van der Pluijm, Gabri [49]

Monitoring metastatic behavior of human breast cancer cells in mice with species-specific PCR and bioluminescent reporter imaging

G. van der Pluijm[1], A. Wetterwald [2], B. Sijmons[1], E. Gautschi[2], I. Que[1], B. Stadler[3], G. Thalmann[2], M. Cecchini[2] & C. Löwik[1]

[1]*Leiden University Medical Center, Dept. of Endocrinology, Leiden, The Netherlands*
[2]*Gene Therapy Laboratory, Department of Clinical Research and Urology Clinic, University Hospital, Bern, Switzerland*
[3]*Institute of Immunology and Allergology, University Hospital, Bern, Switzerland*

Breast cancer metastasizes frequently to the skeleton and causes considerable morbidity. Injection of human MDA-MB-231 breast cancer cells into the left heart ventricle of immunodeficient mice produced multiple osteolytic bone lesions and soft tissue metastases. However, micrometastases are not readily detectable and more sensitive methods are required to detect minimal disease states. Here we describe the use of species-specific PCR (ssPCR) and bioluminescent reporter imaging (BRI) for monitoring the tumor metastatic *in vivo*. We used a CCD camera connected to the Argus-20 image processor (C-2400/VIM, Hamamatsu) to monitor bone metastasis by tumor cells transfected with the luciferase reporter gene. MDA-MB-231 cells were stably transfected with a pCMV-plasmid containing the firefly luciferase gene (MDA-231/luc+). After intracardiac inoclation with MDA-231/luc+ cells, the mice were monitored weekly for the development of bone and soft tissue metastases. For this, the mice were anaesthesized, injected with D-luciferin and photon emission was measured. Micrometastases developed predominantly in the hind limbs and the axial skeleton. Distinct photon emission was detected already after 24 days in bone (detection limit ± 10.000 cells). ssPCR revealed significantly increased expression of VEGF-A, -B and PTHrP by the cancer cells in bone metastases when compared to soft tissues metastases suggesting a causal role in the observed preferential skeletal metastasis. In conclusion, BRI and ssPCR can be used for quantitative detection and growth of micrometastases and may become extremely useful to study the pathogenesis of bone metastasis in living animals, to monitor expression of targeted gene vectors and to determine the efficacy of novel therapeutic agents.

Vandesompele, Jo [50]

Integration of suppression subtractive hybridization, laser capture microdissection and microarray analysis for identification of genes implicated in neuroblastoma pathogenesis

Jo Vandesompele[1], Nadine Van Roy[1], Geneviève Laureys[2], Katleen De Preter[1], Geert Berx[3], Kristin Strumane[3], Anne De Paepe[1], Frans Van Roy[3] & Frank Speleman[1]

[1]*Center for Medical Genetics, University Hospital, Ghent, Belgium*
[2]*Department of Pediatric Oncology, University Hospital, Ghent, Belgium*
[3]*Department of Molecular Biology, VIB, Belgium*

Neuroblastoma is the most frequent extracranial solid tumor in children, showing remarkable clinical and genetic heterogeneity. To make further progress in understanding the genetic basis of neuroblastoma we have applied suppression subtractive hybridization for the identification of differentially expressed transcripts. We analyzed two neuroblastoma cell lines belonging to different genetic subgroups, respectively with and without 1p and 11q deletion and *MYCN* amplification. Northern blot analysis and real-time quantitative polymerase chain reaction with reverse transcription showed differential expression in 75% of the selected genes. We identified both rare and highly abundant differential transcripts. Known and previously unreported genes from the 2p23 and 2p13–14 amplified regions in IMR32 were efficiently selected. From the present set of suppression subtractive hybridization clones, we will select genes relevant to normal neurogenesis through expression analysis of normal fetal neuroblasts isolated by laser capture microdissection (Arcturus, PixCell II). We will select genes implicated in particular subsets of neuroblastoma by microarray analysis (Affymetrix 417) of a large panel of neuroblastoma cell lines and primary tumors.

Vandesompele, Jo [51]

Quantification and normalization of gene expression using SYBR Green I real-time RT-PCR

Jo Vandesompele, Johanna Iso-Oja, Anne De Paepe & Frank Speleman

Center for Medical Genetics 1K5, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium

We have developed a two-step, real-time, quantitative assay, using the polymerase chain reaction with reverse transcription (RT-PCR) and based on SYBR Green I monitoring of PCR product accumulation, for quantification and normalization of gene expression levels. Because housekeeping gene expression can vary considerably among cell types or experimental conditions, our procedure uses multiple internal control genes for more accurate normalization of expression data, compared with traditional use of only one housekeeping gene. Owing to extensive accumulation of primer-dimers when no template control is used during one-step RT-PCR, we introduced a two-step protocol to eliminate this problem. This study further illustrates the prerequisite of DNase treatment of RNA samples before complementary DNA synthesis. The treatment also results in a significantly facilitated primer design for RT-PCR, as the positions of the primers are no longer important to control for genomic contamination. Real-time quantitative RT-PCR of DNase-treated samples and normalization using multiple internal controls is the method of choice for sensitive, accurate and large-scale measurements of gene expression levels.

Varley, Jenny [52]

Genotype and phenotype in carriers of germline *TP53* mutations

Jenny Varley[1], Gareth Evans[2], Jillian Birch[3] & Anna Kelsey[4]

[1]*CRC Cancer Genetics Group, Paterson Institute for Cancer Research, Manchester, UK*
[2]*Department of Medical Genetics, St. Mary's Hospital, Manchester, UK*
[3]*CRC PFCRG and* [4]*Department of Histopathology, Royal Manchester Children's Hospital, Manchester, UK*

We collected data on a large cohort of families with features of Li-Fraumeni syndrome (LFS). To date we have identified germline *TP53* mutations in 28 families: 20 of 25 classic LFS and 8 of 20 Li-Fraumeni–like. In addition we have identified germline mutations in 12 other individuals or families with tumors suggestive of LFS. Most mutations are missense or nonsense, but 7 (18%) are splicing mutations. A survey of the literature showed that this is a considerably higher propor-

tion than has been identified by other groups. The proportion of germline TP53 splicing mutations in LFS families may be markedly under-reported. We have examined the biological consequences of these mutations to determine the functional properties of the mutant p53 and to confirm that they are causative mutations. Previous studies have indicated that five main component tumors are associated with LFS: sarcomas, brain and breast tumors, leukemia and adrenocortical carcinoma. We have analyzed the observed incidence of cancers in first- and second-degree relatives of probands in comparison with that expected, on the basis of United Kingdom national cancer rates. The observed pattern of cancers in germline *TP53* carriers was highly significantly different from that expected across all ages. Carcinoma of the breast, brain and spinal cord tumors, bone and soft-tissue sarcomas, adrenocortical carcinoma, Wilms tumor and malignant phyllodes tumors were all strongly associated with a germline *TP53* mutation. Common carcinomas (such as lung, colorectal, bladder, prostate, cervix and ovarian) did not occur to excess. Germline *TP53* mutations are associated with a subset of specific, mainly rare cancers.

Vidal, Marc [53]

The gene number dilemma: direct evidence for at least 19,000 protein-encoding genes in *Caenorhabditis elegans* and implications for the human genome

David E. Hill, J. Reboul[1], P. Vaglio[1], N. Tzellas[1], C. Jackson[2], T. Moore[2], Y. Kohara[3], J. Thierry-Mieg[4], D. Thierry-Mieg[4], J. Hitti[5], L. Doucette-Stamm [5], J. Hartley[6], G. Temple[6], M. Brasch[6], D.E. Hill[1] & M. Vidal[1]

[1]*Dana-Farber Cancer Institute and Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA*
[2]*Research Genetics, Huntsville, Alabama, USA*
[3]*Center for Genetic Resource Information, National Institute of Genetics, Shizuoka, Japan*
[4]*NCBI, NIH, Bethesda, Maryland, USA*[5]*Genome Therapeutics Corp., Waltham, Massachusetts, USA*
[6]*Life Technologies Inc., Rockville, Maryland, USA*

Estimates of the number of genes for organisms with a wide range of biological complexity will emerge from genome sequencing projects. This information will be crucial to correlate gene number with biological complexity. Computer-based annotations of the genome sequences of *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* predict approximately 6,000, 19,000 and 13,600 genes, respectively. It is seemingly paradoxical that *C. elegans* contains 50% more genes than *D. melanogaster* and that the latter only contains twice as many genes as *S. cerevisiae*. However these numbers should be considered with caution until gene predictions are verified using experimental approaches. To experimentally verify gene predictions, we PCR amplified predicted open reading frames from a *C. elegans* cDNA library, cloned them and generated an ORF sequence tag (OST) for each of them. In a random sample (n = 1,200) of the ~10,000 genes predicted from the entire *C. elegans* genome but experimentally unverified so far, 90% of these predicted genes could be unambiguously verified by OST sequencing. When added to the list of ~9,500 genes previously verified by individual laboratories and/or by EST sequencing, our data confirm the existence of at least 19,000 protein-encoding genes in C. elegans. We suggest that ORFeome cloning coupled with OST analysis could be used to verify gene number predictions for other organisms. Furthermore, the demonstration and identification of 10,000 novel genes provides a resource for comparative genomics and gene expression studies. We also discuss our findings in the context of the current debate on the human gene number.

Villuendas, Raquel [54]

Expression microarrays in the prediction of treatment resistance: The CNIO program

Raquel Villuendas, Jose Palacios, Manolo Morente, Orlando Dominguez, Javier Benitez & M.A. Piris

Centro Nacional de Investigaciones Oncologicas, 28220 Madrid, Spain

The Molecular Pathology Program of the Spanish National Cancer Research Center (CNIO) performs molecular analyses of human tumors, allowing prediction of the treatment response to commonly used drugs and at the same time permitting the identification of relevant cancer genes. Biological therapy has so far been based on the study of unique genes or proteins, such as the administration of tamoxifen to endoplasmic reticulum positive tumors, Herceptin for cancer arising from overexpression of c-*erb-B2,* Cetuximab for cancers associated with overexpressed epidermal growth factor, Rituximab in CD20-positive lymphomas or RAR in progressive multifocal leukoencephalopathy. Although such biologically oriented treatment is making significant progress against different tumor types, it ignores the fact that cancer is a social and multigenic disease. The more common forms of cancer are the result of complex genetic alterations that allow the tumor cells to develop multiple capacities, such as tissue invasion and metastasis, angiogenesis promotion, unlimited replication, apoptosis escape, production of autonomous growth signals, insensitivity to cell cycle control signals and generation of genomic instability. One of the goals of the Molecular Pathology Program is to contribute to an individualized therapeutic strategy, according to the multiple and varied characteristics of the disease. The CNIO is analyzing resistance to commonly used drugs, through molecular study of targets and messenger RNA expression analysis using the microarray technology. The CNIO is also sponsoring the creation of a network of tumor banks in leading hospitals, which will allow massive application of these molecular techniques to large series of patients, diagnosed and treated using standardized protocols. Current projects include the following: (1) A study of the molecular mechanisms of therapeutic failure in cutaneous T-cell lymphoma and malignant melanoma treated with interferon-α, PUVA (a combination of psoralen and long-wave ultraviolet radiation) or both. The objective of this project is to identify the genes involved in interferon-α resistance in myelofibrosis and malignant melanoma. (2) Development of a complementary DNA biochip for the analysis of molecular changes associated with treatment resistance in breast cancer. The CNIO is open to establishing collaborations with other oncology groups to perform integrated analyses of the molecular factors predictive of treatment resistance.

Vinals, Carla [55]

Discovery of new potential antigens for cancer immunotherapy using "custom tissue" screening of a database of expressed sequence tags

Carla Vinals, Swann Gaulis, Jean-Pol Cassart & Thierry Coche

SmithKline Beecham Biologicals, 89 rue de l'Institut, 1330 Rixensart, Belgium

Immunotherapeutic approaches to fighting cancer are based on the principle of mounting an immune response (either an antibody response or a cytotoxic or T-helper response) against a self-antigen expressed by the tumor cells. To reduce potential autoimmunity side effects, the antigens used should be as tumor-specific as possible. A complementary approach to experimental tumor antigen discovery is to screen the human genome *in silico,* particularly the databases of expressed sequence tags, in search of tumor-specific and tumor-associated antigens. We describe a new method of 'customized' screening of a database of expressed