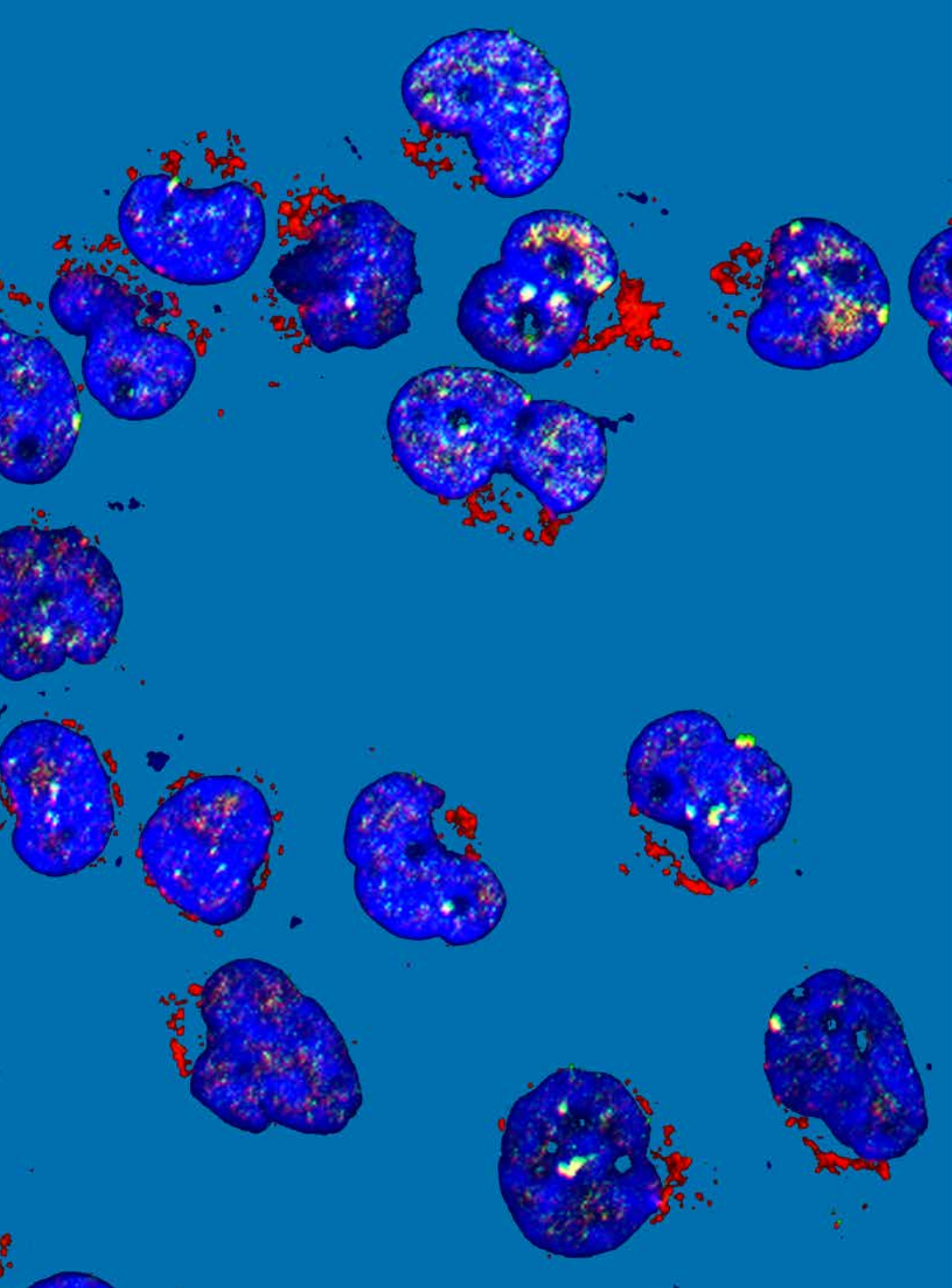

Section of Mechanisms of Carcinogenesis (MCA)

Section head
Dr Zdenko Herceg

Cancers are the consequence of combined genetic and epigenetic changes induced by environmental and lifestyle factors that trigger inappropriate activation or inactivation of specific genes, leading to neoplastic transformation. Although there is consensus that exposures to such factors account for more than two thirds of cancers, meaning that the majority of cancers are potentially avoidable, there is a paucity of evidence about the critical molecular events occurring in the early stages of cancer development or in precursor lesions, as well as the external factors and endogenous cues that trigger these changes. In addition, the challenge posed by cancer genome sequencing efforts is to identify the deregulated genes/pathways and changes in the genome and epigenome that precede and promote tumour development, and to differentiate functionally important drivers from non-functional passenger events. The spectacular advances in genomics and epigenomics have opened up the exciting possibility of simultaneously identifying multiple changes affecting the genome and epigenome of normal, precursor, and cancer cells, as well as their link to the environment. Therefore, it is now possible to improve our understanding of the mechanisms underlying carcinogenesis and define which genetic and epigenetic alterations, or combinations thereof, can be interpreted as reliable biomarkers of exposures.

The broad, long-term goal of the Section of Mechanisms of Carcinogenesis (MCA) is to advance the understanding of mechanisms of carcinogenesis and to contribute to cancer prevention. This is achieved through a multifaceted programme investigating interactions between genes, the epigenome, and the environment. In collaboration with epidemiology groups, MCA contributes to the development of translational studies through the discovery and validation of biomarkers of tumorigenesis and environmental or lifestyle exposures. The Section also aims to promote the development of cancer research relevant to, although not exclusive to, low- and middle-income countries (LMICs) and common cancers related to these regions of the world. Another focus of MCA is the development of genetic/epigenetic methods that are applicable to biobanks associated with case–control and population-based studies. The Section comprises two Groups: the Epigenetics Group (EGE) and the Molecular Mechanisms and Biomarkers Group (MMB), which work in close collaboration with the aim of creating synergies to better exploit and further expand our unique research tools and expertise.

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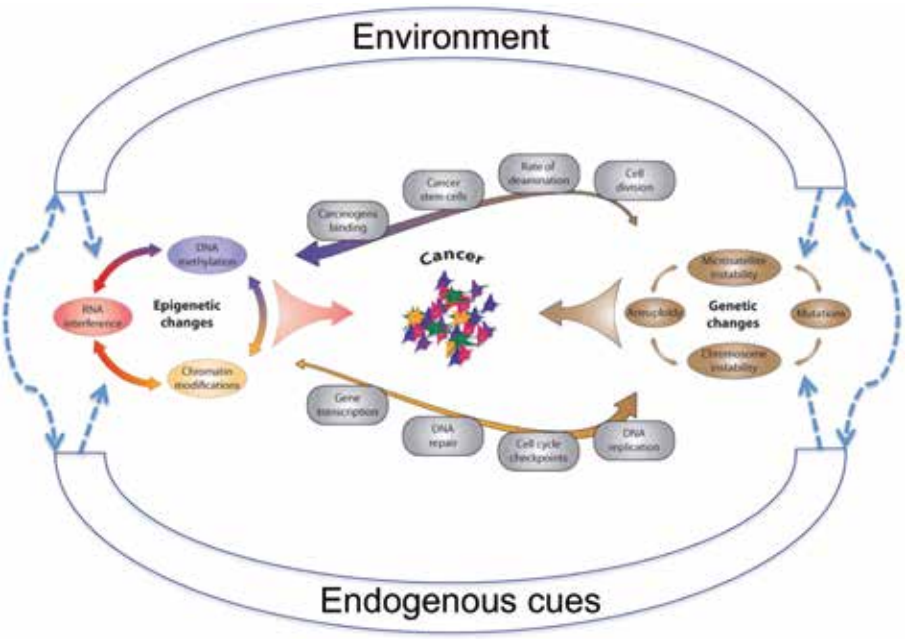
Recent years have witnessed significant advances in our understanding of mechanisms of carcinogenesis. In particular, the importance of epigenetic alterations (methylation, histone modification, small non-coding RNAs) in the development of human cancer opens up new ways in which the environment and lifestyle factors may interact with cells to increase cancer risk (Figure 1) (Herceg *et al.*, 2013). The ubiquity and potential reversibility of epigenetic changes offer interesting opportunities for intervention strategies and biomarker discovery (Herceg *et al.*, 2013; Nogueira da Costa and Herceg, 2012). The Epigenetics Group (EGE) conducts research projects aiming to gain a better mechanistic understanding of tumorigenesis, and 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 and consortia.

Figure 1. Epigenetic mechanisms regulate key cellular processes (such as gene transcription, DNA repair, and differentiation) and play critical roles in cellular responses to environmental exposures and endogenous stimuli. Deregulation of epigenetic mechanisms may promote the development of abnormal phenotypes and cancer. Source: Herceg *et al.* (2013); reproduced with permission from Oxford University Press.



Epigenetic changes associated with risk factors for upper aerodigestive tract (UADT) cancers

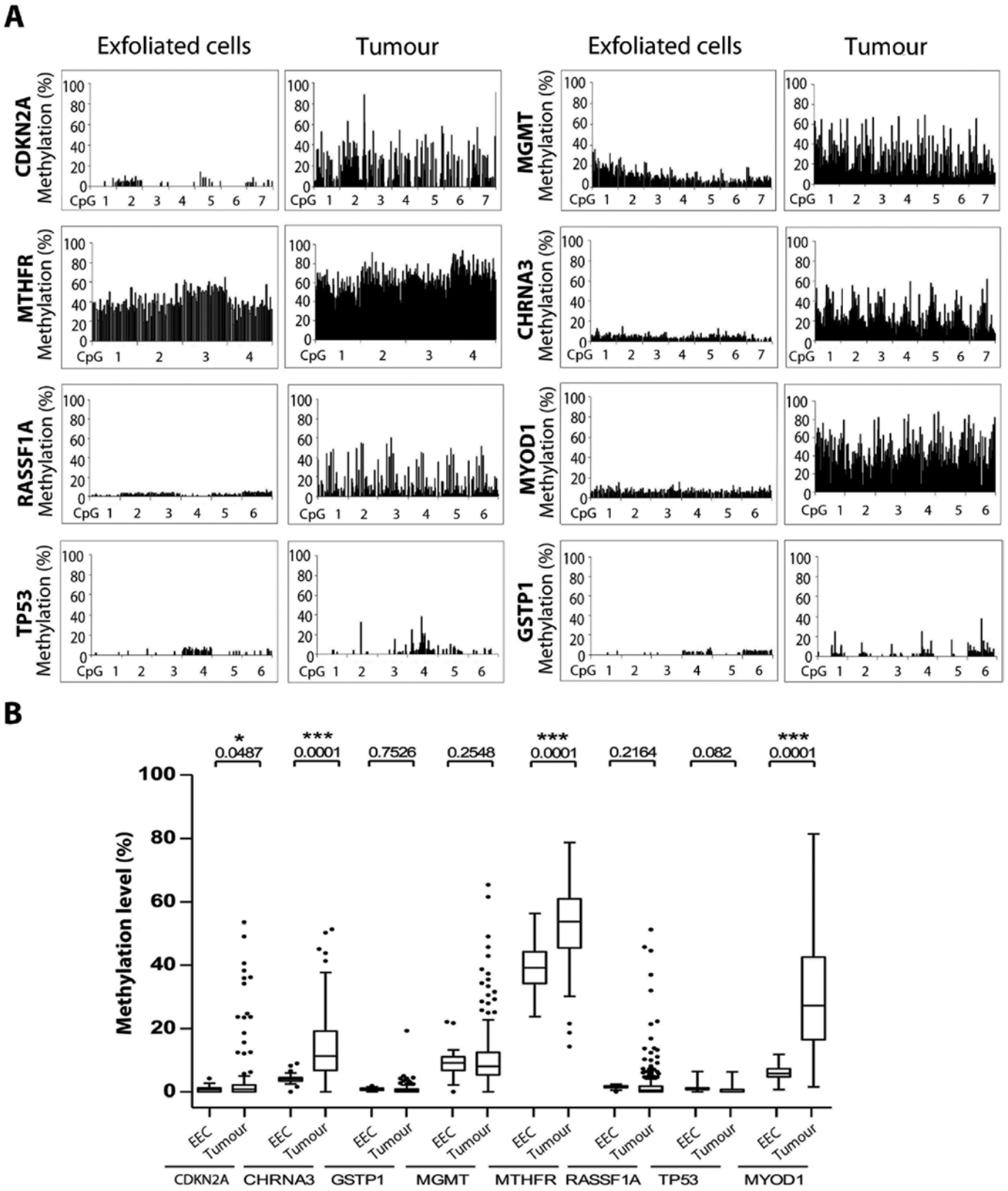
To examine whether the deregulation of the epigenome by environmental, dietary, and lifestyle exposure may disrupt different cellular processes and contribute to cancer risk, we combined quantitative profiling of DNA methylation states in a wide panel of cancer-associated genes using microarray technology and high-throughput pyrosequencing with case–control studies of upper aerodigestive tract (UADT) cancers. Our recent study of promoter methylation in the promoters of more than 800 genes in oesophageal squamous cell carcinoma (ESCC) revealed a panel of differentially methylated genes related to several pathways, including the IL-10 anti-inflammatory signalling pathway and cell communication pathway, indicating that deregulation of these pathways through epigenetic mechanisms may contribute to ESCC.

We further analysed changes in DNA methylation in UADT cancers and their potential association with primary risk factors. To this end, we have taken advantage of a case–control study of UADT cancer involving seven centres in South America, using detailed lifestyle information and quantitative analysis of DNA methylation in a panel of cancer-associated genes. Our analyses revealed a high frequency of aberrant hypermethylation of specific genes, among which we identified new genes (including the nicotinic acetylcholine receptor gene, CHRN3, and the downstream of tyrosine kinase 1, *DOK1*), suggesting that epigenetic deregulation of these genes may promote the development of UADT cancer (Figure 2) (Mani *et al.*, 2012; Siouda *et al.*, 2012). Importantly, we found that sex and age are associated with the methylation states, whereas tobacco smoking and alcohol intake may also influence the methylation levels in specific genes (Mani *et al.*, 2012). Together, these studies identify aberrant DNA methylation patterns in UADT and gastric cancers and suggest a potential mechanism by which environmental factors may deregulate key cellular genes involved in tumour suppression and contribute to these common human cancers.

Epigenetic changes in surrogate tissue as cancer biomarkers

Because DNA methylation profiles of the human genome are tissue-specific, we tested the possibility that global methylation levels in surrogate tissues, such as blood, may be used in epidemiological studies. We used two independent but complementary methods to assess global methylation levels in peripheral blood DNA from a well-characterized population-based case–control study (the Long Island Breast Cancer Study Project, with more than 2100 peripheral blood samples). Our results, obtained by pyrosequencing-based assay (LUMA) and genome-wide methylation (Illumina Infinium arrays) profiling, revealed greater promoter hypermethylation in breast cancer cases, while methylation levels in repetitive elements (as revealed by LINE-1 methylation assay) were not associated with breast cancer risk (Xu *et al.*, 2012). This study shows that global promoter hypermethylation measured in peripheral blood may be used in breast cancer risk assessment. Further studies on the samples from a prospective cohort, the European Prospective Investigation into

Figure 2. The DNA methylation levels in upper aerodigestive tract (UADT) tumours and control samples. (A) Summary of the analysis of DNA methylation of individual CpG sites in eight genes in UADT tumours and exfoliated mouth epithelial cells (EEC) (controls). (B) Graphical representation comparing DNA methylation levels in UADT tumours and control (EEC) samples. Box plots of the summary results obtained by the analysis of the mean levels of all CpG sites analysed for a given gene and the statistical significance for differential methylation in tumours compared with EEC samples. Source: Mani *et al.* (2012); reproduced with the permission of the publisher.

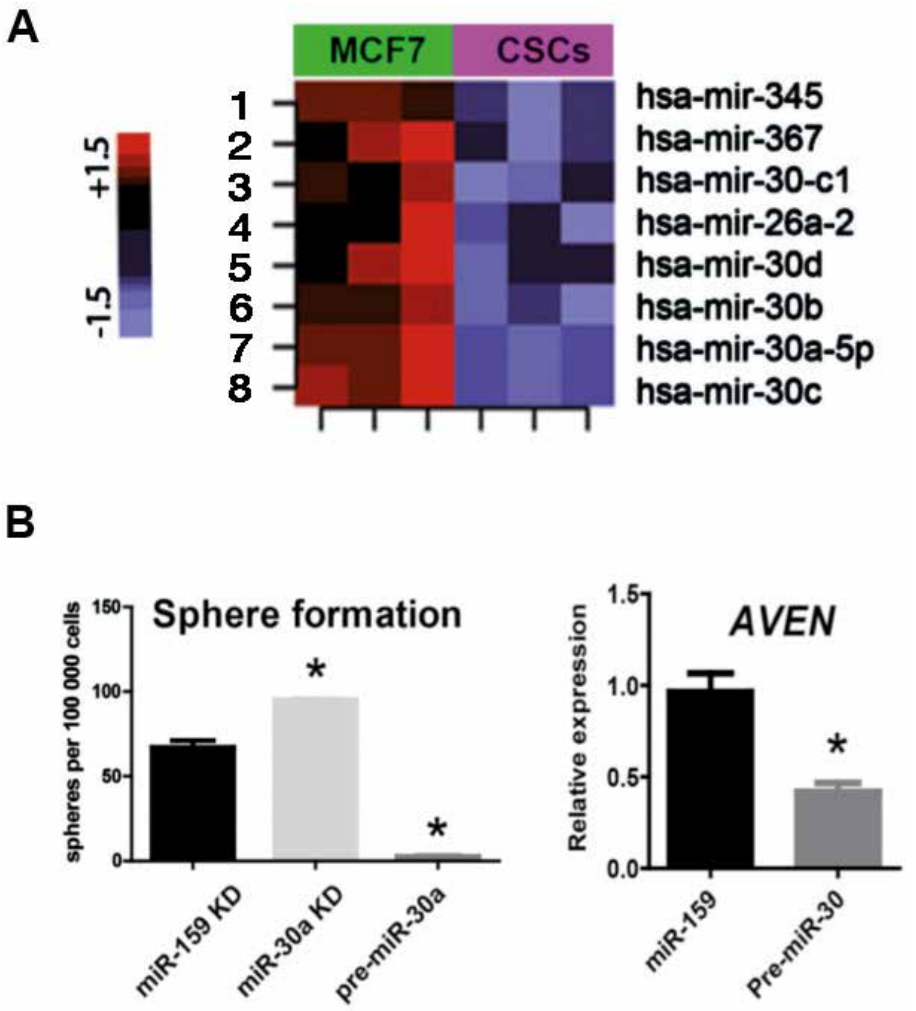


Cancer and Nutrition (EPIC), are under way to assess the value of this marker and address the potential influence of disease onset (reverse causality) and one-carbon metabolism on the methylome of blood DNA.

Identifying oncogenic microRNAs in cancer development and potential new cancer biomarkers

The recent discovery of a new class of small non-coding RNAs, microRNAs (miRNAs), opened up fresh perspectives in studying mechanisms of carcinogenesis and biomarker research. As miRNAs control developmental programmes in normal stem cells, we explored the hypothesis that miRNAs may have a role in sustaining so-called cancer stem cells (CSCs, also known as breast tumour-initiating cells, BT-ICs). To do this, we performed comprehensive profiling of miRNA expression in a model of putative breast CSCs. We found that CSCs display a unique pattern of miRNA expression, highlighted by a markedly low expression of miR-30 family members (Figure 3). We further showed that the miR-30 family regulates non-attachment growth. A target screening revealed that the miR-30 family modulates the expression of apoptosis- and proliferation-related genes and that some of these targets were able to reverse the effect of miR-30a overexpression (Figure 3). Finally, overexpression of miR-30a in vivo was associated with reduced breast tumour progression (Ouzounova *et al.*, 2013). This is the first analysis of target prediction in a whole family of microRNAs potentially involved in the survival of breast CSCs.

Figure 3. Identification of microRNAs involved in survival of breast cancer stem cells (CSCs). (A) Comprehensive profiling of microRNA (miRNA) expression in an in vitro model of putative breast CSCs revealed that the miR-30 family is underexpressed in breast CSCs. MCF7 cells were compared with CSCs (selected by their non-attachment growth). (B) miR-30 inhibition (using miR-30a inhibitor oligonucleotides, KD) enhances non-attachment growth, while overexpression (pre-miR-30a) impairs it (left panel). miR-30 targets the anti-apoptotic protein AVEN (right panel). Source: Ouzounova *et al.* (2013); reproduced with the permission of the publisher.

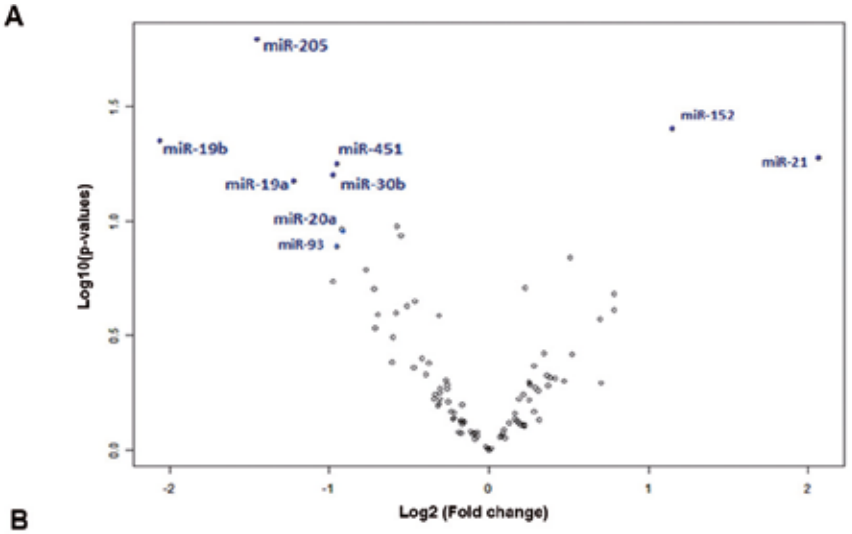


Among different cancer biomarkers, miRNAs are considered the most promising owing to their remarkable stability, their cancer-type specificity, and their presence in body fluids. In a collaborative study between IARC, the N.N. Blokhin Cancer Research Center, Moscow, Russian Federation, and the Hôpital Louis Pradel, Hospices Civils de Lyon, Lyon, France, we compared circulating miRNA profiles in patients with lung squamous cell carcinoma (SCC) before and after tumour removal, assuming that the levels of all tumour-relevant miRNAs would drop after the surgery. Our results revealed a specific panel of the miRNAs (miR-205, -19a, -19b, -30b, and -20a) whose levels decreased strikingly in the blood of patients after lung SCC surgery (Aushev *et al.*, 2013). Interestingly, miRNA profiling of plasma fractions of lung SCC patients revealed high levels of these miRNA species in tumour-specific exosomes; furthermore, several of these miRNAs were also found to be selectively secreted to the medium by cultivated lung cancer cells (Figure 4) (Aushev *et al.*, 2013). These results strengthen the notion that tumour cells secrete miRNA-containing exosomes into circulation, and that miRNA profiling of the exosomal plasma fraction may reveal powerful cancer biomarkers.

Epigenetic mechanisms in control of cellular processes and cancer

We have previously shown that histone modifications and remodelling are important to provide accessibility to DNA lesions and for efficient DNA repair (Gospodinov and Herceg, 2013a; Sawan *et al.*, 2013). In this study, we identified TRRAP, a critical component of histone acetyltransferase (HAT) complexes, as a novel target of proteolytic degradation in a cell cycle-dependent manner. TRRAP overexpression or mutation-induced stabilization resulted in multiple

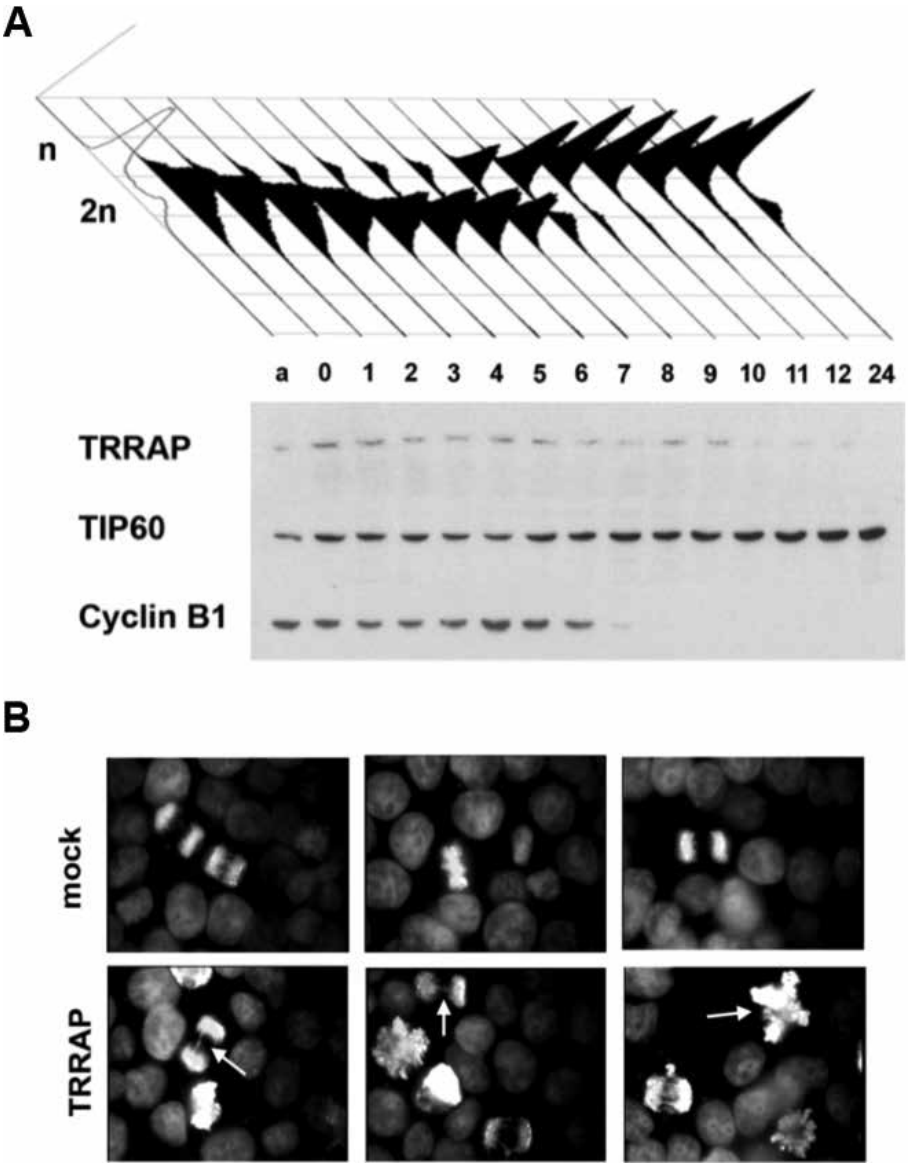
Figure 4. Comparisons of microRNA (miRNA) patterns in plasma before and after tumour removal reveal new biomarkers of lung cancer. (A) Scatter plot of pre-/post-surgery ratios for expression of the analysed miRNAs in patients with lung cancer. Analysis was performed with BRB-ArrayTools software. The group of miRNAs on the left side of the plot (miR-205, -19a, -19b, -451, -30b, -20a, and -93) are the most abundant in the plasma of lung cancer patients and are reduced in the plasma after tumour removal. (B) Enrichment in expression of various miRNA species in the ExoQuick-precipitated fraction (Exosomal fraction) compared with the ExoQuick-depleted fraction (Exosome-free fraction) of the same pre-operative plasma samples from patients with lung cancer. Source: Aushev *et al.* (2013); reproduced with the permission of the publisher.



Rank	Parametric p-value	Fold-change	Unique ID
1	< 1e-07	0.011	**let-7a**
2	< 1e-07	0.037	**let-7c**
3	1e-07	0.0086	**let-7f**
4	3e-07	0.027	**et-7d**
5	6e-07	0.0094	**miR-26a**
6	1.8e-06	0.033	**miR-155**
7	2.4e-06	0.024	**miR-194**
8	2.4e-06	0.012	**miR-30b**
9	3.8e-06	0.024	**miR-150**
10	3.9e-06	0.0034	**miR-103**
11	4.9e-06	0.01	**let-7g**
12	8e-06	0.013	**miR-30c**
13	1.01e-05	0.019	**miR-191**
14	1.43e-05	0.026	**miR-140-5p**
15	1.57e-05	0.034	**miR-29a**
16	1.66e-05	0.033	**miR-143**
17	1.7e-05	0.069	**miR-10b**
18	1.72e-05	0.076	**miR-122**
19	1.93e-05	0.019	**let-7b**
20	2.45e-05	0.043	**miR-21**

mitotic defects, including lagging chromosomes, chromosome bridges, lack of sister chromatid cohesion, and impaired chromosome condensation (Figure 5). We further found that mitotic defects are associated with a global histone H4 hyperacetylation, indicating that TRRAP and TRRAP-mediated histone acetylation are necessary for proper condensation of chromatin, chromosome segregation, and genomic stability. Together with recent findings of recurrent mutations in the *TRRAP* gene in several cancer types, such as melanoma, pancreatic adenocarcinomas, and hepatocellular carcinoma, our results argue that deregulation of TRRAP/HATs and histone acetylation and the resulting changes in chromatin compaction states may represent an important mechanism of chromosome instability and tumorigenesis.

Figure 5. Degradation of TRRAP before cell division is critical for proper condensation of chromatin and proper chromosome segregation. (A) Protein levels of the epigenetic regulator TRRAP are cell cycle-dependent. (B) Aberrant TRRAP degradation leads to aberrant mitotic defects and chromosomal aberrations. Source: Ichim *et al.* (2013). *Oncogene*, 41:1187–1203 http://dx.doi.org/10.1038/onc.2012.570 PMID:23318449; reproduced with the permission of the publisher.



Development of epigenomic methodologies and profiling strategies applicable to biobanks and population-based cohorts

Remarkable advances in epigenomics have tremendously accelerated research on the mechanisms of carcinogenesis and opened up new perspectives in cancer research (Herceg *et al.*, 2013; Umer and Herceg, 2013; Wild *et al.*, 2013; Nogueira da Costa *et al.*, 2012). EGE has been involved in several long-term projects coordinated by IARC that will continue to make important contributions to cancer research and molecular epidemiology in coming years. These include large prospective cohorts, case–control studies, intervention studies, and consortia (such as the EPIC cohort and the International Childhood Cancer Cohort Consortium [I4C]). Therefore, the development of effective genomic and epigenomic methodologies that are applicable to biobanks associated with population-based studies is among our priorities. For a more comprehensive understanding of the functional elements in the normal human epigenome and cancer epigenome, we have exploited improvements in the throughputs and costs of methylation, histone modifications, and microRNA sequencing brought about by the recent establishment of the pyrosequencing and new-generation array platform (Illumina), as well as the next-generation sequencing (NGS) platform at IARC. EGE has been involved in close collaboration with IARC laboratory scientists and epidemiologists, as well as external groups and consortia, to facilitate the application of these new epigenomics methodologies, allowing cancer epigenomics to move from focused approaches to comprehensive genome-wide approaches. These studies advance our understanding of the epigenetic mechanisms involved in cancer development and may prove to be the reference for future studies aimed at identifying potential biomarkers and molecular targets for prevention (Aury-Landas *et al.*, 2013; Genevois *et al.*, 2013; Kaur *et al.*, 2012; Lee *et al.*, 2013a; Rakosy *et al.*, 2013; Sawan *et al.*, 2013; Siouda *et al.*, 2012; Xu *et al.*, 2012; Zoldoš *et al.*, 2012). They also enhance our collaborations with different groups within and outside IARC, contributing to the overall scientific achievements of the Agency.

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National Institutes of Health, National Cancer Institute, USA

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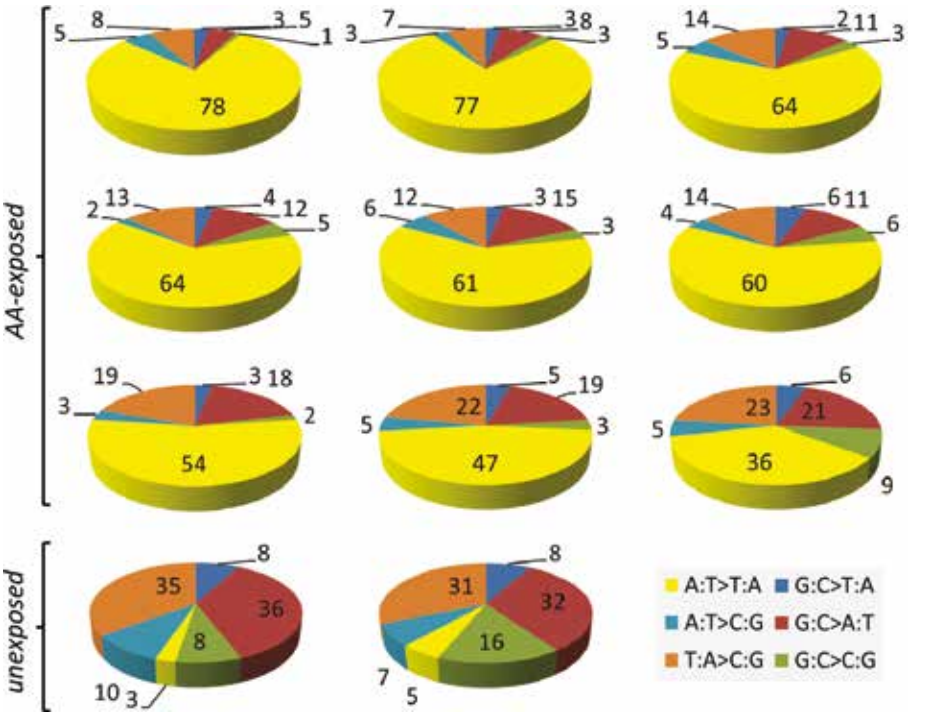
(until May 2012)

The objective of the Molecular Mechanisms and Biomarkers Group (MMB) is to identify genetic alterations, biomarkers, and molecular mechanisms associated with specific environmental exposures underlying carcinogenesis in humans, and, by using model systems, to distinguish the key *driver* events from non-functional or modifying passenger changes. Using molecular epidemiological and mechanistic approaches, MMB currently investigates urinary tract transitional cell carcinomas linked to dietary exposure to aristolochic acid (AA); triple-negative breast cancer (TNBC), a subtype of breast tumours negative for expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. Next, MMB maintains an online resource (http://www-p53.iarc.fr) on *TP53* mutation patterns and corresponding phenotypes in human cancers. This database compiles comprehensive information on candidate biomarkers of diagnosis, prognosis, and relationships between exposure and cancer etiology. The overall conceptual framework and the results of MMB's work form a basis for future translational cancer prevention programmes on specific human cancers conducted in low- and middle-income countries (LMICs). Here we highlight several of the major findings during the biennium.

Molecular epidemiology of aristolochic acid-associated urothelial carcinogenesis

MMB participates in an international collaboration to identify genetic alterations and biomarkers associated with urothelial carcinogenesis linked to dietary exposure to AA. AA is a widespread, potent herbal carcinogen (IARC Group 1) and cytotoxin, causing aristolochic acid nephropathy (AAN) and urinary tract urothelial carcinomas (UTUC), with tens to hundreds of millions of individuals estimated to be exposed worldwide. Using high-throughput tumour DNA sequencing, we identified a genome-wide predominance of A:T → T:A transversions, a specific mutation signature of AA exposure (Figure 1). We next determined the UTUC-specific pattern of microRNAs that are currently explored as non-invasive urinary biomarkers of recurrent carcinogenesis

Figure 1. Identification of the predominant A:T → T:A transversion pattern in aristolochic acid nephropathy (AAN)-associated urinary tract urothelial carcinomas (UTUC) tumour samples. Percentages of individual alterations are shown, as identified by Illumina HiSeq2500 whole-exome sequencing. The average number of mutations per tumour sample was 532 (range, 250–1292).



in AAN. Collectively, our findings provide an evidence-based rationale for efficient preventive measures and lay the groundwork for cost-effective and high-capacity molecular epidemiological approaches to identify new populations at risk, namely in the LMICs in south and east Asia, and to decrease the AAN-associated cancer burden globally.

Integrated molecular analysis of triple-negative breast tumours

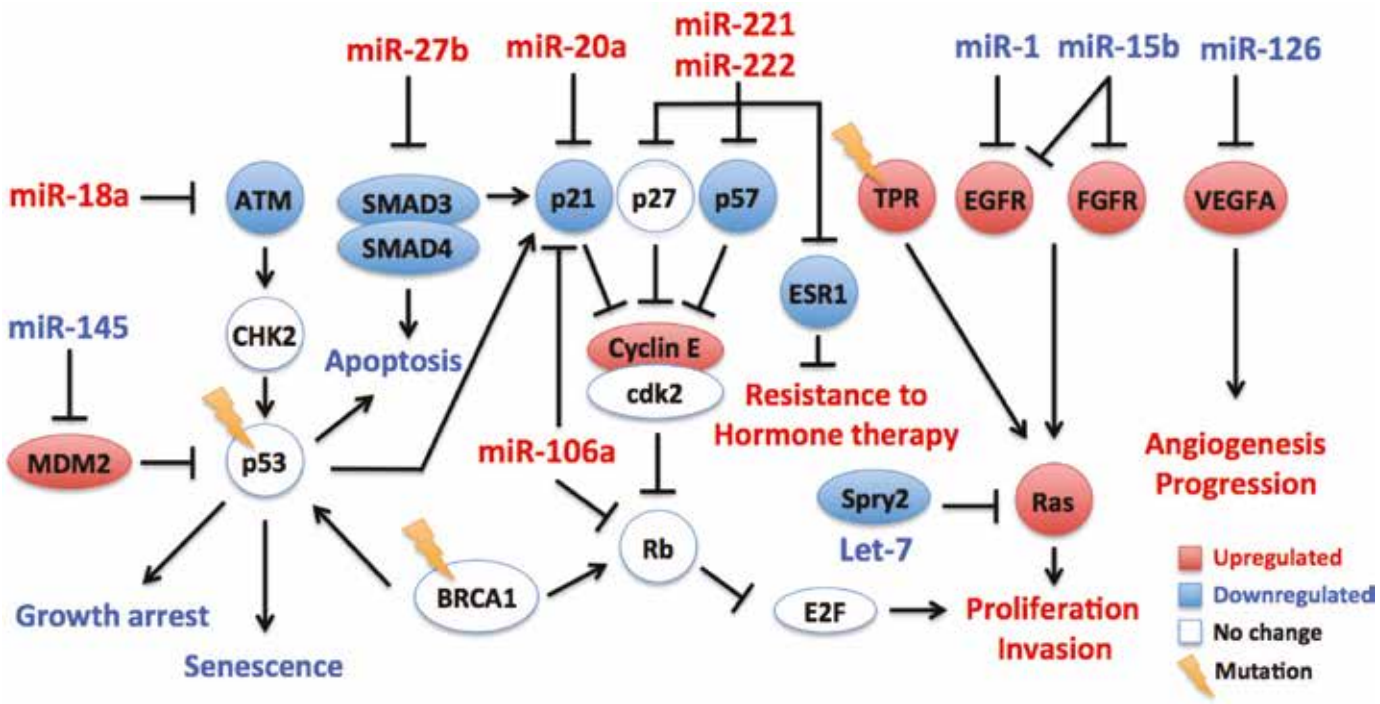
Representing 10–20% of breast cancer subtypes, triple-negative breast tumours (TNBC) is marked by molecular heterogeneity, poor prognosis, and lack of targeted treatment. TNBC is more prevalent in certain populations, such as in Mexicans. We investigated the tumour-specific molecular landscapes of TNBC by integrated high-throughput analyses of archived tumour samples from Mexican patients, combining whole-exome sequencing, transcriptomic (miRNA and mRNA) profiling, and complex bioinformatic analyses. We found deleterious alterations in cancer driver genes such as *TP53* (Table 1), *BRCA1*, *HIF1A*, *RELA*, *PRKG1*, and *KDM6A*, and mutations in DNA-repair genes, consistent with the increased genomic instability observed in TNBC. While the transcriptomic profiling identified signatures of tumour-initiating and -promoting processes, the global aberrant molecular program revealed a degree of ambivalence involving

Table 1. *TP53* gene mutations in TNBC tumours

Mutation type	Alteration	Genomic position (hg19)
SBS	c.G396C:p.K132N	chr17: 7 578 534
Deletion	c.183_201del:p.61_67del	chr17: 7 578 252–7 578 270

SBS, single base substitution; TNBC, triple-negative breast cancer.

Figure 2. Global gene regulation programs in triple-negative breast cancer (TNBC) tumours in Mexican women, identified by whole-exome sequencing and integrated transcriptomic analysis.

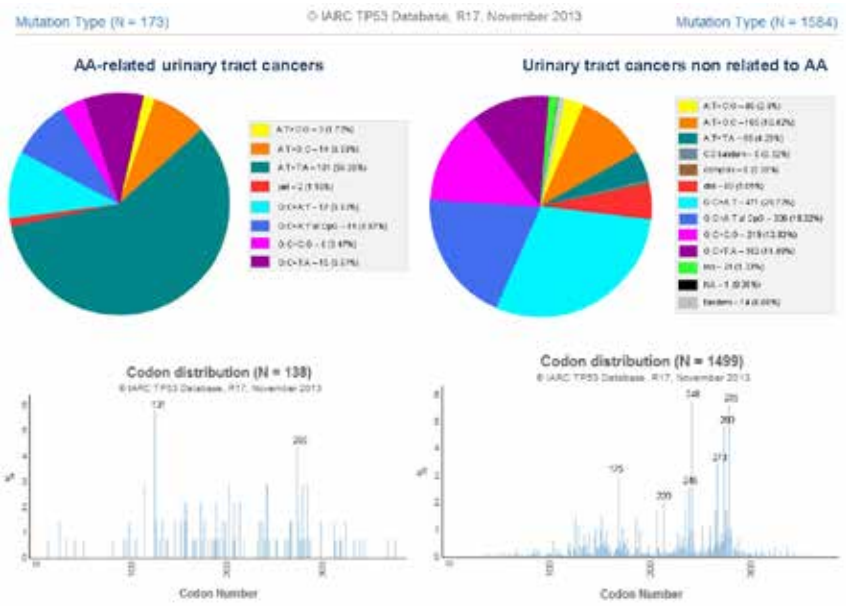


repression of cell cycle control and apoptotic signals and activation of growth- and tumour-promoting cascades (Figure 2).

The IARC TP53 database of *TP53* gene alterations in human cancers

MMB maintains the IARC *TP53* Database (http://p53.iarc.fr), a public, fully searchable and downloadable online resource with data on gene variations in the most frequently mutated cancer gene. The database allows interpretation of the clinical and biological significance of more than 5000 distinct *TP53* gene variations and provides a wide range of annotations on their structural and functional impacts and on associated tumour pathology, patient demographics, risk factors, and exposures. It compiles data on both germline and somatic variations as well as experimental data on the functional impacts of mutations. The database is a popular resource, which has been cited more than 3700 times in scientific publications and is used worldwide by scientists, clinicians, and trainees. In 2012, the database web site was redesigned to improve its interactivity and allow new data sets to be analysed. MMB also published studies on how research on *TP53* has advanced our understanding of the molecular basis of human cancer, and how this knowledge translates into cancer management, therapy, and epidemiology (Figure 3) (Fernández-Cuesta *et al.*, 2012; Hainaut *et al.*, 2013; Hollstein *et al.*, 2013).

Figure 3. The IARC TP53 Database web site provides graphical tools for the analysis of mutation patterns in human cancers. It is used as a data mining tool and a reference data set for molecular epidemiology studies. The example graphs show the different mutation patterns observed in urinary tract cancers from acid aristolochic (AA)-exposed patients (left panels showing a predominance of A:T → T:A at specific hotspots) and from patients non-exposed to AA (right panels showing a predominance of G:C → A:T mutations in different hotspots).



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