

# Chapter 2

## Evolution of Cytogenetic Methods in the Study of Cancer

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### Abstract

Cytogenetic methods have not changed greatly over the last 50 years since Nowell and Hungerford's description of the Philadelphia chromosome but the clinical utility of these methods has evolved dramatically. The multicentre clinical studies that have identified major clinical applications for cytogenetic analysis in different cancers and the development of in situ hybridization have contributed to an explosion in cytogenetic testing for cancer patients.

**Key words:** Cytogenetic analysis, Fluorescence in situ hybridization, Translocations, Array comparative genomic hybridization, SNP arrays

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### 1. Introduction

Cytogenetic analysis has become an integral part of the diagnosis and management of many malignancies. Theodor Boveri was the first to suggest that malignant tumours could be due to an abnormal chromosome constitution (1). His hypothesis stated that the cell of a malignant tumour has an abnormal chromosome constitution and that any event leading to an abnormal chromosome constitution will result in a malignant tumour. He also postulated the existence of enhancing or suppressing chromosomes, suggesting that malignant growth would result from loss of suppressing chromosomes or the predominance of enhancing chromosomes. Thus, prior to the concept of genes, Boveri foreshadowed the existence of oncogenes and tumour suppressor genes.

The term chromosome was first coined by Waldeyer in 1888 but it took nearly 70 years for the chromosome complement of a normal human cell to be reliably determined. The birth of cytogenetics is generally dated from Tjio and Levan's identification of

the true chromosome complement in human cells as it is from this time that abnormalities of chromosome number and subsequently chromosome structure were reported (2). Their discovery was made possible by a number of advances. In his delightful book, Hsu divided the study of human cytogenetics into four periods: the pre-hypotonic period (or Dark Ages), the period from 1952 to 1959 that included the discovery of hypotonic solution pre-treatment for cytological preparations, the third period (1959–1969) during which time chromosome abnormalities were linked to clinical syndromes, and the post-banding (modern) period (3).

During the pre-hypotonic era, chromosomes were studied in mouse and rat cancers and camera lucida drawings of metaphases suggested the presence of many more chromosomes than normal and of structural abnormalities within these chromosomes. The drawings were taken from squash preparations, a technique that was used to flatten metaphase spreads of chromosomes into a two-dimensional configuration, but still resulted in crowded overlapping aggregations of chromosomes that were very difficult to count. Colchicine, an extract of the autumn crocus, was used to arrest the cells in the metaphase stage of the cell cycle and increase the number of mitoses available for analysis.

The use of a hypotonic pre-treatment method was an enormous step forward in the production of analysable chromosome preparations. The chromosomes could now be separated and viewed individually. Counting chromosomes was simplified and gross structural abnormalities could be discerned. Hsu describes the discovery of the utility of a hypotonic pre-treatment as a laboratory accident, the perpetrator of which never owned up to the error; thus, a major discovery in the history of cytogenetics was apparently made by an unknown technician.

It was in this era, in 1956, that Tjio and Levan finally answered the question that had been plaguing investigators for more than 30 years, when they reported that there were 46 chromosomes in the human cell rather than 48. Subsequently, a number of researchers were able to identify chromosome abnormalities that appeared specific for clinical syndromes. Lejeune and his colleagues published the chromosomal nature of Down syndrome in 1959 (4). Their observation of an extra G group chromosome in patients with a specific congenital malformation syndrome showed for the first time that cytogenetic analysis could be used to diagnose a human condition. From this time, there was a stream of publications describing chromosome aneuploidies associated with other malformation syndromes. A number of the early, seminal papers in the field have been reproduced in Peter Harper's excellent study of the beginnings of human cytogenetics (5). The study of the constitutional karyotype was aided by the discovery that phytohemagglutinin (PHA) could induce peripheral blood lymphocytes

to divide (6). This method was adopted by Moorhead et al. (7) for the study of human chromosomes and remains one of the mainstays of modern cytogenetic analysis.

The confirmation of the correct chromosome number in human cells led in the late 1950s and early 1960s to a flood of publications describing numerical and structural abnormalities of chromosomes. The resulting confusion in the literature made clear that there was a need for a common nomenclature to describe these rearrangements in a manner that was intelligible to other workers in the field. Thus, a small group met in Denver, CO, to establish a system of describing chromosome abnormalities. They published the results of their deliberations in a report entitled “A Proposed Standard System of Nomenclature of Human Mitotic Chromosomes,” also known as the Denver Conference (1960), and this report has formed the basis for all subsequent nomenclature reports, now published as An International System for Human Cytogenetic Nomenclature (ISCN) (8).

The ability to create a banding pattern on human chromosomes understandably complicated the nomenclature. Meetings in Paris and Edinburgh proposed a basic system for designating chromosome regions and bands, resulting in a report of the Paris Conference (1971). Crucially, this report provided a way of describing structural rearrangements in terms of the band composition and the breakpoints involved in the rearrangement. The subsequent ISCN publications have been updated to encompass the various advances in the field, including fluorescence in situ hybridization (FISH) and arrays, but their core function remains the description of chromosome abnormalities in a manner that allows a cytogeneticist to interpret the report of a colleague immediately from anywhere in the world. Whilst the complexity of chromosome rearrangements in cancer cells frequently tests this system to the limit, as a scientific form of Esperanto, it has been spectacularly successful over the years.

The advent of banding enabled chromosomes to be individually identified and the normal homologues paired. Initially, banding patterns along the length of each chromosome were induced by preparations stained with quinacrine mustard and visualized via a fluorescence microscope (9) or depended upon a method whereby slides were incubated in warm saline or buffer solutions prior to staining by Giemsa. The initial Giemsa staining method required 3 days for completion. Seabright’s rapid banding technique was therefore embraced as the whole procedure could be carried out at room temperature using air-dried slides and producing G-banded chromosomes ready for observation within 10 minutes (10). Once banding became available, there were numerous publications describing recurrent chromosome abnormalities that appeared to be found in specific tumour types.

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## 2. Cancer Cytogenetics

The history of cancer cytogenetics is not a long one but it has been eventful and much knowledge has been accumulated in the 50 years since Peter Nowell and David Hungerford published their finding of a small marker chromosome in the chromosome complement of cells cultured from seven patients with chronic myeloid leukaemia (CML) (11). Nowell and Hungerford made their landmark discovery in 1960, only just beating another group from Edinburgh who had also noted the same marker chromosome in their CML patients (12).

Although the hypothesis that malignant cells were derived from normal tissue cells that had acquired an abnormal chromatin content was first proposed by Boveri, it was not until Nowell and Hungerford's description of the Philadelphia chromosome that a revolution in our understanding of the processes underlying the development of malignancy began. Nowell and Hungerford called the marker chromosome the Philadelphia chromosome 1, Ph<sup>1</sup>, after the city in which they worked and the number 1 superscript signalled that they fully expected that there were many more cytogenetic markers of cancer to be discovered. This was an exciting finding and an exciting time that effectively launched the field of cancer cytogenetics but the following years were frustrating as abnormalities were observed in various cancers but the inability to identify specific chromosomes by any method other than their basic shape limited researchers' abilities to link abnormalities with different morphological subtypes of haematological or solid tumours. All this changed with the advent of banding techniques.

Banding allowed the chromosomes to be clearly distinguished from one another and, most importantly, revealed the nature of structural abnormalities: balanced translocations of material between chromosomes, deletion of part of a chromosome, duplication of another segment, or an inversion of a chromosome segment. In 1973, Janet Rowley reported that a reciprocal translocation between chromosomes 9 and 22 resulted in the Philadelphia chromosome (13). Since then, hundreds of rearrangements have been identified including not only translocations but also deletions and additions of part or all of chromosomes and also inversions of genetic material within chromosomes.

From the descriptions of chromosome rearrangements, long before the Human Genome Project shone a light on the location of genes strung along our chromosomes, the molecular biologists were able to discover critical genes at the breakpoints of translocations. Banding continues to allow us to identify new chromosome abnormalities in both haematological and solid tumours and these abnormalities provide the sign posts to the critical genetic changes that underlie the transformation of normal cells into cancer cells.

The first cytogenetic abnormality to have its genetic secrets unlocked was the 8;14 translocation which characterizes Burkitt lymphoma/leukaemia. Researchers identified that the translocation caused two genes, *MYC* on 8q24 and the immunoglobulin heavy chain gene, *IGH* on 14q32, to come together (14, 15). It is now known that two classes of translocations are found in malignancies. The first type is epitomized by the t(8;14) in Burkitt lymphoma, one gene which is already actively transcribed in the cell type, such as *IGH* in B lymphocytes, is juxtaposed to a gene such as *MYC* which is, by virtue of its resulting proximity to *IGH*, up-regulated. Other translocations such as the t(8;21) in acute myeloid leukaemia (AML), also described by Janet Rowley in 1973 (16), and the t(9;22) in CML, form fusion genes with a “new” gene product which incorporates part of the normal genes broken at the sites of translocation. In the case of t(8;21), the genes involved are the *RUNX1T1* (originally named ETO after “Eight Twenty-One”) gene on 8q22 and the *RUNX1* (*AML1*) gene on 21q22 (17); the t(9;22) causes a fusion of the *BCR* gene on 22q11.2 and *ABL1* on 9q34 (18, 19). It is the altered function of these “new” fusion genes that appears to transform the cell, as shown by the development of CML-like disorders in mice into which *BCR-ABL1* constructs have been inserted (20).

It took time for the medical and scientific worlds to realize the importance of the cytogenetic discoveries of the 1960s and 1970s. It was necessary to convince clinicians that the chromosome changes being described in the marrow and peripheral blood of their patients with a variety of malignancies could provide valuable information about the type and prognosis of these disorders. Many important clinical correlations were either identified or confirmed in the International Workshops on Chromosomes in Leukaemia. These constituted gatherings of physicians and scientists from around the world who brought together case studies of chromosome analyses together with clinical and laboratory data relating to each case.

The first of these was held in Helsinki, Finland in August 1977 (21). Laboratories participated from Belgium, Finland, Sweden, England, Germany, and the USA and the participants reviewed the data of 223 patients with Ph<sup>1</sup>-positive CML and 279 patients with acute non-lymphocytic leukaemia. A number of further workshops were held and information regarding the incidence and prognostic significance of rearrangements in CML, AML, acute lymphoblastic leukaemia (ALL), and myelodysplastic syndromes (MDS) provided by these workshops formed the basis for all future studies. Subsequently, national and multinational clinical trial groups have incorporated cytogenetic studies into their prospective trials and provided a wealth of data to show that cytogenetic analysis is of diagnostic and prognostic importance in most haematological malignancies and a number of solid tumours.

It has only been by the careful observation of chromosome abnormalities and their correlations with clinical features that true insights have been obtained as to the underlying genetic basis of malignancy.

Whilst the basic cytogenetic methods used in laboratories around the world today are very similar to those first described in the 1960s and 1970s, there are areas where improvements have been made. Mitogens were introduced into cultures to induce chronic lymphoid malignancy cells to divide in the late 1970s (22) but further refinements and combinations of mitogens are still being discovered. For example, Chapter 9 describes a recently discovered method that enables chromosome abnormalities to be identified in the majority of cases of chronic lymphocytic leukaemias (23).

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### 3. Introduction of FISH Testing

The identification of the genes involved in chromosome translocations paralleled the development of *in situ* hybridization (ISH) and so allowed the most significant advancement in the field of cytogenetics to come into being. Early ISH studies used tritiated thymidine to label the DNA fragments that were used as probes (24). Slides were prepared by dropping fixed cytogenetic suspension onto the slide. After the subsequent application of probe, the slide was immersed in photographic emulsion, wrapped in foil, and stored away in a light proof box for up to 2 months. The localization of the probe was identified by “developing” the slide so that silver granules were deposited at the site of the tritiated thymidine emissions. In expert hands, this method worked well and many of the early gene localizations were made using this method. However, the necessity of performing most of the steps in total darkness and the time required for hybridization made this a most frustrating method as, after 2 months, it was entirely likely that the test had been unsuccessful and determining the reasons for failure after such a time period was extremely difficult.

The development of FISH was therefore very welcome indeed. FISH did not require total darkness for successful completion of testing and a result could be obtained in 24 h. The ease with which routine diagnostic laboratories could establish FISH techniques now enabled them to be used in the routine diagnostic setting for the first time. Moreover, FISH probes were developed that allowed the cytogeneticist to determine the presence or absence of extra copies of chromosomes, translocations, and deletions in non-dividing cells. Initially, the probes were home-grown with single colour fluorescent signals for gains and losses of chromosomes and the translocation probes produced only a single fusion signal. However, the increasing use of commercial FISH

probes in the clinical setting ensured that the probe designs evolved. False-positive and false-negative results were reduced by designing probes with built-in controls or with a resulting signal pattern that could not be readily duplicated by accidental co-localization in a normal patient control slide.

The ability to identify chromosome rearrangements in non-dividing cells has proved particularly useful in the chronic lymphoid malignancies. FISH has been used with panels of probes to identify prognostic subgroups within CLL (25) and plasma cell myeloma (26). FISH has also identified cryptic translocations such as the t(12;21) in paediatric ALL (27) and t(4;14) in myeloma (28) and cryptic deletions such as the 4q12 deletion that results in a *PDGFRA-FIP1L1* fusion gene (29). Both translocations and deletions are invisible microscopically and could only be found by molecular methods.

Further refinements of FISH methods enabled the effective painting of each chromosome a different colour so that complex karyotypes could be elucidated (30, 31) and the combination of FISH probes with fluorescent-labelled antibodies to identify individual cell types has proved invaluable in identifying chromosome abnormalities in disorders with variable marrow infiltration such as myeloma (32). Such strides have been made in the last 20 years in the use of FISH in cancer diagnosis that it now seems inconceivable for cytogenetics laboratories not to use FISH routinely.

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#### 4. Array-Based Karyotyping Methods

One of the innovative uses for FISH testing that has evolved has been the development of comparative genomic hybridization (CGH). CGH involves the labelling of patient DNA and normal control DNA with green and red fluorochromes, respectively. The two DNAs are then allowed to compete for hybridization on a slide containing normal chromosome preparations. The concept relies on a computer “reading” each chromosome and assessing the proportion of green- and red-labelled DNA that has hybridized along the length of each chromosome. If there are no gains or losses of DNA in the patient sample there should be an equal proportion of patient and control DNA hybridized to each chromosome and an equal mixture of green and red fluorescence rendering each chromosome yellow. In the event that there is loss of part of a chromosome in the patient genome, there is a disproportionate amount of control DNA hybridizing to that chromosome and so it appears red. In contrast, an extra segment of DNA in the patient sample causes that segment of the normal chromosome to appear green. CGH has been used largely in the research setting and achieved only limited use in diagnostic laboratories.



It produced a picture of genetic abnormalities across the genome without the need to produce metaphase spreads but its major drawback was the resolution only allowed the detection of very large gains and losses of DNA.

However, the application of CGH to arrays of bacterial artificial chromosomes (BACs) or oligonucleotides dotted onto slides or “chips” has overcome the problem of resolution and made CGH an enormously powerful tool in cytogenetics. Array CGH is capable of mapping deletions or amplifications measured in kilobases rather than megabases. It is also possible to design arrays that target specific areas of interest or cover the entire genome. To date, these arrays are not capable of detecting balanced translocations but their ability to detect changes in copy number is extraordinary. For the cancer cytogeneticist, the challenge will be how to interpret the vast amount of information generated by these arrays. A leukaemia karyotype may appear to contain a simple chromosome abnormality but the array CGH applied to the same genome may uncover hundreds of sub-microscopic rearrangements. Only large clinical trials incorporating array data collection will enable us to determine what is important and what is not from these vast repositories of information.

Another refinement has been added to the use of arrays. It is now possible to detect uniparental disomy or, as those who work in the field of acquired abnormalities in cancer prefer, copy number neutral loss of heterozygosity (LOH) or acquired isodisomy. LOH without loss of one copy of a DNA segment refers to regions of cancer genomes where it appears that one chromosome has lost a region but has replaced it with a duplication of the identical segment from the other homologue. The regions of LOH can be identified by the use of single nucleotide polymorphism (SNP) arrays. SNP arrays detect the presence of thousands of polymorphisms along the length of each chromosome, and so a stretch of DNA without any variation observed between the two homologues indicates either that only one copy of the region is present or, if it is clear that there is no deletion, that duplication of one copy has occurred. The biological impetus for this action appears to be, in many instances, to achieve a doubling of a gene mutation such as *TET2* mutations in chronic myelomonocytic leukaemia (CMML). CMML usually has a normal karyotype but SNP arrays have shown copy number neutral LOH involving a region of the long arm of chromosome 4 in up to 35% of cases and most of these have been shown to carry homozygous *TET2* mutations (33). The power of these arrays appears likely to reveal many more genetic rearrangements in different cancers.

Ultimately, the challenge for cytogeneticists and for clinicians will be how to use the current and future technologies to best serve the needs of our patients. Conventional cytogenetics remains a powerful and affordable test that is integral to the management



of patients with a wide variety of malignancies. FISH, too, has become an important tool both for diagnosis and to predict outcome in many cancers. The potential of the array technologies cannot be under-estimated but their role in the care of cancer patients remains to be defined. And thus, just as the 1960s and 1970s were exciting decades for cytogeneticists, so too will be the coming years as we cope with integrating existing and emerging technologies.

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