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Review

Epigenetics and cancer, 2nd IARC meeting, Lyon, France, 6 and 7 December 2007[☆]

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ARTICLE INFO

Article history:

Received 28 February 2008

Received in revised form

14 March 2008

Accepted 17 March 2008

Available online 27 March 2008

Keywords:

Epigenetic changes

Epigenomics

DNA methylation

Histone modifications

Cancer

Environment

Molecular epidemiology

ABSTRACT

It is becoming widely accepted that epigenetic alterations are universally present in human malignancies and that cancer is as much a disease of abnormal epigenetics as it is a genetic disease. The potential utility of epigenetics and epigenomics in cancer research and cancer control is highlighted by the fact that many funding agencies put cancer epigenetics on the priority list. The goal of this meeting, held at the offices of the International Agency for Research on Cancer in Lyon, France, 6 and 7 December 2007, was to discuss recent conceptual and technological advances in cancer epigenetics and epigenomics, the future research needs in the field, and their implications for early detection, risk assessment and prevention of cancer.

While epigenetics has been historically linked to phenomena that do not follow normal genetic principles of heritability, recent mechanistic advances have begun to change our understanding of complex diseases including cancer, traditionally viewed as genetic in origin. It is now known that epigenetic mechanisms play critical roles in regulation of many cellular functions and their deregulation may disrupt the control of fundamental processes leading to tumour formation. A flurry of screening and functional studies revealed that most key processes found in cancer cells, such as silencing of tumour suppressor genes, activation of oncogenes, aberrant cell cycle processes, defects in DNA repair, and deregulation of cell death, can be triggered by epigenetic deregulation. Two important features that distinguish epigenetic changes from genetic alterations are the gradual appearance and reversibility of epigenetic events. These features make epigenetic alterations an attractive target for therapeutic intervention and the development of preventive strategies. For example, aberrant patterns of DNA methylation and histone acetylation and methylation can be targeted by drugs aiming to re-activate epigenetically silenced genes. Until now, most studies on epigenetic changes in cancer were generally focused on specific genes. However, this meeting also stressed the need to take advantage of recent progress in

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doi:10.1016/j.molonc.2008.03.005

epigenomics and emergence of powerful technologies for detection of epigenetic changes in high throughput and genome-wide settings. This may further advance our capacity to evaluate the contribution of epigenetic changes induced by environmental epimutagens to human cancer. This information may prove critical for the design of efficient strategies for early diagnosis, therapy, and prevention of cancer.

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1. Introduction

In recent years, we have witnessed an explosion of interest in epigenetics and recent spectacular advances in the field have changed our understanding of various biological phenomena and complex diseases, most notably cancer. Historically, the term “epigenetics” was used to describe different biological phenomena that did not follow normal genetic principles of heritability. It was first coined by Conrad Waddington in early 1940s to describe the studies of “the interactions of genes with their environment that bring the phenotype into being” (Waddington C: the epigenotype. *Endeavour* 1942, 1:18–20). Since the introduction of this term, a number of biological events that are not coded in the DNA sequence have been classified as epigenetic. These included X chromosome inactivation (in females), paramutations (mostly in plants), imprinting (in mammals), and position-effect variegation (in the fruit fly). A number of recent studies in the field of epigenetics revealed that these distinct biological phenomena share common underlying mechanisms involving DNA methylation, histone modifications, or microRNAs. These advances in understanding have turned academic, medical, and public attention towards the potential application of epigenetic modifications in biomedical research.

Currently, the term “epigenetics” refers to all stable changes of phenotypic traits that are not coded in the DNA sequence itself (Baylin, 2005; Feinberg et al., 2006; Rountree et al., 2001). In a broader sense, epigenetics can be considered as an interface between genotype and phenotype. Epigenetic mechanisms play a critical role in normal physiological responses to environmental stimuli allowing the establishment of appropriate gene expression patterns. However, adverse and prolonged exposure to environmental, physical, chemical and infectious agents, as well as lifestyle factors, may induce epigenetic changes leading to a neoplastic process. Recent laboratory-based and epidemiological studies have implicated epigenetic changes induced by environmental exposures and lifestyle factors in the development of human malignancies. Functional and screening studies revealed that most critical processes found in cancer cells, such as silencing of tumour suppressor genes, activation of oncogenes, aberrant cell cycle processes, defects in DNA repair, and deregulation of cell death, can be triggered directly or indirectly by epigenetic deregulation. However, the contribution of deregulated epigenetic states to specific human cancers remains unknown.

Epigenetic inheritance includes three distinct and self-reinforcing mechanisms: DNA methylation, histone

modifications, and RNA-mediated gene silencing. The methylation of DNA refers to the covalent modification (attachment of a methyl group) of cytosine bases in CpG dinucleotides carried out by DNA methyltransferases. DNA methylation plays an important role in different cellular processes including gene expression, silencing of transposable elements, and defense against viral sequences (Bird, 2002; Jaenisch and Bird, 2003). A large number of studies have shown that aberrant DNA methylation is closely connected to a wide variety of human cancers, although underlying mechanisms remain poorly understood. Additionally, it has been proposed that aberrant levels and patterns of DNA methylation may induce other epigenetic changes, notably histone modifications. However, there are some findings to suggest that it actually works the other way round: abnormal histone premarking (disruption of specific patterns of histone modifications) during early embryonic development may predispose certain genes to cancer promoting DNA methylation events (Schlesinger et al., 2007; Widschwendter and Menon, 2006). Histone modifications refer to post-translational marking of core histones and include acetylation, methylation, phosphorylation, and ubiquitination, a combination of which may constitute the “histone code” (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Histone marks are believed to play critical roles in key cellular processes such as gene transcription and DNA repair. Accumulating evidence indicates that histone modification patterns may be altered in human cancer and that they can be exploited in clinics and biomarker discovery. RNA-mediated gene silencing involves microRNAs molecules (miRNAs) that may also control gene expression in a stable manner (Calin and Croce, 2006). miRNAs appear to be able to act as either tumour suppressors or oncogenes by affecting distinct genes involved in cell proliferation and differentiation. Several recent studies indicated that miRNA profiles differ significantly between cancer and normal tissues but also between different tumours (Calin and Croce, 2006; Volinia et al., 2006), and that these changes can be exploited in therapeutic, diagnostic and prognostic purposes, although much remains unknown.

In addition to recent progress in understanding the epigenetic mechanisms controlling normal cellular processes and cancer, we have witnessed the development of a number of technical methodologies allowing robust, quantitative and genome-wide analysis of epigenetic alterations. These new techniques set the stage for the development of novel strategies for prevention, epigenetics-based diagnostics, and treatment. The purpose of this meeting was to assess the state of research and discuss possible future priorities

in epigenetics and epigenomics that may lead to a better cancer control.

2. Genome-wide screening of epigenetic changes

Epigenetic events play a key role in regulation of crucial cellular processes and thus epigenetic alterations may disrupt the control of fundamental cellular mechanisms leading to tumour formation. Until now, studies on epigenetic changes in cancer were generally focused on specific genes or even restricted to promoter regions. However, the need to assess the impact of epigenetic events in tumour progression led to the development of novel approaches and strategies that allow genome-wide profiling of epigenetic changes. In this context, Ben Tycko's (Institute for Cancer Genetics) keynote lecture on DNA methylation profiling in cancer and normal tissues described an interesting concept in which changes in DNA methylation of non-island sequences may also be associated with tumorigenesis. DNA methylation is a heritable change, established during embryogenesis, and transmitted from one cell to its daughter cells through the activity of DNA methyltransferases. However, the spreading of DNA methylation is accurately regulated in each DNA sequence allowing establishment of different methylation patterns: tissue-specific and allele-specific. Several attractive hypotheses linking these processes to cancer were discussed by Tycko, including the probable alteration of non-island sequences in tumours and how stable methylation changes are correlated with gene expression and the gain of aberrant DNA

methylation in specific malignancies (e.g., Wilms tumours) (Valk et al., 2004). Tycko also described a single nucleotide polymorphism (SNP) chip-based method for profiling DNA methylation. This approach allows the identification of genome-wide DNA methylation patterns using commercially available (Affymetrix) SNP microarrays (Yuan et al., 2006) and can evaluate both net DNA methylation (averaging the two alleles) and allele-specific methylation, in which the methylation status of each allele is queried independently. Thus, this technique provides information on differences in net methylation at thousands of loci enabling comparison of normal and cancer tissues, and different cancer types.

Stephan Beck (UCL Cancer Institute) discussed a system approach involving epigenetic events to complex phenotypes and diseases, such as cancer. The determination of phenotype is influenced by both endogenous and exogenous (environment, diet) factors, a combination of which regulates gene expression. While over 45,000 SNPs responsible for inter-individual differences have already been characterized in the human genome, DNA methylation constitutes an additional level of variability that is not "written" in DNA sequences. In the human genome, the distribution of CpG dinucleotides is not uniform and their methylation represents a regulatory element for gene expression. Thus, in addition to genome-based characteristics shared between all cells in an organism, there is also an "epigenome", formed by a combination of epigenetic marks that are specific for each cell. Interestingly, Beck also pointed out that recent study on DNA methylation patterns seems to suggest these features differ only marginally between ages and sexes, implying that DNA

methylation is more stable than previously thought (Eckhardt et al., 2006).

He went on to discuss the Human Epigenome Project, launched in 1999, which is similar to the International HapMap Project, intended to establish a map of the common human DNA sequence variations through the use of a single microarray for the analysis of SNPs, and aims to identify and quantify genome-wide epigenetic variants. Indeed, like SNPs, methylation variable positions may dictate the status of gene expression and thus constitute informative epigenetic markers, which offer new avenues for better understanding of mechanisms underlying human diseases. Beck referred to the Nimblegen and Solexa IG platforms (MeDIP sequencing) as tools to integrate SNPs and methylation patterns (Eckhardt et al., 2006; Raval et al., 2007), but stressed that the development of novel strategies will be essential for further identification and quantification of epigenetic variants. He concluded by highlighting the need for integration of genetic and epigenetic data to identify potential candidate “hepitypes” (Baylin, 2005; Murrell et al., 2005).

Gerd Pfeifer (Beckman Research Institute) presented a novel approach to study the DNA methylation in the mammalian genome named the Methylated-CpG Island Recovery Assay (MIRA). MIRA is based on the high affinity of the methyl binding proteins (MBPs) for methylated DNA (Rauch et al., 2006). This strategy has been applied, in combination with Agilent CpG island arrays and NimbleGen tiling arrays, to analyse methylation patterns in lung squamous cell carcinoma and matched normal lung tissue. This study revealed that CpG methylation may represent a promising biomarker for early detection of lung cancer (Rauch et al., 2006). The concept of chromosomal methylation hotspots and the role of extensive DNA hypomethylation in tumours were also discussed, highlighting the significance of methylated-CpG islands as diagnostic markers.

3. Epigenetic inheritance, biological processes and stemness

The fact that all cells in any given organism share an identical genome, yet they exhibit strikingly different morphological and functional properties, testifies to the importance of epigenetic features. All developmental and differentiation processes are guided largely by epigenetic mechanisms with such a key role, epigenetic events are therefore likely to define the identity and proliferation potential of different cells. It should be noted, however, that epigenetic inheritance is less stable than DNA-based inheritance and typically results in a non-Mendelian pattern of inheritance.

Genomic methylation patterns are determined during embryogenesis by the activity of DNA methyltransferases (Rountree et al., 2001). While X chromosome inactivation is random in somatic cells, the expression of imprinted genes is controlled by parental origin, suggesting that epigenetic mechanisms are not arbitrary but rather precisely regulated (Jaenisch and Bird, 2003). Genomic imprinting is defined as monoallelic gene expression that is dictated by parental origin. It is a phenomenon through which epigenetic information (in the form of a DNA methylation pattern) is imposed to

DNA sequence. This information is erased at meiosis and re-established during embryogenesis to allow genes to be properly transcribed. This process is controlled by regulatory element called as “imprinting control region”, which responds to DNA methylation (Delaval and Feil, 2004). Many of the imprinted genes are involved in important cellular processes including cell growth, signalling, the cell cycle, and embryonic development (Jaenisch and Bird, 2003). Robert Feil (Institute of Molecular Genetics) illustrated how epigenetic alterations of imprinted genes can give rise to several congenital diseases such as Silver–Russell Syndrome and Beckwith–Wiedemann Syndrome. Interestingly, while all epigenetic marks are usually removed to allow gene expression in embryo, imprinted genes escape the demethylation programme following fertilization. In somatic cells, the allelic DNA methylation at imprinting control locus was related to tri-methylation of H4 lysine 20 and H3 lysine 9 (Delaval et al., 2007; Fournier et al., 2002; Umlauf et al., 2004; Wagschal et al., 2008), suggesting that these regions may be involved in the somatic maintenance of their allelic methylation. Currently, the potential of human embryonic stem cells as a therapeutic application for degenerative diseases such as Parkinson disease is much talked about. However, culturing these cells may cause epigenetic changes that lead to disruption in gene expression and phenotype (Pannetier and Feil, 2007). Consequently, a better insight into changes in epigenetic mechanisms during embryonic development is needed before considering potential therapeutic purposes of these cells.

Dirk Schübeler (Friedrich Miescher Institute) discussed the interesting viewpoint of the involvement of epigenetic modifications during terminal differentiation of murine stem cells and the cross talk between RNA polymerase and chromatin. The transition from undifferentiated to differentiated status of embryonic stem cells is characterized by loss of pluripotency that correlates with setting up of epigenetic repressive marks including DNA methylation. In order to analyse genome-wide distribution of DNA methylation and its effect on gene expression, Schübeler’s team has generated an epigenomic map of DNA methylation, RNA polymerase II occupancy and chromatin status on promoters in murine somatic and germline cells. This analysis has revealed that promoter regions may be classified according to their concentration in CpG sites: strong, weak, and poor CpG islands (Weber et al., 2007). He also suggested that the frequency of promoter DNA methylation and its effect on gene expression are generally established by structure of cis-regulatory regions rather than by transcriptional activity.

Although it is well accepted that epigenetic information is centrally connected to the abnormal events that lead to oncogenic transformation and tumour development, the underlying molecular mechanisms remain to be defined. Epigenetic information in chromatin is an essential mechanism by which cells regulate accessibility to chromatin DNA, hence allowing critical processes that utilise DNA as a template, such as gene transcription or DNA repair, to occur. Chromatin modifications occur at the level of histones that are targets for several post-translational covalent modifications, including acetylation, phosphorylation, and methylation (Jenuwein and Allis, 2001). Factors that mediate chromatin modifying activities include: histone acetyltransferases (HATs), histone

deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases, and adenosine triphosphate (ATP)-dependent nucleosome-remodelling factors (Carrozza et al., 2003; Roberts and Orkin, 2004). Despite remarkable progress in identifying different forms of epigenetic information in chromatin and the molecules/complexes responsible for these modifications, their biological function in normal cellular processes and disruption in cancer remain largely unknown. Zdenko Herceg (IARC) gave some insights into the mechanisms by which histone modifying factors, such as TRRAP (TRansformation/tRanscription domain Associated Protein)/histone acetyltransferases, and histone acetylation may control chromatin-based processes including gene transcription, DNA repair, and DNA damage response (Murr et al., 2007; Raval et al., 2007). He discussed possible impact of the deregulation of chromatin modifications on oncogenic transformation and tumour development and new insights into possible mechanisms by which histone acetylation and other chromatin modifications may regulate cell renewal and differentiation, and how deregulation of histone marking may lead to tumourigenesis.

4. DNA methylation as biomarker in cancer

In 1983, the discovery of involvement of epigenetic mechanisms in human cancer opened up the way to innovative approaches for understanding tumour processes and development of potential therapeutic applications (Feinberg and Tycko, 2004). The coexistence of both genome-wide hypomethylation and local hypermethylation is the hallmark of tumourigenesis. Therefore, DNA methylation represents an attractive biomarker of tumourigenesis that can be exploited in clinics and molecular epidemiology.

Human colorectal cancer is one tumour type that could benefit from this approach. This cancer can be effectively treated and often cured by surgical resection, if the malignancy is detected at early stage. Until now, screening tools that detect such early stage lesions use invasive methods (e.g., colonoscopy), which are costly and uncomfortable for patients. Manon van Engeland (Maastricht University) talked about the identification of two promising biomarkers that could lead to non-invasive tests for colorectal cancer, namely GATA-4 and GATA-5. Hypermethylation and consequent silencing of GATA-5 and GATA-4 has been described as the most sensitive and most specific marker (Akiyama et al., 2003). The stability of these epigenetic markers has been confirmed in a prospective study involving 2000 patients being treated at the University Hospital Maastricht, with the aim of evaluating the efficiency and cost-effectiveness of use of methylation markers compared with colonoscopy.

Lung cancer is another tumour for which early detection is crucial for improving treatment effectiveness. In his presentation, Triantafyllou Liloglou (University of Liverpool) proposed the use of DNA methylation as an attractive diagnostic tool based on the fact that frequent epigenetic alterations are found in lung cancer. He outlined a strategy for identifying good epigenetic markers. First, potential biomarkers should be selected by use of high throughput analysis, with, for instance,

microarray based methylation analysis to identify prospective candidates. Then, an accurate analysis is performed on clinical samples by using quantitative methods, such as pyrosequencing or real time quantitative methylation specific polymerase chain reaction, that provide consistent diagnostic results.

5. Towards clinical applications

Robin Ward (POW Clinical School) focused her discussion on the methylation of the hMLH1 promoter and the germline epimutation inheritance in colorectal cancer. Epimutations have been described as all changes in the epigenetic state of a gene that leads to inappropriate activation or silencing in the absence of DNA sequence changes. In colorectal cancer, promoter hypermethylation of both alleles of hMLH1 arises as a somatic event in about 15% of cases. Cancer cells are characterized by loss of hMLH1 (MutL homolog-1) expression and microsatellites instability caused by the defect in DNA mismatch repair. It has been shown that individuals with hypermethylation of a single allele of hMLH1 in all somatic cells have a predisposition for the development of cancer in a pattern typical of hereditary nonpolyposis colorectal cancer, suggesting a germline epimutation inheritance. While genetic mutations are transmitted from one generation to the next in a Mendelian pattern, epimutations are relatively unstable because of the epigenetic reprogramming process during embryonic development, that virtually removes all epigenetic marks (Hitchins et al., 2007). The fact that epimutations may be inherited highlights the importance of epigenetic mechanisms in cancer predisposition.

Gastrointestinal cancer is a progressive process, resulting in multiple genetic but also epigenetic changes that alter expression of genes involved in several cellular mechanisms including cell cycle regulation, apoptosis, and DNA repair. Minoru Toyota (Sapporo Medical University) discussed how simultaneous genetic and epigenetic analysis in primary colorectal cancer may reveal specific DNA hypermethylation events leading to the establishment of a CpG island methylator phenotype with distinct clinicopathological features (Shen et al., 2007; Toyota et al., 1999). Toyota also discussed the link between epigenetic events and environment. It has been proposed that *Helicobacter pylori*, a major risk factor for development of gastric cancer, causes DNA methylation alterations, leading to carcinogenesis. This evidence highlights how epigenetic mechanisms, under the influence of environmental infection agents, can be disrupted resulting in an abnormal phenotype.

6. Conclusions and perspectives

This meeting provided an overview of recent conceptual advances in cancer epigenetics and technology advances in epigenomics that allow genome-wide and high throughput analysis of epigenetic changes in cancer samples (Figure 1). It is now widely recognized that, in addition to genetic mutations, deregulated epigenetic mechanisms are also involved in virtually every step of cancer development and progression – as evidenced by the fact that funding agencies put cancer epigenetics on their priority list. For example, at the end of

Figure 1 – Epigenetics and cancer: an interface between genotype and phenotype. Epigenome extends and modulates genome and thus dictates phenotypic outcomes. The epigenome is under constant influence of endogenous cues and exogenous (environmental) stimuli and its deregulation (changes in DNA methylation and histone modification patterns) may lead to the disruption of critical cellular processes (such as gene expression and DNA repair) and cancer development. In contrast to genetic alterations, epigenetic changes occur gradually and are in principle reversible, features that can be exploited for the development of therapeutic and preventive strategies. Early appearance and universal presence of epigenetic alterations in virtually all human malignancies make them attractive tools in clinical diagnostic settings and population-based screening. Recent progress in epigenomics and emergence of new technologies for detection of epigenetic changes in high throughput and genome-wide settings will improve our capacity to evaluate the contribution of epigenetic changes in specific human cancers and identification of potential biomarkers. The fact that major funding agencies (including NIH) has identified cancer epigenetics as a priority area of cancer research should facilitate these endeavors.

2007, the National Institute of Health (NIH, USA) launched a comprehensive programme on cancer epigenetics as a part of new NIH Roadmap Initiative (nihroadmap.nih.gov/epigenomics/index.asp), which was discussed at the meeting by Mukesh Verma (Methods and Technologies Branch). Recent studies provided important information on the mechanisms by which epigenetic inheritance mediates critical regulatory processes and how epigenetic patterns are disrupted in human malignancies. Numerous physical and chemical carcinogens and infectious agents in the environment are believed to promote neoplastic process through disruption of epigenetically maintained patterns of gene expression and chromatin structure and function. The list of environmental epimutagens is likely to grow rapidly with the completion of large-scale molecular epidemiology studies aiming to identify new cancer risk factors in the environment.

Epimutagens appear to be capable of deregulating a number of critical processes found in cancer cells, such as silencing of tumour suppressor genes, activation of oncogenes, aberrant cell cycle control, defects in DNA repair and detoxification of carcinogens. However, further studies are needed to elucidate

molecular mechanisms by which epimutagens impair the establishment and maintenance of normal patterns of epigenetic modifications and to identify associated abnormal epigenetic events causally involved in cancer development.

Importantly, epigenetic changes may occur early in tumour development and may trigger a spectrum of genetic alterations such as mutations and chromosomal aberrations. Therefore, it is of great interest to identify what are the primary and what are the secondary genetic and epigenetic events induced by specific environmental exposures. This will be important for a better mechanistic understanding of the process of tumourigenesis but also for the development of novel strategies for cancer prevention. Two important features that distinguish epigenetic changes from genetic alterations are gradual appearance and reversibility of epigenetic events. These features make epigenetic alterations an attractive target for therapeutic intervention and the development of novel preventive strategies. Many drugs capable of targeting changes in DNA methylation and histone modifications are currently in clinical trials. Current efforts in clinic will likely to result in the emergence of many other drugs

capable of efficient targeting epigenetic changes in different cancer types. Epigenetic drugs may also prove efficient in preventing cancer development in individuals at high risk.

Emerging evidence supports the role of epigenetic changes induced by environmental factors in cancer development; however, epigenetic epidemiology is in its infancy and we know little on the contribution of environment-induced epigenetic changes to cancer burden. Recent advances in epigenomics and emergence of powerful technologies for detection of epigenetic changes in high throughput and genome-wide settings (Callinan and Feinberg, 2006; Esteller, 2007) will further advance our capacity to evaluate the contribution of epigenetic changes induced by the environmental epimutagens to human cancer. These powerful tools will be applied to large population-based and case-control studies which offer some of the most exciting opportunities to study the contribution of epigenetic events to specific human cancers. Application of epigenetic markers to epidemiological studies requires careful considerations in the design of such studies. Specifically, random and systematic errors should be avoided through the enrolment of a sufficiently large series of cancer cases and controls. Sample size should be estimated based on power calculations that depend on the background rate of the marker and the expected strength of the association because the major obstacle in establishing a relationship between epigenetic changes and exposure to putative environmental “epigenetic carcinogens” (epimutagens) in cancer is the fact that case-control studies tend to be too small and to lack the statistical power needed to identify the interactions between epigenetic changes and dietary, environmental and lifestyle factors.

Acknowledgements

We are grateful to Carla Sawan and Thomas Vaissière and other members of Epigenetics Group for a great assistance and Susan Anthony for an excellent administrative organisation. We acknowledge the financial support of the meeting from Dr. Peter Boyle (the director of IARC). Our work is supported by grants from Association for International Cancer Research (AICR), United Kingdom; the National Institutes of Health/National Cancer Institute (NIH/NCI), United States; Institut National du Cancer (Epigenetic profiling Network, EpiPro), France; L'Association pour la Recherche sur le Cancer (ARC), France; la Ligue Nationale (Française) Contre le Cancer, France; the European Network of Excellence Environmental Cancer Risk, Nutrition and Individual Susceptibility (ECNIS), and the Swiss Bridge Award (to ZH).

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