, Somchai Niruthisard and Apiwat Mutirangura Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
Significant amounts of free DNA circulate in the plasma of cancer patients. Characteristics of tumor DNA have been found in genetic material extracted from the plasma of cancer patients. These features include decreased strand stability, mutations of specific oncogenes or tumor suppressor genes, gene rearrangements and amplifications, microsatellite alterations and hypermethylation of several genes. Moreover, free viral DNA such as EBV has been detected circulating in the plasma. Finally, mDNA characteristic of tumor cells has also been found in the serum of cancer patients. Malignancies studied include breast, colorectal, pancreatic cancer, head and neck, lung, kidney, breast, ovarian, bladder, nasopharyngeal, liver cancer, non-Hodgkin’s lymphoma, acute B cell leukemia and melanoma. The results obtained, sometimes prior to clinical diagnosis, have opened a new research area indicating that plasma DNA might eventually be a suitable target for the development of non-invasive diagnostic, prognostic and follow-up tests for cancer.

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Quantitative analysis of EBV DNA in nasopharyngeal carcinoma Y.M. Dennis Lo Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China
The recent demonstration of circulating tumor-derived DNA has opened up new possibilities for cancer detection and monitoring. Nasopharyngeal carcinoma (NPC) is a common cancer in South China and Southeast Asia and it is well-established that Epstein-Barr virus (EBV) has a close association with this malignancy. It has recently been shown that EBV DNA can be found in a significant proportion of plasma and serum samples from NPC patients. Our group has used real time polymerase chain reaction (PCR) techniques to establish fundamental quantitative parameters of circulating EBV DNA in NPC. Our results have shown that the levels of plasma/serum EBV DNA are higher for patients with advanced, compared with early disease. Serial sampling of plasma EBV DNA during treatment has shown a rapid decline in circulating EBV DNA level, with a median half-life of 3.8 days. We believe that this type of kinetic analysis will be useful for the evaluation of novel therapeutic modalities for NPC. Following treatment we have also shown that EBV DNA measurement allows the earlier detection of tumor recurrence than conventional methods. Finally, we have also shown that plasma/serum EBV DNA is an independent prognosticator for NPC, both with regard to early event (within the first year after treatment) and also for long-term survival. We believe that a strong case can be made for including circulating EBV DNA measurement in the routine work-up and follow-up of NPC patients.
Circulating nucleosomes in serum
S. Holdenrieder1, Petra Steiber1, H. Bodenmiller1, M. Busch1, J. v Pawel2, A. Schallhorn1, D. Nagel1, D. Sedel1, Kliniken Grosshadern, Munich1, Roche Diagnostics, Penzberg and Asklepion-Klinik, Gauting, Germany

Nucleosomes are aggregates consisting of histone proteins and cellular DNA and are products of apoptotic cells. They are released into circulation in patients with malignant diseases and during chemo- and radiotherapy. We developed a modified version of the Cell Death Detection®-ELISA (Roche Diagnostics) for the application in serum and established a standardized protocol for the prognostic analytic handling.

In patients with malignant tumors (N=418), we found higher concentrations of nucleosomes in serum (mean=350AU) compared to healthy persons (N=63; mean=36AU) and patients with benign diseases (N=41; mean=46AU). In the monitoring situation during chemotherapy, we observed an early increase of the values after the first application followed by a slow decrease. During chemotherapy, remission was associated with a 50% decrease of the nucleosomal baseline values (23% to 25 pts.), which were determined before each therapeutic cycle. Correspondingly, patients with progression showed an increase of 50% (8/14). During radiotherapy, remission correlated with an early start of decrease after the peak (±1d) to very low levels (±100AU) (9/10 pts.), whereas disease progression correlated with a later start of decrease (1d) to higher minimum levels (100AU (4/5 pts.).

In order to estimate the efficacy of chemotherapy during the early course of treatment, we investigated sera of 124 patients with lung cancer (101 NSCLC, 23SCLC) who were special risk to the first line treatment of nucleosomes were determined at days 1, 3, 5, 8 and 21 (1st day of the 2nd cycle). In patients with NSCLC, responders to therapy (remission of disease) showed significantly lower values for the AUC (days 1-8) (p=0.024) and day 22 (p=0.011) than non-responders to therapy (stable disease and progressive disease).

Thus, the efficacy of chemo- and radiotherapy can earily be predicted by the course of the nucleosomes in serum.

Proteomic approaches within the National Cancer Institute’s Early Detection Research Network for the discovery and validation of cancer biomarkers
Sudhir Srivastava National Institutes of Health, National Cancer Institute, U.S.A.

In the post-genomic era, proteomics offers a new approach to studying normal and transformed cell functions, disease detection targets, and novel novel chemoprevention approaches. The proteomic platforms allow to genotype profiling challenges nearing completion, elucidation of the entire human genome sequence is going to take some time. However, the genomic information cannot give information about what is actually occurring at the protein level within a given cell type and a given time. This information is even further removed from the in vivo condition when juxtaposed to the context of a cell existing as an entity linked to the complex tissue and organ system orchestration, where cells act in concert with each other and the vasculature in a threedimensional cellular structure. The present state of genomic information can only be considered a master “inventory list” about what proteins any cell could make. The National Cancer Institute’s Early Detection Research Network is employing proteomics, or “protein walking,” in the discovery and evaluation of biomarkers for cancer detection and for the identification of high risk subjects. Armed with the powerful use of Laser Capture Microdissection (LCM) to procure pure populations of cells directly from human tissue, the Network is facilitating the development of technologies that can overcome the problem of tissue heterogeneity. LCM, when coupled with downwstream proteomics applications such as two-dimensional polyacrylamide gel electrophoresis and SELDI (surface enhanced laser desorption ionization) separation followed by mass spectrometry analysis, can greatly facilitate the characterization and identification of protein expression changes which track with the normal and diseased phenotypes. We will discuss the recent works from Network investigators to demonstrate the potential of SELDI and MALDI (matrix-assisted laser desorption ionization) to identify relevant cancer screening proteins present in tissues and bodily fluids. We will also discuss the complementarity of proteomics approaches to plasma-based circulating RNA assays in cancer detection and risk assessment.

Fetal cells in maternal blood: from pregnancy to autoimmunity disease
Diana L. Bianchi Department of Pediatrics, Tufts University School of Medicine, Tufts New England Medical Center, Boston, Massachusetts 02111, USA

The isolation of fetal cells from maternal blood is currently under active investigation as a noninvasive method of prenatal genetic diagnosis. In the context of this work, we discovered that fetal cells from a prior pregnancy could persist for decades postpartum. This led to the realization that following pregnancy, a woman becomes a chimera.

The relationship between fetal cell microchimerism and maternal disease is currently being investigated. During pregnancy, fetal cells in the maternal skin are associated with polymorphic eruptions of pregnancy and increased fetomaterial trafficking is detectable in cases of pre-eclampsia. After delivery, we have shown that more male DNA of presumed fetal origin is present in the blood of women with scleroderma as compared to healthy controls. Fetal cells are also detectable in autopsy tissue specimens from women with scleroderma and as well as in surgical specimens from women with inflammatory diseases of the thyroid. Fetomaterial cell trafficking provides a potential explanation for the increased prevalence of autoimmune disorders in adult women following their childbearing years. Remarkably, some of the fetal cells are capable of further differentiation into mature organs and may thus play an additional therapeutic role.

Fetal DNA in maternal plasma: towards non-invasive prenatal diagnosis
Y.M. Dennis Lo Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China

With the discovery of fetal DNA in maternal plasma in 1997, a new approach for non-invasive prenatal diagnosis has become possible. Through the use of quantitative molecular methods, fetal DNA has been found in maternal plasma in much greater relative concentrations than fetal nucleated cells in maternal blood. Data from multiple groups in the world have confirmed the robustness of fetal DNA detection in maternal plasma. Fetal genetic characteristics and diseases which have been detected include gender for the investigation of sex-linked diseases, rhesus D blood group status, myotonic dystrophy, achondroplasia and paternally-inherited microsatellite polymorphisms. An exciting development recently is the demonstration of apoptotic fetal cells in maternal plasma. Using these “plasma-derived fetal cells” our group has achieved the prenatal detection of fetal Down’s syndrome by fluorescence in-situ hybridization analysis from maternal plasma. Apart from the qualitative detection of fetal genetic characteristics, quantitative fetal DNA abnormalities have also been detected in a number of diseases, including Down’s syndrome, pre-eclampsia and preterm birth. Such findings offer new strategies for the detection and monitoring of these disorders. With further research in this emerging area, it is expected that the clinical applications of fetal DNA in maternal plasma will continue to expand.

Circulating RNA as a tumor marker
Michael S. Kopreski and Christopher D. Gocke OncoMEDs, Inc., Columbia, Maryland, USA

Cancer is commonly associated with the inappropriate mRNA expression of non-coding genes. Recent advances in several areas of toxicology, Westermark et al., 2003, have generated the hypothesis that RNA (or DNA) from cells of fetal origin could be found in maternal circulation as a marker of fetal-maternal cell trafficking. In a recent study we examined fetal genetic characteristics, quantitative fetal DNA abnormalities have also been detected in a number of diseases, pre-eclampsia and preterm labor. This led to the realization that following pregnancy, a woman becomes a chimera. The isolation of fetal cells from maternal blood is currently under active investigation as a noninvasive method of prenatal genetic diagnosis. In the context of this work, we discovered that fetal cells from a prior pregnancy could persist for decades postpartum. This led to the realization that following pregnancy, a woman becomes a chimera.

Circulating mRNA as a tumor marker
A. Zippelius, R. Lutterbuese, G. Oeser, E. Henrich, K. Pfaff, M. Muller, Onkologische Universitaetsklinik, Eppendorf, Hamburg, and Institut fur Immunologie, MLU, Munchen, Germany

Early systemic spread of occult tumor cells that may develop into founders of incurable distant metastasis has been identified in cancer patients by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of mRNA species encoding for tumor-associated proteins such as cytokeratin-19 and 20, carcinoembryonic antigen or prostate-specific antigen. Nevertheless the introduction of this new staging tool into the clinical setting has been hampered by the disparate and contradictory data on the sensitivity and specificity of the RT-PCR methods. We therefore used PSA RT-PCR, as a model, to examine the influence of analytical variables such as priming and enzyme of RT reaction, temperature and time of primer annealing, primer extension and denaturation as well as the concentrations of magnesium chloride, Taq polymerase, dNTP, primers and bovine serum albumin (BSA) on the amplification process. By systematically varying these chemical and physical components, we could demonstrate a significant increase in amplification yield and in stringency of the assay that may explain the wide variation of results on molecular staging of prostate cancer and other epithelial tumors, which currently impedes the clinical introduction of RT-PCR assays into clinical practice.
practice. Thus, methodological analyses are needed for standardization and quality assurance to achieve reproducible molecular methods.

Presence of fetal RNA in maternal plasma
Leo L.M. Poon1, Tse N. Leung2, Tse K. Lau1, Yu M. Dennis Lo3
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The discovery of fetal DNA in maternal plasma has opened up new possibilities for prenatal molecular diagnosis. Many groups have since shown that fetal genetic traits can be determined from fetal DNA in maternal plasma. However, it is not known whether fetal RNA is also present in maternal plasma. Using a two-staged reverse transcription (RT)-PCR assay, we demonstrated the presence of fetal-derived, male-specific ZFY mRNA in plasma of pregnant women carrying male fetuses. The detection rate of plasma fetal ZFY mRNA in early (N=9) and late pregnancies (N=29) were 22% and 63%, respectively. In addition, of 9 filtered plasma samples collected from women carrying male fetuses in late pregnancies, positive ZFY mRNA signals were detected in 6 samples, indicating that at least a portion of fetal RNA in maternal plasma exists in cell-free form. These results suggest that maternal plasma might be a source for noninvasive detection of fetal gene expression.

Fetal and tumor-derived DNA in urine: A new generation of ultra-non-invasive tests
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From 10³ to 10⁵ cells die daily in the human body. The vast majority of these cells die by apoptosis. Apoptosis is accompanied by chromatin condensation, internucleosomal cleavage of DNA, formation of so-called apoptotic bodies, which frequently contain DNA, and cell fragmentation. Cell fragments are engulfed and finally digested by macrophages or by neighboring cells. The presence of cell-free DNA in the blood plasma suggests that a portion of DNA from the apoptotic cells escapes complete degradation. Detection in this cell free DNA of "alien" sequences, which are different from the bulk cell DNA, can be used for diagnostic purposes. However, the concentration of cell-free DNA in plasma is low, equivalent to 10³-10⁵ cells/μl. This can seriously limit the sensitivity of plasma DNA based technology. We have recently shown that cell-free DNA derived from a fetus, tumors or transplanted cells is also detectable in urine at concentrations comparable to those observed in plasma. In many instances, availability of urine samples can represent a significant advantage for diagnostic purposes. Several important questions should be addressed in order to facilitate clinical applications of this technology: (i) Are different genome sequences equally represented in circulating cell-free DNA? (ii) How is DNA of various tissues represented in plasma and urine? (iii) Is DNA from dying cells present in plasma and urine as a free nucleoprotein, as a part of apoptotic bodies or in other form?

Molecular medicine in the emergency room: plasma DNA in trauma
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Circulating cell-free plasma DNA is useful for the clinical diagnosis and prognosis of cancer patients, pregnant women and organ transplantation recipients, and may be liberated into plasma either as a result of apoptosis or direct injury. As bodily trauma involves considerable tissue damage, DNA may be liberated from tissues within minutes to hours after such injury. There is a positive correlation between early plasma DNA levels and injury severity, and levels are highest in those patients who develop the acute respiratory distress syndrome, multiple organ dysfunction syndrome and those who eventually die.

Plasma DNA can be measured within minutes to hours after injury using a real-time, quantitative polymerase chain reaction assay. Using DNA and other markers entered into a Classification and Regression Tree, it is possible to derive a prediction rule for post-traumatic complications with high sensitivity, specificity, and positive predictive values. These guidelines require prospective validation before they find a place as research tools.

On the fate of plant or other foreign genes upon the uptake in food or after intramuscular injection
Ute Hohlweg, Walter Doerfler Institute of Genetics, University of Cologne, Weigerst 121, 50631 Koenigswinter, Germany.

Uptake and persistence of the DNA of bacteriophage M13 and the cloned gene for the green fluorescent protein (GFP) as test genes for food-ingested DNA have previously been traced from intestinal contents, via the gut wall, Peyer patches and peripheral white blood cells to spleen and liver and via the placenta to fetuses and newborn animals. We have now chosen a natural scenario and fed soybean leaves to mice. The distribution of the plant-specific gene for the nucleus-encoded ribulose-1,5-biphosphate-carboxylase (rubisco) has been studied in the mouse organism. Plant-associated, naturally fed DNA is more stable in the intestinal tract than naked DNA. Rubisco gene-specific PCR products have also been amplified from spleen and liver DNA. There is no evidence for the expression of orally administered genes, as assessed by RT-PCR method. Moreover, mice have been continuously fed daily with GFP DNA for 8 generations and have been examined for the transgenic state. The results have been negative and argue against germline transfer of orally administered DNA. Upon intramuscular injection of GFP DNA, authentic GFP DNA fragments have been amplified by PCR up to 17 months post injection. GFP fragments can also be retrieved from the intestinal contents up to 6 h post injection. Apparently, the organism eliminates injected foreign DNA via the liver-bile-intestinal route.

DNA microsatellite markers with LOH in plasma from stage III melanoma patients is associated with a decreased survival
D.S.B. Hoon, B. Taback, D.L. Morton, Y. Fujiwara. John Wayne Cancer Institute, Santa Monica, CA, USA

DNA microsatellite markers in plasma from stage III melanoma patients is associated with a decreased survival. LOH for at least one marker was present in 17 of 27 stage III patients' plasma. In conclusion, these results suggest that plasma DNA markers can possess clinical utility. DNA microsatellite LOH markers in plasma may serve as a valuable surrogate molecular marker for rapid disease progression identifying patients who may benefit from potential therapies.
genomic repetitive elements. The cloned lmdNA is enriched with G/C-pairs (48% against 40% in rodent genome).

The application of the protocol for identification of nucleosome-positioning sequences in eukaryotes is an ideal tool for the study of nuclear DNA organization and for the detection of nucleosome-based repetitive elements in the genome. The protocol is able to provide high-resolution information on the distribution of nucleosomes within the genome.

The length of cloned DNA fragments following irradiation by 100 Gy was very different in sizes in comparison with the influence of 8 Gy. It may be caused by the difference in the conditions of irradiation. The results in the comparison of lmdNA after irradiation at these doses consist of the change in the levels of dinucleotides CpG and CpT. This DNA in question originates from different cell populations.

Data-based detection of prostate cancer in blood, urine, and ejaculates

Poster Session Abstracts

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DNA-based detection of prostate cancer in blood, urine, and ejaculates

Carsten Goessler, Markus Müller, Rüdiger Heicappell, Hans Krause, Kurt Miller

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Promoter hypermethylation of the glutathione-S-transferase P1 gene (GSTP1) characterizes more than 90% of prostate cancers but has not been found in benign prostate tissue. Therefore, methylation-specific polymerase chain reaction (MSP) targeting GSTP1 promoter sequences was applied for DNA-based detection of prostate cancer in various human body fluids.

We investigated GSTP1 promoter hypermethylation in DNA isolated from serum, plasma, nucleated blood cells, ejaculate, urine after prostate massage, and dissected prostate tissue from 33 patients with prostate cancer and 26 controls identified by benign prostatic hyperplasia (BPH). After methylated lmdNA products were analyzed on an automated gene sequencer.

Whereas GSTP1 promoter hypermethylation was not detectable by MSP in prostate tissue and body fluids from patients with BPH, we found it in 94% of tumor samples. The detection rate of plasma or serum samples was 82% (14 of 40) and 36% of urine samples (4 of 11) from patients with prostate cancer. Additionally, MSP identified circulating tumor cells in 30% (10 of 33) of prostate cancer patients.

Analysis of GSTP1 promoter hypermethylation by MSP provides a specific tool for molecular diagnosis of prostate cancer in body fluids complementing information obtained with the conventional serum tumor marker PSA.

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Detection of gene promoter hypermethylation in serum samples of gastric cancer patients


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Ablation gene promoter hypermethylation is recently found to be an alternative mechanism for gene silencing. This study aims to investigate the feasibility of detecting such epigenetic changes in serum of gastric cancer patients. A total of 54 serum samples from gastric cancer patients with confirmed tumor tissue sample were analyzed for promoter hypermethylation of DAP kinase, E-cadherin, GSTP1, p15 and p16 by methylation-specific PCR (MSP). Thirty serum samples were included as controls. Promoter methylation of DAP kinase, E-cadherin, GSTP1, p15 and p16 was found in 70.4% (38/54), 74.1% (40/54), 68.5% (37/54) and 64.8% (35/54) of tumor tissue samples. For the corresponding serum, aberrant methylation was also detected for DAP kinase (48.1%, 26/54), E-cadherin (51.9%, 28/54), GSTP1 (56.6%, 30/54), p15 (55.6%, 30/54) and p16 (51.9%, 28/54). Moreover, for all of the mentioned marker genes, serum positive cases were found in patients with corresponding gene methylation also demonstrated in the paired gastric tumor tissue samples. None of the 30 normal serum controls contained aberrant hypermethylation. These findings suggested that detection of aberrant methylation by MSP in serum is feasible. The potential roles in cancer diagnosis and disease monitoring deserve further evaluation.

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Remarkable application of serum quantitative PCR of EBER1 DNA in monitoring the response of nasopharyngeal carcinoma patients to salvage chemotherapy

Roger KC Ngan, WH Lau, Timothy TC Yip, WCS Cho, WW Cheng, CKP Lam, KK Wan, Eagle Chu, JS Joab, V Grunewald, WF Yoon, and JHC Ho

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Relapse of disease is frequently encountered in nasopharyngeal carcinoma (NPC) patients. Previous retrospective analysis in our hospital by Lee et al. (1992) showed that, among NPC patients with complete tumor regression to primary radiotherapy (RT), more than half of them will later develop relapse. Chemotherapy (CT) is a common salvage modality employed to treat NPC relapse. However, conventional clinical indicators for treatment response to salvage CT are neither sensitive nor specific. In this paper, we investigated whether determining EBV DNA level in the patients’ sera by TaqMan real time-quantitative polymerase chain reaction (Q-PCR) could be a useful indicator to monitor the response of salvage CT. Twenty-one patients, who were recruited in a phase II clinical trial of salvage CT for relapse using a new drug combination of cisplatinum and gemcytobine were entered into this study. Longitudinally collected sera from this cohort of patients were analyzed by Q-PCR for EBV DNA encoding Epstein-Barr virus (EBV) small RNA (EBER1) at the beginning of relapse and at intervals of 1 to 3 months during/after salvage CT for a period of 1 to 5 years. Among them, 6/21 (29%) patients had local or loco-regional (LR) recurrence, 6/21 (29%) developed distant metastasis (DM) in single site and 9/21 (43%) had DM in 2 or more sites. EBER1 DNA was present in patients’ sera was substantially elevated with relapse in 17/21 (81%) patients. On salvage CT, 17/21 (81%) patients had dramatic drop of EBER1 DNA level corresponding presumably to tumor regression in response to the drug. The specificity of the test was demonstrated by a total of 1 EBER1 DNA negativity in 1/5 (20%) patient before who was EBV in situ hybridization negative. Detailed follow-up of the clinical course in these patients revealed an excellent correlation of EBER1 DNA level with the events of disease progression/regression. In conclusion, Q-PCR amplification of EBV gene has great potential to be developed into a routine test for monitoring salvage treatment in NPC.

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DNA of human papillomavirus detected by PCR in cervical cancer patients: a potential marker of residual disease

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Background

Cervical cancer is the leading cause of death from gynecological cancers in the world. Tumor markers, especially when specific and sensitive, are of great value in the management of cancer patients. For cervical cancer there are no known tumor markers with these characteristics that could be used as a free tumor marker. However, circulating DNA has been identified as a marker for the presence of HPV in patients with cervical cancer. Additionally, HPV DNA sequences specific to those found in primary tumors are also detected in the plasma. This has been shown for mutated K-ras, microsatellites and IgH chain arrangements in tumors such as colon, pancreas, head and neck and lymphomas. The type of mutation found in the primary tumor is always the same as that detected in serum or plasma from these patients. The fact that HPV DNA is not only present but also detectable in blood, even before the diagnosis is made, strongly suggests that viral DNA sequences may be present in the plasma of cervical cancer patients and to prove it useful as a marker to predict response or tumor recurrence.

Methods: The plasma of 70 cervical cancer patients with histologically proven cervical cancer irrespective of clinical stage had HPV DNA sequences detected by PCR amplification of β-globin sequences to avoid false negative results due to poor DNA quality. The β-globin positive cases were then analyzed for HPV16 sequences. The association between the PCR findings in cancer and control groups was analyzed with the Fisher’s exact test.

Results: We found HPV DNA sequences in the plasma of 49 out of 70 patients (70%) with cancer, while no DNA amplification was observed in the blood of healthy subjects. HPV DNA was not detected in any of the plasma of cancer patients and body fluids from patients with BPH. HPV DNA sequences were also found in plasma with clinical stage. Several cancer patients already treated had been followed and the results showed a high correlation between the presence of HPV DNA and the occurrence of recurrent disease. In conclusion, HPV DNA sequences were detected in serum of cervical cancer patients and proved useful as a marker for the detection of residual disease. Further follow-up is needed to confirm its utility in management of cervical cancer patients.

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Gross elevation of TT virus genome load in the peripheral blood mononuclear cells of cancer patients

Sheng Zhong, Winnie Yeo, Mandy W Tang, Xiaoyong Lin, Frankie Mo, Wai-Yee Pun, Hai Huang and Philip Johnson

Department of Clinical Oncology, Sir Y. K. Pao Centre for Cancer, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N. T., Hong Kong

TT virus (TV) is a recently described circular DNA virus of about 3.8 kb, which integrates into the chromosome, over a period of 1⁄2 to 5 years. Among them, 6/21 (29%) patients had local or loco-regional (LR) recurrence, 6/21 (29%) developed distant metastasis (DM) in single site and 9/21 (43%) had DM in 2 or more sites. EBER1 DNA was present in patients’ sera was substantially elevated with relapse in 17/21 (81%) patients. On salvage CT, 17/21 (81%) patients had dramatic drop of EBER1 DNA level corresponding presumably to tumor regression in response to the drug. The specificity of the test was demonstrated by a total of 1 EBER1 DNA negativity in 1/5 (20%) patient before who was EBV in situ hybridization negative. Detailed follow-up of the clinical course in these patients revealed an excellent correlation of EBER1 DNA level with the events of disease progression/regression. In conclusion, Q-PCR amplification of EBV gene has great potential to be developed into a routine test for monitoring salvage treatment in NPC.
including breast cancer, non-Hodgkin’s lymphoma, colon cancer, hepatocellular carcinoma, nasopharyngeal carcinoma and other cancers was measured. 30 amplicons were randomly selected and cloned into pGEM-T Easy Vector and subsequently sequenced to identify the TTV genotypes in PBMCs of the cancer patients. TTV DNA was detected in 69 (69%) of 100 plasma samples of cancer patients tested, and 39 (39%) of 100 plasma samples randomly selected from 1000 plasma samples from blood donors (p<0.05). TTV DNA was detectable in the plasma of the cancer patients. When 88 isolates from the 30 patients were analyzed, 71.6% were confirmed in at least one of the body fluids and with at least one of the six primers used (88%). No relation between the detected DNA alterations on the investigated loci, the frequency of alterations respectively and the tumor staging and grading could be confirmed. The study indicates that the simultaneous and multiple use of tumor-specific microsatellite markers could have a clinical relevance as a non-invasive tool for diagnosis and screening of bladder cancer in patients.

Circling DNA and Cancer Poster Presentations

23 Persistence of K-ras mutations in plasma three days after colorectal tumor resection U. Lindforss, L. Dungner, H. Zetterquist, H. Olivecrona

As an initial step in studying the dynamics of tumor specific DNA in plasma after radical surgery, we extracted DNA from plasma samples taken pre- and post-operatively after three days in 25 patients with colorectal cancer. All patients were free of macroscopical and histological residues of the tumor after surgery. DNA was extracted (QIAAmp, Qiagen) from plasma and the corresponding tumors. The K-ras gene codon 12 were amplified and mutations analyzed with TGGE (temperature gradient gel electrophoresis). 16/25 (64%) of the tumor biopsies, carefully evaluated as neoplastic before sampling, harbored K-ras mutations. Of these, 9 patients (56%) had K-ras mutations in their plasma DNA. 8 of these patients still had a K-ras mutation in plasma post-operatively on day 3. These results are surprising since the half-life of nucleic acids in the circulation presumably is much shorter. However, in cancer patients this has not been studied. This may suggest that the half-life of free DNA and oligonucleotides in the circulation is prolonged in cancer patients.


Microsatellite analysis of circulating tumor DNA in urine and blood as a possible non-invasive tool for the detection of bladder cancer Michael Utting*, W. Werner*, J. Schubert*, H. Malke*, K. Junker* Department of Urology, Friedrich-Schiller-University Jena, Germany Department of Molecular Biology, Friedrich-Schiller-University Jena, Germany

We performed microsatellite analysis of urine, serum and plasma of 42 bladder cancer patients. Six microsatellite markers were selected to detect shifts or LOH on chromosomes 4, 9 and 17. Blood and urine specimens were collected pre-operatively by venipuncture and spontaneous micritation. DNA was extracted using a modified protocol of a commercial DNA extraction kit. The DNA was analyzed and compared to peripheral lymphocytes. For DNA-analysis we used supernatants only. Tumor specimens were obtained by transurethral resection.

Genetic alterations were detected in 26 of the 36 histologically classified bladder tumors (72%). Tumor specific alterations on chromosome 4 were confirmed in 54% of urine, 18% of serum and in 36% of plasma; on chromosome 9 in 61% of urine and both in 48% of serum and plasma; and on chromosome 17 in 45% of urine, 3% of serum, and in 5% of plasma samples. Genomic instability was observed in 25 of 29 tumors with genetic alterations could be confirmed in at least one of the body fluids and with at least one of the six primers used (88%). No relation between the detected DNA alterations on the investigated loci, the frequency of alterations respectively and the tumor staging and grading could be confirmed. The study indicates that the simultaneous and multiple use of tumor-specific microsatellite markers could have a clinical relevance as a non-invasive tool for diagnosis and screening of bladder cancer in patients.

26 The molecular detection of prostate cancer K Patel, SA Burchill and PJ Selley

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Sensitive methods for the detection of prostate cancer may be of prognostic significance and may lead to earlier diagnosis. If therapeutic intervention is successful in the treatment of low level disease. In this study the sensitivity and specificity of reverse transcriptase polymerase chain reaction (RT-PCR) and microsatellite analysis for the detection of prostate cancer were compared. RT-PCR for PSA was very sensitive, detecting 10 LNCAP cells spiked in 2 ml of PB, although PSA mRNA was only detected in PB from 2/11 patient samples. Furthermore, PSA mRNA was detected in normal PB (4/24 samples). Microsatellite instability and LOH were detected in 5/9 prostate tumors, but only LOH was detected in plasma of 2/9 patients. The study indicates that the simultaneous and multiple use of tumor-specific microsatellite markers could have a clinical relevance as a non-invasive tool for diagnosis and screening of bladder cancer in patients.

Circling DNA microsatellites with LOH in early stage breast cancer patients serum is associated with increased tumor proliferation B. Taback, A.E. Giuliani, N. Hansen, D.S.B. Hoorn. John Wayne Cancer Institute, Santa Monica, CA, USA

LOH of DNA microsatellites is a common genetic event occurring in primary tumors and can be detected in blood. However, clinicopathologic correlations are limited and inconsistent which may be due to the varied patient populations assessed and differences among techniques. The purpose of this study was two-fold: 1) to demonstrate the presence of LOH in the serum from early-stage breast cancer patients (EBC), and 2) to determine whether correlations exist with known histopathologic parameters.

Preoperative serum was obtained from 56 patients (stages I and II) undergoing segmental mastectomy and sentinel lymphadenectomy, and assessed for LOH with 8 microsatellite markers: TP53, D16S421, D17S855, D17S849, D8S321, D10S197, D14S62 and D14S51. Twelve of 56 (21%) patients demonstrated LOH for at least one marker, the most frequent being D16S421 (11%) No LOH was detected in 5 patients with DCIS or those with tumors <1 cm. In all, but two cases, circulating LOH was associated with primary tumors demonstrating abnormal ploidy, increased diploid index and MIB-1 fraction. This comprehensive study provides evidence demonstrating the presence of free tumor-related genetic markers in EBC serum. The association of these findings with tumors manifesting known pathologic features consistent with increased proliferation suggests a possible etiology for their presence.

27 Downregulation of p53 and p53-ab in plasma of chemoresistant colorectal carcinoma patient after chemogene treatment consisting of Adwtp53+5-FU

J. Giannios, P. Ginopoulos Department of Clinical Oncology, Peripheral Hospital of Patras, St. Andrews*, Greece

We reported a colorectal cancer patient who had developed chemoresistance due to p53 mutations which inhibited induction of PCD. The presence of mutant type p53 proteins induced the anti-p53 antibody production by human...
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CD4+ T cells, as detected by ELISA, immunoblotting and immunoprecipitation. Thus, high p53 antibody levels in plasma were correlated with mutant p53. Tumor DNA was extracted and purified from plasma and direct sequencing of PCR products in a wide range of patients with adenovirus containing wt-p53 DNA and S-PUF which resulted in a significant decrease in tumor volume due to extensive tumor cell death by induction of D2 apoptosis. In contrast, to a biopsy specimen, circulating anti-p53 antibodies were detected in the plasma of the cancer patient with ELISA, immunoblotting and immunoprecipitation. Additionally, p53 sequence analysis by PCR in tumor DNA from plasma detected no mutations in the whole region of exon 7 due to Adwtp53 transformation. In conclusion, wt-p53 function has eradicated anti-p53 antibody production and p53 mutations in the plasma of the colorectal carcinoma patient where chemotheraphy reduced the tumor volume by induction of PCD.

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Tumor markers in cancer patient blood plasma DNA A.S. Belokhvostov, A.E. Bartnovskiy, K.V. Goranin, A.A. Novik, P.V. Perestenko Russian Military Medicine Academy, St. Petersburg 194044, Russia

The cloning of tumors has resulted in the mutational identity between the primary tumor and its associated metastasis, as well as any DNA that the tumor has released into the plasma. The latter phenomenon has raised the possibility that plasma DNA can be used for early cancer detection and disease monitoring.

We analyzed codon 12 mutations in the K-ras gene by enriched PCR. Mutations in exons 5, 6, 7, and 8 of the p53 tumor-suppressor gene were also assessed by SSCP analysis of PCR-amplified products. No mutations in codon 12 of the K-ras gene were observed in tumor or blood plasma in 15 lymphoma and 12 breast cancer patients. In contrast, mutations in the p53 gene were observed by SSCP analysis in blood plasma DNA samples and the corresponding tumor DNA in 8 of 12 breast cancer patients and 7 of 13 lymphoma patients. The level of mutant plasma DNA was reduced at two months after tumor removal and chemotherapy, and became undetectable in a proportion of patients at 6 months. In 3 cases, clinical follow up at 1 and 1.5 years revealed no evidence of residual disease. In 5 breast cancer patients, mutant p53 sequences were persistently detectable in blood plasma DNA.

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Molecular genetic markers—tumor mutant genes in plasma of cancer patients after Glutoxim treatment L.A. Kozhemyakin, A.S. Belokhvostov, A.E. Bartnovskiy, BAM Ltd., Sankt-Peterburg, Russia

Tumor mutant genes can be found in blood plasma independent of tumor cell circulation in blood. Codon 12 mutations of the K-ras gene were found in plasma of pancreatic cancer patients, but not in those with breast cancer. SSCP analysis detected mutant p53 gene (exons 5, 6, 7, 8) in both cancer patient groups. In 15 lymphoma and 12 breast cancer patients, no mutations were detected in the tumor removal (group 1), but a proportion of the women were additionally treated with Glutoxim, an analog of oxidized glutathione with stable disulfide bond (group 2). Glutoxim causes apoptosis in malignant cells but not in normal lymphocytes. After 3 chemotherapy courses, mutant p53 was present in 12 of 32 patients (23%) in group 1, but only in 5 of 43 (11.6%) patients in group 2. Together with the disappearance of mutant p53, a diminution of hypomethylated species of LINE mobile elements was found. Our data, therefore, demonstrated a positive effect of Glutoxim therapy.

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Cirulating cell-free Epstein-Barr virus DNA levels in patients with EBV-associated lymphoid malignancies Kenny L.K. Le, Lisa Y.S. Chan, Wing-Yee Chan, Philip J. Johnson, Y. M. Dennis Li Departments of Clinical Oncology, Chemical Pathology and Anatomical & Cellular Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong SAR, China

Cell-free Epstein-Barr virus (EBV) DNA has been detected in the plasma and serum of patients with Hodgkin’s disease, post-transplant lymphoproliferative disease (PTLD), and acquired immunodeficiency syndrome-related lymphoma. We have recently described a strong correlation between plasma EBV DNA levels and therapeutic response in 9 patients with EBV-associated lymphoid malignancies during therapy. The present study is aimed to update our results and to determine the role of measuring plasma EBV DNA in disease monitoring. Using a real-time quantitative polymerase chain reaction assay, we studied the plasma EBV DNA levels in 35 patients with EBV-associated lymphoid malignancies (14 Hodgkin’s disease, 14 natural killer/T-cell lymphoma (PTLD and 3 Burkitt’s lymphoma) at presentation and during therapy. Plasma EBV DNA was detected in 28 patients at presentation (median: 1,968 copies/ml; interquartile range: 150-1,172,122 copies/ml) but not in any of 35 healthy control subjects (p <0.0001). Serial measurements of plasma EBV DNA levels were performed in 21 of the patients during the course of therapy. Sixteen patients who responded to therapy demonstrated a significant reduction of plasma EBV DNA to low or undetectable levels. One patient with progressive disease during therapy demonstrated rapid increase of plasma EBV DNA levels, whereas in one patient with persistent disease the plasma EBV DNA levels remained elevated. We concluded that plasma EBV DNA is a useful tool for monitoring therapeutic response in EBV-associated lymphoid malignancies. As plasma EBV DNA levels correlate well with the therapeutic response, such analysis may be a valuable tool for monitoring clinical progress.

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Cirulating Epstein-Barr virus DNA in the serum of patients with gastric carcinoma Y.-M. Dennis Lo, Wing-Yee Chan, Enders K.W. Ng, L.S. Chan, Paul B.S. Lai, S.C. Sydney Chung Departments of 1Chemical Pathology, 2Anatomical & Cellular Pathology and 3Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China

We investigated the detectability of Epstein-Barr virus (EBV) DNA in the serum of gastric carcinoma patients in Hong Kong. Previous data have shown that approximately 10% of gastric carcinomas in Hong Kong are associated with EBV. We recruited 51 patients with gastric carcinoma from the Prince of Wales Hospital, Hong Kong and blood samples were taken from these individuals prior to surgery. Following operation, the resected tumor samples were subjected to in-situ hybridization for EBER (small EBV encoded RNA). In this cohort, 5 gastric carcinomas were EBER-positive and 32 cases were negative. In the remaining 14 cases, the tumor cells were EBER-negative but there were occasional infiltrating lymphocytes which were EBER-positive. These 14 cases were classified as having ‘background positivity’. Serum EBV DNA was detected in only one of the EBV-seronegative gastric carcinoma patients (EBV DNA concentration: 1063 copies/mL). No serum EBV DNA was detected in any of the 32 negative cases. Cases demonstrating ‘background EBER positivity’ had an intermediate median serum EBV DNA concentration of 50 copies/mL. Our data thus indicate that serum EBV DNA reflects tumoral EBER status and opens up the possibility that circulating EBV DNA may be used as a tumor marker for the EBER-positive gastric carcinomas. The biological and clinical significance of serum EBV DNA detectability in the cases with ‘background EBER positivity’ requires further elucidation in future studies.

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Detection of circulating gastric cancer associated antigen MG7-Ag in human sera with McAb and bisppecific ScFv-streptavidin fusion protein-based immuno-PCR technique Wen-Chao Liu, Jian-Rong Chen, Le-Daaming Fan and Xiao-Yong, Center of Oncology, Xijing Hospital, The Fourth Military Medical University, Xi’an 710032, China.

Background: In 1994, a novel sensitive method termed Immuno-PCR for the detection of the gastric cancer associated antigen MG7-Ag in gastric cancer cell line KATO III was reported.

Methods: The sera of patients were immobilized on wells and a specific DNA molecule, which could be amplified by PCR, was employed as a marker. The chimeric molecule used in the detecting system consists of two forms. One is made by inserting a free streptavidin which is capable of binding the biotinylated McAb and biotinylated recombinant plasmid DNA marker, respectively. The second form is that a genetically prepared fusion protein of ScFv and streptavidin. The bound biotinylated DNA was amplified by PCR. For comparison, CEA, CA50, CA19-9 and TAG-72 were detected concurrently by radioimmunoassay and immunoradiometric assay in the same serum samples from 86 patients with gastric cancer and 83 with relevant benign diseases of stomach.

Results: Using the Immuno-PCR assay, positive results were obtained in 164 out of 198 cases with gastric cancer (82.8%). The rates of positivity in other cancers were: esophageal ca. (15/86, 17.4%), colon cancer (40/90, 44.4%), liver ca. (30/84, 36.5%), ovarian ca. (1/45, 2.2%), uterine ca. (0/27, 0%), and lung ca. (4/46, 6.1%). The positive results obtained from those with benign diseases were: peptic ulcer (6/78, 7.7%), chronic gastritis (7/118, 5.9%), chronic colitis (2/60, 3.3%) and normal blood donor (2/236, 0.8%).

Conclusions: The application of Immuno-PCR in the serological diagnosis of carcinoma has the significant advantages that it can be readily applied to the clinic as well as can serve as a potential screening tool in mass survey of high-risk population for gastric cancer.

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Etiological association of genetic instability and mismatch repair alterations in non-small cell lung cancer Y.-C. Wang1, J.-W. Chang2, Y.-P. Lu1, Y.-C. Chen2, C.-Y. Chen3, J.-T. Chen3 Department of Biology, National Tsing Hua University, Taichung; 1Institute of Toxicology, Chung Shan Medical College, Taichung; 2Department of Chest Medicine and Pathology, Taichung Veterans General Hospital, Taichung, Taiwan, R. O. C.

To investigate the etiological association of genetic instability and mismatch repair alterations in non-small cell lung cancer (NSCLC), we investigated the presence of the microsatellite instability (MI) of the hMSH2 and hMLH1 genes in 68 NSCLC patients. We also explored the association between acquisition of a replication error phenotype and mismatch repair alteration. The expression of...
Circulating Fetal DNA and Fetal Cells

Oral Presentations

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Quantitative analysis of fetal DNA in maternal plasma in pathological conditions associated with placental abnormalities L Cremonesi1, A Vasallo1, M Ferrari1, A Ferrari2, F Lagona3, L Valsecchi1, MT Castiglioni1, R Caïrone2, G Almirante2, L Maniscalco2, L Danti3, A Lojacono3, C Slompo3, L Touliatou4 and C. Metaxotou4

Kolialexi1, G. Th. Tsangaris2, A. Mavrou1, A. Antsaklis3, F. Tzortzatou2, V. A.

Our data imply that care should be taken when considering diagnostic applications of this technology.

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Both maternal and fetal cell free DNA in plasma fluctuate Sinuhe Hahn, Xiao Yan Zhong, Martin R. Bürk, Carolyn Troeger, Anjeung Kang and Wolfgang Holzgreve Dept. OB/GYN, University of Basel, Schanzenstrasse 46, CH 4031, Basel, Switzerland.

Real time quantitative analyses of extracellular fetal and maternal DNA in maternal plasma have indicated that the levels of these molecular analytes are elevated in pregnancies with preeclampsia. In addition elevations in free fetal DNA concentrations were noted in pregnancies affected by preterm labor and in those with aneploid fetuses.

The possible diagnostic use of these new observations depends on the stability of the levels of these analytes in the maternal circulation. For this purpose we have examined the levels of total free DNA in blood samples taken longitudinally from normal healthy donors as well as the quantities of free fetal and free maternal DNA in samples obtained from normal pregnancies. Our data show that MI plays a significant role in NSCLC tumorigenesis in Taiwan. In addition, MI may play a role in the pathogenesis of MI. We have previously shown that multiple paternally inherited fetal loci, such as the RhD and SRY genes, can be determined in a non-invasive manner using free fetal DNA in the maternal circulation by multiplex PCR. In order to optimise this procedure for diagnostic applications we have developed an efficacious multiplex Taqman assay. By the use of real time PCR we have also observed that the levels of free fetal DNA are elevated in pregnancies affected by preeclampsia, similar to what we have observed for fetal cell traffic. We, however, also observed that the levels of free fetal DNA were elevated in preeclamptic pregnancies and that the increments in both free DNA species correlated to each other and to the degree of disease severity. Our data, therefore, indicate that in preeclampsia both the fetal and maternal compartments are affected in a co-ordinated manner.

Since elevated numbers of fetal cells have been noted in pregnancies with certain aneuploid fetuses, we have quantified free fetal DNA levels in such pregnancies. Our data showed that free fetal DNA levels are elevated in pregnancies with trisomy 21 fetuses, but not in those with trisomy 18 fetuses. In conclusion, the quantitative difference of FM observed in whole blood from women with SSc compared to controls appears to be cellular in origin. Further studies are necessary to determine which cell populations are specifically involved.

Circulating Fetal DNA and Fetal Cells

Poster Presentations

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Comparison between plasma and PBMC to quantify fetal microchimerism by real time quantitative PCR in healthy women and women with scleroderma NC Lamberti, YMD Lo, TD Erickson, TS Tylee, W. Leisenring, D Furst and JL Nelson. Fred. Hutchinson Cancer Res Ctr, Seattle USA; The Chinese University of Hong Kong SAR, Virginia Mason Res Ctr and University of WA, Seattle USA.

More frequent and quantitatively greater fetal microchimerism (FM) was found in whole blood from parous women with scleroderma (SSc) compared to healthy controls. However, it is not known whether this quantitative difference is due to circulating cells of fetal origin or free fetal DNA in the peripheral circulation reflecting damage in disease-affected tissues.

To distinguish the origin of FM, we tested PBMCs and plasma from healthy women and women with SSc who had previously given birth to a son, by a sensitive real time quantitative PCR assay for the Y-chromosome specific sequence DYS14. Preliminary results indicate FM was higher in PBMCs from patients compared to controls with a range of 0.00-21.15 and 0.00-1.32 copies of fetal DNA/ million maternal DNA copies respectively. PBMCs results were significantly higher in patients compared to controls (p=0.04, two-sided Student’s t-test). In contrast, there was no quantitative difference of FM between patients and controls in plasma (p=0.84, two-sided Student’s t-test).

In conclusion, the quantitative difference of FM observed in whole blood from women with SSc compared to controls appears to be cellular in origin. Further studies are necessary to determine which cell populations are specifically involved.

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Free DNA in plasma: prenatal diagnosis of fetal genetic loci and pregnancy related disorders Sinuhe Hahn, Xiao Yan Zhong, Martin R. Bürk and Wolfgang Holzgreve Dept. OB/GYN, University of Basel, Schanzenstrasse 46, CH 4031, Basel, Switzerland.

We have previously shown that multiple paternally inherited fetal loci, such as the RhD and SRY genes, can be determined in a non-invasive manner using free fetal DNA in the maternal circulation by multiplex PCR. In order to optimise this procedure for diagnostic applications we have developed an efficacious multiplex Taqman assay.

By the use of real time PCR we have also observed that the levels of free fetal DNA are elevated in pregnancies affected by preeclampsia, similar to what we have observed for fetal cell traffic. We, however, also observed that the levels of free fetal DNA were elevated in preeclamptic pregnancies and that the increments in both free DNA species correlated to each other and to the degree of disease severity. Our data, therefore, indicate that in preeclampsia both the fetal and maternal compartments are affected in a co-ordinated manner.

Since elevated numbers of fetal cells have been noted in pregnancies with certain aneuploid fetuses, we have quantified free fetal DNA levels in such pregnancies. Our data showed that free fetal DNA levels are elevated in pregnancies with trisomy 21 fetuses, but not in those with trisomy 18 fetuses, remarkably paralleling previous reports on fetal cell numbers.

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HELLP-syndrome as complication of preeclampsia increases the amount of cell-free fetal and maternal derived DNA in maternal plasma D.W. Swinkels1, J.B. de Kok1, E. Wiegierink2, P.L.M. Zusterzeel2 and E.A.P. Three1

1Departments of Clinical Chemistry and 2Obstetrics and Gynecology, Universi-

In a previous study we demonstrated that apoptosis increased according to gestational age, accounting partly for the presence of free fetal DNA in maternal plasma and serum. Using simultaneous TUNEL assay and FISH analysis we identified the fetal origin of part of the apoptotic cell population but very few TUNEL+ cells showed hybridization signals since they were in a late apoptosis stage and nuclei were distorted.

In the present study apoptotic cell population was identified immunocytochemically using Annexin V, a marker of cells in early stage of apoptosis. Apoptosis was determined in mononuclear cells isolated from the peripheral blood of 20 pregnant women in the 16th-19th week of pregnancy. The mean apoptosis rates using Annexin V was 6.8±0.2% (range 4.2-8.1%).

FISH using X and Y chromosome specific probes was applied in 11 cases known to be carrying male fetuses. The proportion of apoptotic cells showing X/Y signals was 7.8% (range: 5-12%) while 75% of Annexin V+ cells showed hybridization signals.

Although our results are still preliminary, it seems that use of Annexin V antibody to detect apoptotic cell population improves FISH analysis and allows a more accurate determination of the proportion of fetal cells among the apoptotic cell population.

Poster Presentations
Fetal DNA diagnosis from maternal blood: PEP-TaqMan PCR analysis of a single nucleated erythrocyte (NRBC) K. Ikawa, K. Yamafuji*, T. Ukita*, S. Kuwabara*, T. Igarashi**, H. Takabayashi***, Takeda Health Service Association, Ueda Hospital†, Fetal cells and DNA are known to circulate in maternal blood. It is uncertain how fetal cells were transferred into maternal circulation, despite the fact that the maternal immune system could be induced to eliminate the fetal cells when fetal cells cross into maternal circulation. It is reported that nucleated erythrocytes (NRBC) in maternal blood, obtained at the time of delivery, can be identified using fluorescent PCR amplification of highly polymorphic short tandem repeat DNA probes. The advantages of PEP-TaqMan PCR system are high specificity and sensitivity. This system seems a powerful tool for fetal DNA diagnosis from maternal blood.

Elimination mechanism of fetal nucleated erythrocytes in maternal circulation Tetsuro Kondo, Akihiko Sekizawa, Akira Watanabe, Hiroshi Saito, Takashi Oka* Department of Obstetrics and Gynecology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, 142-8666, Japan

Objectives: To assess the possibility of fetal gender diagnosis with conventional PCR using maternal cord venous blood obtained at the 1st trimester and to quantify the fractional concentration of fetal DNA in them.

Materials and Methods: Blood samples were obtained from 81 pregnant women at 5 to 10 weeks of gestation. DNA was extracted with QIAamp blood kit. Conventional PCR of fetal gender diagnosis, 40 cycles of PCR were performed. The Y-specific DYS14 gene was used. To quantify the fractional concentration of fetal DNA in maternal serum, 50 cycles of real-time quantitative PCR of SRY and beta-globin genes were carried out, respectively.

Results: We found 40 pregnant women carrying a male fetus. The DYS14 sequence was detected in each serum sample collected from 38 of the male-bearing pregnant women (95%). The concentration of SRY gene ranged from 1.7 to 148.7 copies per ml (mean: 37.2), and the fractional concentration of fetal DNA in maternal serum ranged from 0.046 to 4.18% (mean: 1.01).

Conclusions: It is possible to diagnose the gender of a fetus with conventional PCR using maternal serum obtained at the early gestational periods. We also showed that the fractional concentration of fetal DNA in maternal serum obtained at the early gestational periods was about 1% on average.

Detection of fetal NRBCs in maternal blood of pregnant carriers of β-thalassemia using anti γ and anti ε monoclonal antibodies A. Mavrou1, A. Kolalevi1, A. Ambaklis2, X. Kriken2, A. Koratzi2, and G. Mystakidou3 1Medical Genetics, 2nd Department of Obstetrics and Gynecology, 2nd Department of Pediatrics, Athens University School of Medicine, 3rd Department of Obstetrics and Gynecology, University of Patras School of Medicine, Greece

Objective: Umbilical cord blood obtained at time of birth in umbilical cord blood contains fetal cells and DNA. umbilical cord blood is a source of hematopoietic stem cells for transplantation. Although the first clinical applications were encouraging, concern has been raised about contamination of umbilical blood by maternal cells, which might constitute a theoretical risk of graft versus host disease. The aim of this study was to assess the frequency of fetal cell contamination in umbilical cord blood using fluorescent PCR amplification of highly polymorphic short tandem repeat DNA markers.
Study Design: 57 mother/child pairs were tested for the presence of maternal DNA sequences in cord plasma. Maternal specific alleles were detected using PCR amplification of 9 highly polymorphic short tandem repeat markers (D21S11, D21S1411, D21S1412, D18S586, D18S555, MBPA and B, D13S631, D13S634).

Results: All 57 mother/child pairs were informative for the identification of uniquely maternal alleles. Maternal specific alleles were found in 42 of 57 (73%) cord plasma samples.

Conclusion: The results of our study demonstrated that maternal DNA is present in the majority of umbilical cord blood plasma samples. The technique described here might have applications in the screening of umbilical cord blood samples for the purpose of containing maternal genetic material.

Rapid determination of RhD zygosity by real-time quantitative polymerase chain reaction: implication for fetal RhD status determination by maternal plasma Rossa W.K. Chiu1, Michael F. Murphy2,3, Carrie Fidler4, James S. Wainscoat2, Y.M. Dennis Lo1

Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, 2Department of Haematology, John Radcliffe Hospital, Oxford, OX3 9DQ, United Kingdom and 3National Blood Transfusion Service, Oxford, United Kingdom

Despite the widespread use of prophylactic Rh immunoglobulin, Rh immunization during pregnancy and hemolytic disease of the newborn (HDN) still occurs. For the prenatal investigation of sensitized rhesus negative patients with and without detectable virus replication Hepatitis B virus RNA circulating in the blood of chronically infected cells from healthy individuals.

We established a RT-PCR-based amplification system for the detection of a chain reaction: implication for fetal RhD status determination by maternal plasma Rossa W.K. Chiu1, Michael F. Murphy2,3, Carrie Fidler4, James S. Wainscoat2, Y.M. Dennis Lo1

We established a RT-PCR-based amplification system for the detection of a panel of five different genes, known to be over-expressed in normal and malignant lung tissues, respectively. The aim of our study was to find out whether it is possible to use this system for the detection of extracellular, free circulating, tumor-related mRNA in the plasma/serum of patients with lung cancer, and in the cells from Ficoll-purified peripheral blood cells from the same patients. We analyzed a small number of patients with lung cancer (4 NSCLC, 1 SCLC pat.) for the expression of five markers, known to give a false positive signal when tested with RNA from Ficoll-purified peripheral blood cells from healthy individuals.

The messages for β-actin (control of integrity of the RNA) and CK-19 were detected in all analyzed sera and cells from patients with lung cancer (100% concordance). The expression of MAGE-2 and TFF-1 genes was not observed in any of the patients in both compartments. Expression of the PGP 9.5 gene was observed in the cells of all 5 patients, but mRNA in the serum was only detectable in one case. The hBNP B1 mRNA was detectable in 4/5 sera and in the cellular compartment from all patients. The clinical and biological meaning of these observations and their potential usefulness for patients with lung cancer has to be explored in larger sample studies.


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Hepatitis B virus mRNA circulating in the blood of chronically infected patients with and without detectable virus replication Qin Su1,2, Shu-Fang Wang4, Tian-En Chang5, Raul Breitkreutz1, Holger Hemmi6, Kunio Takegoshi7, Lutz Edler1, Claus H. Schröder1, 1Virus-Host Interactions, 2Biostatistics, Deutsches Krebsforschungszentrum, Heidelberg, Germany, 3Pathology and Clinical
Other Aspects of Circulating Nucleic Acids

Oral Presentations

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Since cell-free fetal DNA in maternal plasma has a short half-life we have examined whether it is eliminated from the circulation via the urinary system. No free fetal DNA was detected in any of the urine samples obtained from women pregnant with a male fetus, using either a nested PCR approach or a real-time assay for a single copy gene on the Y chromosome. On the other hand we were able to detect both recipient and donor derived cell free DNA in the urine of kidney transplant recipients, confirming a previous report by Lo and colleagues. By quantifying these two molecular analytes we also observe that increments can correlate with periods of graft rejection. However, no correlation was possible with other parameters such as creatinine clearance. Our data suggest that cell-free DNA may aid in the monitoring of kidney graft acceptance but that its use may be limited when considering prenatal diagnostic applications.

Detection of Plasmodium falciparum DNA in plasma S Gal1, C Fidler1, S Turner1, YMD Lo2, D Roberts1 and JS Wainscoat1

Plasmodium falciparum

DIAGNOSIS

DNA in plasma: the detection of parasite DNA in plasma. We have investigated the presence of Plasmodium falciparum DNA in the blood cells and plasma of patients with malaria.

8 blood samples of malaria patients and 8 blood samples of healthy volunteers were collected. Plasma was separated and DNA was extracted from both plasma and blood cells. DNA samples were subjected to a nested PCR for the small sub-unit rRNA gene of Plasmodium falciparum (Snowou et al) and to PCR of the human rRNA gene as a control.

All six cases positive for microscopy for Plasmodium falciparum were positive by PCR on DNA extracted from blood cells and plasma. Two cases negative for microscopy for malaria were positive by PCR - one case 7 days after successful treatment of malaria and one case at the onset of the malaria illness.

The quantitation of Plasmodium DNA in plasma may prove to be useful prognostic measurement in malaria, and the detection of Plasmodium falciparum DNA in archival stored plasma samples may enable the reconstruction of the recent historical evolution of the parasite genome.

Poster Presentations

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Extracellular DNA as prognostic criterion of radiation effects S.S. Sherlina

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At present, facts are accumulating in relation to increases in DNA level in blood plasma at a variety of pathological states and exposure of the body to extreme factors. However, the biological significance of this phenomenon remains unclear. At the same time a possible association of these alterations with activation, not only destructive, but adaptogenic processes under these conditions is discussed. Exposure to ionizing irradiation at various doses (from sub- to absolutely lethal) is a suitable model to search for differentiation criteria of radiation effects. It is established that after exposure of rats to irradiation, a dose-dependent increase in blood plasma DNA level with peak up to 2.5 h and normalization of these indices at the close of the first day occurs. Kinetics of the above processes decreases with increasing radiation dose. Besides this, the selectivity and specificity features of a part of genome presented extracellularly and also the genetic polymorphism are detected by restriction enzyme digest and blot hybridization analysis.

The found shifts are likely to be related to the disturbance of ligand-receptor interactions with DNA-linking proteins, known for liver cells and significant in the homeostatic sense. In this regard, the coincidence of time periods of DNA level normalization in blood plasma and resynthesis of receptors proteins (3 days), in particular, for receptor cytokines is of interest. The extent of DNA structure polymorphism apparently determines the length of the normalization period in blood plasma. The answer as to whether it is a critical process for survival or serves as an adaptogenic process, remains unclear.

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Alterations of blood plasma DNA fractional composition due to inflammatory processes K.V. Goranin, A.S. Belokhvostov

Russian Military Medicine Academy, St. Petersburg, Russia

DNA was obtained by phenol deproteinization of blood plasma and precipitation with ethanol. Fractional composition of DNA samples was revealed by agarose gel electrophoresis. Healthy donor blood plasma DNA does not contain low-molecular fractions. Plasma DNA of rheumatoid, cancer, hepatitis and other patients sometimes carry low-molecular fractions. These fractions are of size equal to DNA mononucleosome or oligonucleosome. No correlation between diagnosis and low-molecular DNA macromolecule was noted. Considerable correlation between low-molecular DNA appearance and conventional inflammatory markers was found.

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Plasma DNA as a marker for post-traumatic acute lung injury T.H. Rainer Accident and Emergency Medicine Academic Unit, Room G05, Cancer Centre, The Prince of Wales Hospital, Shatin, Hong Kong

Highly sensitive and specific predictors of post-traumatic complications allow early risk-stratification of trauma patients. The purpose of this study was to investigate the potential of cell-free (plasma) DNA as an early predictor of post-traumatic acute lung injury (ALI). Ethical approval was obtained from the Chinese University of Hong Kong Research Ethics Committee. Blood withdrawn from patients within 4 hours of injury (median 60 minutes; range 30 - 240; IQR 50 – 90) was analysed using real time polymerase chain reaction (PCR) to quantify the plasma DNA. Median plasma DNA results from patients (median age 37 years, range 12 to 86; 83% male) and controls were as follows: control (n = 30) – 3154 genome-equivalents/mL, major (n = 37, ISS ≥ 16) – 18130 genome-equivalents/mL (Kruskal-Wallis test p <0.0001; a 58-fold increase between major and control values). In the major trauma group alone, median plasma DNA values in ALI-ve and ALI+ve subjects was 144563 genome-equivalents/mL and 398225 genome-equivalents/mL (Mann-Whitney (MW) test p<0.05). The area under the Receiver Operator Characteristic (ROC) curves for ALI was 0.88 (95% confidence limits (CI) 0.79 – 0.94). For ALI, plasma DNA values of ≥230 000 genome-equivalents/mL gave a sensitivity of 100% (CI 100.0 – 100.0), a specificity of 81% (CI 70.6 – 89.0) and a positive likelihood ratio of 5.27. Cell-free plasma DNA quantified using real time PCR may be used to whether a patient is likely to develop post-traumatic ALI.

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Possible origin of extrachromosomal DNA from human lymphocytes treated in vitro with phytohemaggutinin J. John S. Chen and Maria Giulia Menesini

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It is well known that lymphocyte responds to the chemical/pathological challenge by synthesizing epitomes/extrachromosomal DNA (eDNA) (Patel et al., N. Engl. J. Med. 2000, 342:1325). For T-lymphocytes, phytohemaggutinin (PHA) is a mitogen and can stimulate T-cells to increase the synthesis of epitomes and the release of DNA (Rogers and Kerstiens, Immunol. 1983, 126:703), interleukin-2 and other factors (Oddera et al, Ann. Allergy Asthma Immunol. 1995, 75:333). However the origin of this eDNA is largely unknown. In this study attempts were made to shed light on this enigma. Lymphocytes obtained from a healthy donor were cultured in F10 medium in the presence of PHA/lectin (Sigma) at 37°C, 5% CO2 for 72 hrs. A bovine acrosomal DNA obtained from a healthy donor were cultured in F10 medium in the presence of PHA/lectin (Sigma, 37°C) and the hybridization analysis. Results show that a strong fluorescence in situ hybridization (FISH) analysis. Results show that a strong fluorescence was localized to the nucleoid and in cytoplasm of some T-cells. This preliminary result seems to confirm the observation made in previous immunocytochemical study (Chen et al, 12° CAPE Convention, July 2000 Brussels, pp 134-135) suggesting that the lymphocyte eDNA would possess some common genes with bovine acrosomal DNA and was probably organized/synthesized in the nucleolus.