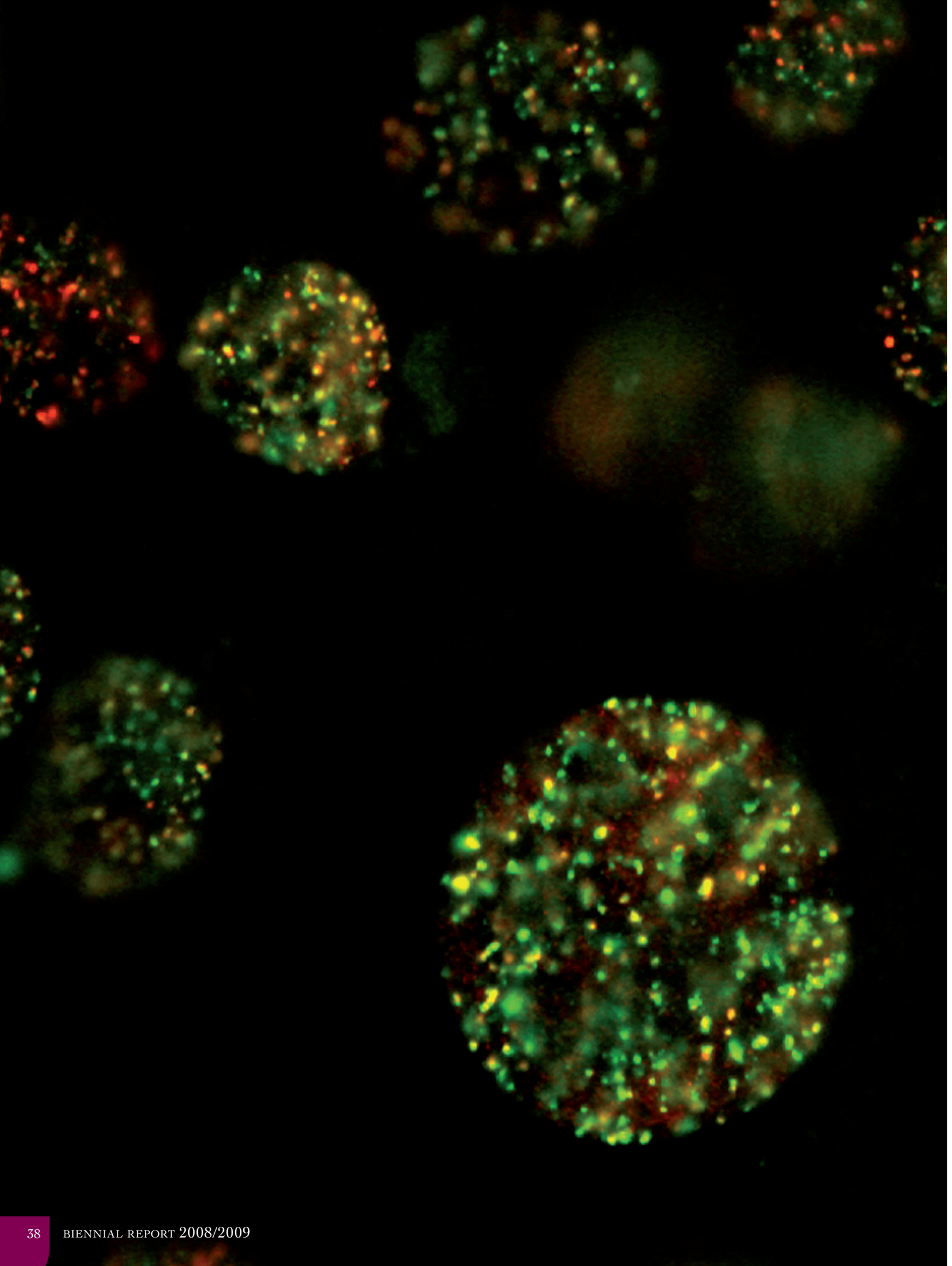
Section of Mechanisms of Carcinogenesis (MCA)

Section Head:
Dr Pierre Hainaut

The overall aim of the Section is to contribute to cancer prevention and control through a better understanding of mechanisms of carcinogenesis. This includes investigating interactions between the environment, the genome and the epigenome. Most of the Section's work involves translational studies on biomarkers of effects of environmental exposures and biomarkers of early cancer, focusing on cancers common in low-resource countries, such as hepatocellular carcinoma (HCC), squamous cell carcinoma of the aero-digestive tract (SCC) and breast cancer.

Highlights of the Section's work during this biennium include (1) the development of techniques and processes that allow the application of multi-loci mutation and epigenetic studies to large, molecular pathology and molecular epidemiology studies; (2) novel lines of mechanistic research on the contribution of TP53 mutations to specific cancers (lung, breast, liver) and on the molecular basis of epigenetic regulation of stem cells, based on the use of elaborated *in vitro* cell culture systems; (3) the development and coordination of an international consortium on liver cancer (International Liver Cancer Study, http://ilcs.iarc.fr/); and (4) further studies on the coordination of molecular databases, including further development of the IARC TP53 database (http://www-p53.iarc.fr) and establishment of a pilot for an international cancer epigenetics database. The Section has also carried out the development and management of a large biobanking infrastructure at IARC that has gained international recognition, in particular through the publication of Guidelines and Standard Protocols now recognised as a worldwide standard for biobanks.



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Epigenetics: an emerging field in molecular carcinogenesis

The field of cancer epigenetics has become increasingly "mainstream", as it promises to further advance our understanding of the etiology of human cancer and mechanisms of carcinogenesis, and to facilitate the development of novel strategies for cancer detection, treatment and prevention. The intrinsic reversibility and ubiquity of epigenetic changes in virtually all types of human cancer make them attractive subjects for biomarker discovery and strategies for cancer prevention. The Epigenetics Group (EGE) conducts both mechanistic studies and epigenetic profiling, aiming to gain a better mechanistic understanding of tumourigenesis and to discover and validate new epigenetic biomarkers. This programme exploits new concepts in cancer epigenetics and recent technological advances in epigenetics and epigenomics, and is carried out in close collaboration with IARC laboratory scientists and epidemiologists as well as external groups. EGE activities can be divided broadly into three major areas: (1) studies aiming to elucidate the role of epigenetic changes induced by major risk factors in specific human cancers, (2) studies aiming to investigate epigenetic changes for the mechanistic understanding of cancer development and progression, and (3) studies aiming to discover and validate new epigenetic biomarkers.

DNA methylation changes in lung cancer and their association with environmental risk factors

We have applied quantitative profiling of DNA methylation in a large panel of cancer-associated genes in a case–control study of lung cancer. Our analyses revealed a high frequency of aberrant hypermethylation of MTHFR, RASSF1A and CDKN2A in lung tumours as compared to control blood samples, whereas no significant increase in methylation levels of GSTP1 and CDH1 was observed, consistent with the notion that aberrant DNA methylation occurs in a tumour-specific and gene-specific manner (Vaissière et al., 2009a). Importantly, tobacco smoking, sex, and alcohol intake had a strong influence on the methylation levels of distinct genes (RASSF1A and MTHFR), whereas folate intake, age and histological subtype had no significant effect. We observed a strong association between MTHFR hypermethylation in lung cancer and tobacco smoking, whereas methylation levels of CDH1, CDKN2A, GSTP1 and RASSF1A were not associated with smoking, indicating that tobacco smoke targets specific genes for hypermethylation. We also found that methylation levels in RASSF1A, but not the other genes under study, were influenced by sex, with males showing higher levels of methylation. This study identifies aberrant DNA methylation patterns in lung cancer and thus exemplifies the mechanism by which environmental factors may interact with key genes involved in tumour suppression and contribute to lung cancer (Vaissière *et al*., 2009a).

Methylome analysis reveals deregulation of specific pathways in putative breast cancer stem cells and human sporadic breast tumours

Growing evidence supports the existence of a subpopulation of cancer cells with stem cell characteristics within breast tumours. We used the mammosphere model combined with DNA methylation bead arrays to characterise the epigenetic mechanisms involved in the regulation of developmental pathways in putative breast cancer stem cells. Our results revealed that these cells exhibit distinct CpG promoter methylation profiles in a specific set of genes, including those involved in Jak-STAT and T-cell receptor signalling pathways. Remarkably, aberrant methylation of Jak-STAT pathway gene promoters was also observed in human breast cancer samples relative to its matching surrounding tissue, and hypermethylation in tumours was consistently correlated with reduced gene expression of Jak-STAT-related transcripts. These results favour the concept that the expression of cancer stem-like pathways and the establishment and maintenance of the defining properties of cancer stem cells are orchestrated by epigenetic mechanisms (Hernandez *et al*., submitted).

DNA methylation profiles as potential biomarkers in hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a malignancy characterised by late detection and fast progression, and epigenetic disruption may be the cause of molecular and clinicopathological distinction of subsets of HCC tumours. To further investigate this possibility, we characterised the changes in promoter methylation in a series of HCC tumours and their respective surrounding tissue. A wide panel of cancer-related gene promoters (1505 CpG sites in 807 gene promoters) was analysed using bead array technology (in collaboration with Florence Le Calvez-Kelm [GEN/GCS] and Sean Tavtigian [GEN/GCS]), and CpG sites were selected according to their ability to classify clinicopathological parameters. An independent series of HCC tumours and matched surrounding tissue was used for validation of the signatures. We identified a signature that distinguished HCC from surrounding tissue and from other tumour types. Differentially methylated promoters were significantly enriched in the Wnt, TGF-beta, Hedgehog and Notch signalling pathways. The results also revealed a set of genes aberrantly methylated in HCC, including imprinted genes. In addition, methylation of an independent panel of gene promoters was strongly correlated with survival after cancer therapy (Hernandez Vargas *et al*., 2009b, submitted).

Epigenetic mechanisms in control of critical cellular processes and tumourigenesis

While it is well established that aberrant epigenetic events can cause incorrect gene activation and improper gene silencing, recent evidence argues that deregulated epigenetic states may contribute to cancer development by compromising other critical cellular processes such as DNA repair, replication, cell cycle, and stem cell features (“stemness”). We have discovered a novel mechanism for ubiquitination of b-Catenin, the central player in the canonical Wnt pathway that is frequently deregulated in human cancers (Finkbeiner *et al*., 2008). The Wnt pathway is a key regulator of embryonic development and stem cell self-renewal, and hyperactivation of Wnt/b-Catenin signalling is associated with many human cancers. We found a new mechanism of b-Catenin ubiquitination acting in the context of chromatin, which is mediated by the histone acetyltransferase (HAT) complex component TRRAP and Skp1, an invariable component of the Skp-Cullin-F-box (SCF) ubiquitin ligase complex. Our results demonstrate that there is a distinct regulatory mechanism for b-Catenin ubiquitination/destruction acting in the nucleus that functionally complements cytoplasmic destruction of b-Catenin and prevents oncogenic stabilisation of b-Catenin and chronic activation of the canonical Wnt pathway (Finkbeiner *et al*., 2008).

In another study, we have identified a role for HATs in the mechanism that balances self-renewal and differentiation of embryonic stem cells (ESC) and adult stem cells (hematopoietic stem cells, HSC). Conditional deletion of TRRAP in mice resulted in unscheduled differentiation of these cells as judged by morphological, biochemical and gene markers. TRRAp-deficient mouse stem cells showed a loss of histone acetylation to be associated with condensation of chromatin into distinct foci (heterochromatisation), loss of hyperdynamic properties of chromatin, and uncoupling of H3K4-dimethylation and H3K27-trimethylation, markers believed to be important in the establishment of the bivalent chromatin domains in stem cells. These findings establish histone acetylation and HATs

as a part of common mechanisms that restrict differentiation and promote the maintenance of embryonic and adult stem identity (self-renewal and pluripotency), and underscore the importance of histone modifications and chromatin signature in the control of "stemness" and differentiation fates (Loizou *et al.*, Journal of Immunology, 2009, in press).

Development of epigenetic methods applicable to large-scale epidemiology studies

Cell-free circulating DNA isolated from the plasma of individuals with cancer has been shown to harbour cancer-associated changes in DNA methylation, and thus represents an attractive target for biomarker discovery. We have developed a novel combination of methods that allows quantitative and sensitive detection of DNA methylation in minute amounts of DNA present in body fluids (**q**uantitative **M**ethylation **A**nalysis of **M**inute DNA amounts after whole **B**isulfitome **A**mplification, qMAMBA) (Vaissière *et al*., 2009b). This method involves genome-wide amplification of bisulphite-modified DNA template followed by quantitative methylation detection using pyrosequencing, and allows analysis of multiple genes from a small amount of starting DNA. qMAMBA offered high efficacy in the analysis of methylation levels and patterns in plasma samples with extremely small amounts of DNA and low concentrations of methylated alleles. Therefore, qMAMBA will facilitate methylation studies aiming to discover epigenetic biomarkers, and should prove particularly valuable in profiling a large sample series of body fluids from molecular epidemiology studies as well as in tracking disease in early diagnostics (Vaissière *et al*., 2009b).

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Mutations in cancer-related genes are the cornerstone of carcinogenesis. While many mutations accumulate during tumour progression, some of them may occur in normal cells as the result of improper DNA repair processes or exposure to environmental mutagens. The most frequently and most diversely mutated gene in human cancer is TP53, encoding an all-round tumour suppressor that controls cell proliferation, apoptosis, DNA repair and senescence (Hainaut and Wiman, 2009). Studies in the MOC Group address the role of TP53 mutations as markers of exposure to mutagens and as biomarkers for tumour progression, prognosis and response to therapy. Most of the work focuses on common cancers (breast, lung) and in particular on cancers that show wide geographic variations in incidence and etiological mechanisms (liver, oesophagus). Experimental laboratory studies are carried out to understand the mechanistic basis of mutant p53 contribution to carcinogenesis and to elucidate new potential mechanisms that regulate p53 function.

Somatic TP53 mutations and role of p53 in mechanisms of carcinogenesis

Studies on TP53 mutations have focused on cancers of the breast, lung, oesophagus and liver. In breast cancer, we further assessed the value of TP53 mutations as independent prognostic markers (Zalcman et al., 2008). Using cultured breast cancer cells, we have shown that cells with mutant TP53 have altered responses to estrogens and anti-estrogenic drugs, providing a biological basis for the previously reported observation of an interaction between TP53 and hormone receptor status (Fernandez et al., submitted). In lung cancers, following our previous studies on the correlations between EGFR or HER2 mutations and TP53 mutations in never-smokers, we further characterised the specific pathological and molecular profiles of cancers in never-smokers (Aranda et al., 2007; Clement-Duchene et al., 2009; Paris et al., 2009). The prognosis/predictive value of TP53 mutations was investigated in 783 patients of the International Adjuvant Lung Cancer Trial (IALT). TP53 mutations predicted response to therapy, with a significant trend toward benefit in patients with wild-type TP53 and toward worse prognosis in patients with mutant TP53 (P for interaction: 0.05) (Ma et al., submitted; Stacher et al., submitted). In oesophageal cancer, we have investigated patterns of TP53 mutations in relation to expression of inducible nitric oxide synthase (NOS2) and accumulation of nitrotyrosine in patients with gastroesophageal reflux disease (GERD), Barrett's oesophagus or primary ADC. Our results show a correlation between elevated levels of inflammation markers and TP53 mutations at CpG dinucleotides (83% vs. 11%; P=0.008), providing further evidence for a link between chronic inflammation and oesophageal malignancy (Vaninetti et al., 2008). Additional studies identified an association between p53 functional status and expression of a novel, interferon-inducible gene, GBP2, in squamous cell carcinomas (Duarte et al., 2009; Guimaraes et al., 2009). We also investigated the effect of bile acids on the expression of differentiation markers in normal oesophageal mucosa. We found that this treatment induces rapid proteasome-dependent degradation of p63, a protein required for the formation of squamous epithelium. Additional studies using RNA interference demonstrated that loss of p63 induces a major change in cell adhesion patterns, providing a molecular mechanism for initial steps in the formation of intestinal metaplasia in response to GERD (Thépot et al., submitted).

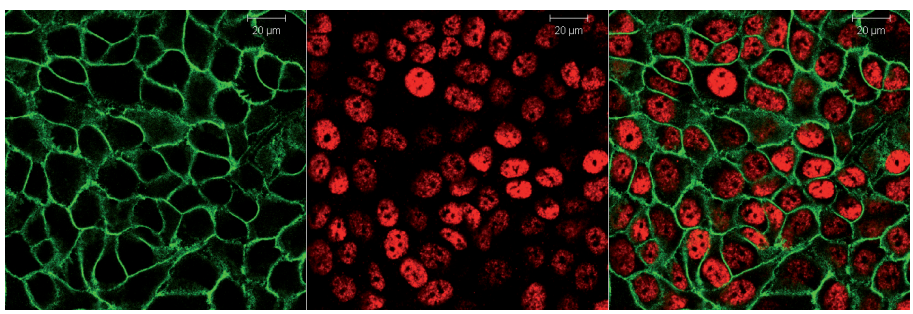

Figure 1. Immunofluorescent staining for the cell adhesion molecule p-Cadherin (green, left), for the differentiation protein p63 (red, center) and for both (right) in cultured human oesophageal cancer cells

In liver cancer, in collaboration with Gerd Pfeifer (Duarte, CA) we further assessed the mechanisms of TP53 mutagenesis by aflatoxin (Besaratinia et al., 2009) and analysed the significance of p.R249S TP53 mutation in the plasma of chronic HBV carriers from Egypt, Nigeria, Gambia and China (Hosny et al., 2008; Igetei et al., 2008; Kuniholm et al., 2008; Szymanska et al., 2009). In a cohort from China, we found that the mutation was detectable ahead of cancer diagnosis in a subset of subjects (Szymanska et al., 2009). Using cell line model systems, we showed that the candidate therapeutic drug PRIMA1 could at least partially reactivate the suppressive function of p.R249S, suggesting a possible mechanism for intervention in patients carrying this mutation (Gouas et al., 2009; Shi et al., 2008). In collaboration with Klas Wiman (Stockholm, Sweden), we demonstrated that PRIMA1 operates through a redox-dependent mechanism of action (Bykov et al., 2009; Lambert et al., 2009). Overall, our work on liver cancer contributed to a better understanding of the interplay between risk factors and viral infections, and may have useful application in preventive interventions (Pujol et al., 2009; Viviani et al., 2008; Hainaut and Boyle, 2008; Pujol et al., 2009).

Germline TP53 mutations and Li-Fraumeni Syndrome

Li-Fraumeni Syndrome is a complex familial predisposition to multiple early cancers. We found that this syndrome was more common than previously recognised (Palmero et al., in press). In collaboration with Maria Isabel Waddington Achatz (Sao Paulo) and Patricia Ashton Prolla (Porto Alegre, Brazil), we developed studies of specific inherited (germline) TP53 mutations in

Figure 2. A gathering of subjects from cancer prone families, proudly showing their familial tree spanning 8 generations

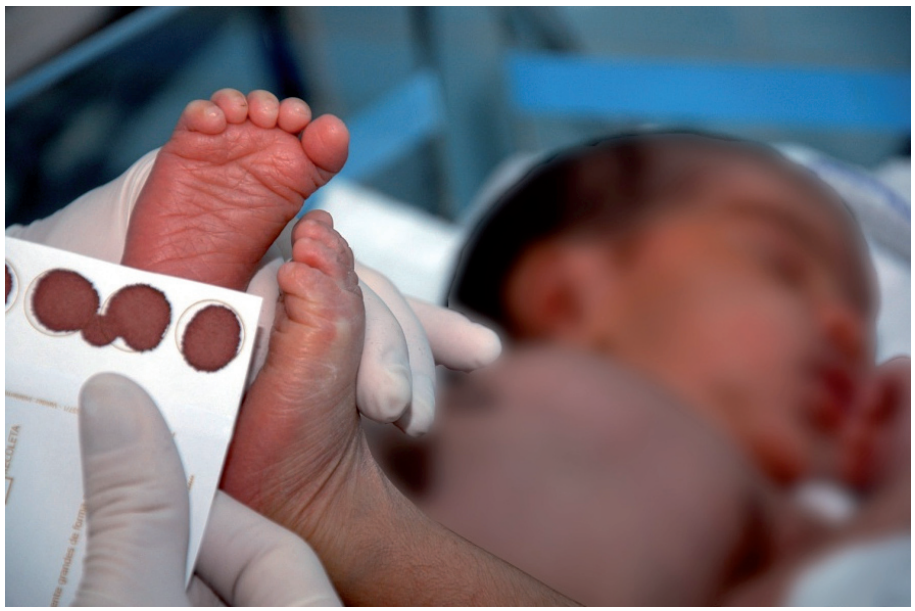

Figure 3. TP53 R337H mutation and Newborn screening

Presence of a common TP53 mutation in 0.3% of the population of South Brazil has led to questions about whether it was appropriate to screen newborns for this mutation in order to better detect subjects at high risk of cancer. Studies by IARC and collaborators have argued against this approach, considering that there is not enough evidence to predict the risk of cancer over lifetime. Childhood predictive genetic testing for R337H should not be carried out in mass screening programs, although it may represent a suitable approach in some families, on a case-by-case basis and within counseling and follow-up strategies that take into account the wide diversity of tumour patterns in mutation carriers

Achatz et al. 2009 Lancet Oncology 2009

Southern Brazil (Palmero et al., 2008). Together with Sean Tavtigian (GCS) and Stephano Landi and Raphaela Gemigniani (Pisa, Italy), we generated a fine haplotype map of TP53 and used it to demonstrate a frequent founder mutation, p.R337H (Garritano et al., in press). This mutant carries a lifetime risk of cancer of 70% at age 60 and is predicted to cause up to 2000–3000 annual cancers currently not identified as familial in Brazil, potentially identifying an opportunity for cancer risk detection via genetic screening of newborns in this area (Achatz et al., 2009). Two genetic modifiers were identified, including an already known polymorphism in MDM2 promoter (SNP309), and an intragenic TP53 polymorphism in intron 3. The latter modulates the age of cancer onset by, on average, 20 years (Marcel et al., 2009). *In vitro* studies demonstrated that this polymorphism modifies the structure of a secondary motif in p53 mRNA and regulates p53 alternative splicing, thus generating different levels of p53 isoforms. These isoforms appear to act as potential inhibitors of p53 function, suggesting a novel genetic mechanism of regulation of p53 activity (Hall et al., 2009; Marcel and Hainaut, 2009).

TP53 Mutation Database

The IARC TP53 Database (http://www-p53.iarc.fr/) is a popular web resource that has been maintained at IARC since 1994. It is both a research and educational tool that contains information and data related to *TP53* gene variations in human cancers. The aim of the database is to provide data and tools that may be used to characterise the impact and phenotypes of *TP53* mutations in human cancers. Available data and annotations include *TP53* mutation frequency, spectrum, phenotype and biological activities of mutant proteins. Data are compiled from the peer-reviewed literature and other online databases. Over the last two years, several developments have been made, including the addition of new annotations on the predicted effect of mutations on splicing and on the production of altered p53 isoforms. We have also been actively promoting the use of standards for database annotations by publishing guidelines for improving mutation data collection, distribution and integration (Olivier et al., 2009b). Within a FP6 European network project on mutant p53 (http://www.mutp53.com/) that has supported the database for the last 5 years, we have organized a series of International workshops on mutant p53, the most recent one being held in Israel (4th International workshop on mutant p53, http://www-p53.iarc.fr/P53meeting2009/P53meeting2009.html). A review on 'Recent advances in p53 research' based on new findings presented at the 3rd workshop was published in 2008 (Olivier et al., 2009a).

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