Clinical Medical Research Award

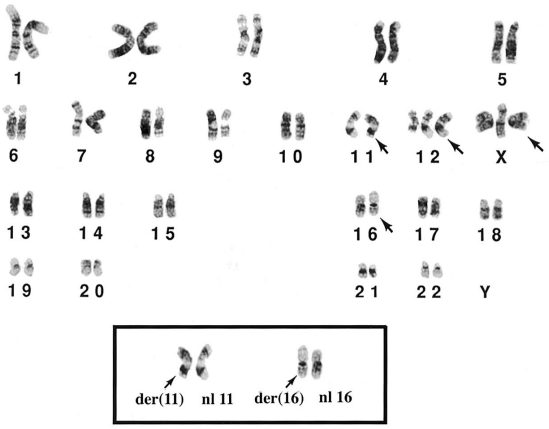
Cancer genetics, cytogenetics—defining the enemy within

PETER NOWELL, JANET ROWLEY & ALFRED KNUDSON

Suggestions of a significant relationship between chromosome abnormalities and tumor development came first from several German pathologists in the late nineteenth century[1]. It was, however, the biologist Theodor Boveri, who worked with sea urchins, not tumors, who first posited several hypotheses (subsequently proved correct) on the importance of somatic genetic changes in tumor development[2]. Boveri observed developmental defects in sea urchins with mitotic abnormalities and suggested that mammalian tumors might be similarly initiated by aneuploid chromosome complements. Remarkably, he also suggested the importance of genetic instability in tumor cell populations, the possible unicellular origin of tumors and the significance of specific chromosomal or submicroscopic changes. Unfortunately these hypotheses preceded the techniques required to test them and so Boveri's concepts lay dormant for several decades.

In the 1930s and 1940s a few studies in both experimental and human tumors did suggest that chromosome numbers were usually abnormal in neoplastic cells and the concept of a 'stemline' or clonal nature of tumors, with acquisition of additional genetic changes over time, was explored, primarily in transplantable rodent tumors[1]. However the 'modern' era of cytogenetics did not begin until the mid 1950s when improved cell culture and slide preparation techniques made it possible to accurately enumerate the number of human chromosomes as 46.

A second source of insight into genetic changes associated with cancer was a series of pre-twentieth-century reports of uncommon families with an excess of cancer. By 1930, dominantly inherited breast and colon cancers had been described, as had hereditary retinoblastoma and neurofibromatosis. The relationship between rare heritable cancers and common nonhereditary cancers was puzzling and mostly beyond investigation in the absence of the ability to map and clone genes. Meanwhile, there could be only speculation about the determinants and mechanisms of penetrance in heterozygous carriers of mutant genes. Early studies of cancer inheritance in mice pointed towards multigenic determination of carcinogenesis and away from strongly predisposing single genes.



Identifying translocations.

PETER NOWELL

I inadvertently entered the field of tumor cytogenetics shortly after the determination of the correct human chromosome number. Joining the pathology department at the University of Pennsylvania in 1956, I extended an interest in leukemia developed during my limited residency training and two years at the US Naval Radiological Defense Laboratory (NRDL) and began studying the growth and differentiation of human leukemic cells in short-term cultures. The cells were grown on slides and, in accordance with my pathology training, rinsed under tap water and stained with Giemsa prior to examination. Unaware that this procedure was an accidental rediscovery of the technique of hypotonic cytogenetic preparation, I simply noted that my slides contained metaphase stage chromosomes. I knew nothing of cytogenetics, but this seemed worth pursuing and I was soon directed to a graduate student, David Hungerford, who was attempting to find material for his thesis project. We promptly began a collaboration: I obtained the cells and cultured them, and Dave (using the 'squash' technique of the time) examined both acute and chronic leukemic cells from a number of patients. The first positive finding was his identification of a characteristic small chromosome in the neoplastic cells of two male patients with chronic myelogenous leukemia[3] (CML). Subsequently, using an improved air-drying technique of slide preparation[4], we identified the same tiny chromosome in other patients. As this was the pre-banding era, the specific chromosome involved could not be identified and so, in accordance with the nomenclature suggested by the First International Conference on Cytogenetics (1960), it was designated the Philadelphia chromosome (Ph).

Because this specific somatic genetic alteration was present in all of the neoplastic cells of nearly every case of typical CML examined, we felt that these findings strongly supported Boveri's suggestion that tumors arise from a critical genetic alteration in a single cell, allowing its progeny to expand in a clonal fashion, and that the Philadelphia chromosome might represent the essential genetic change that led to this particular form of human leukemia. However, we (and other groups) had a very frustrating time as we attempted to confirm and extend these conclusions over the next decade. With the techniques then available, no consistent cytogenetic changes were demonstrated in other neoplasms, leading some investigators to suggest that the Philadelphia chromosome was an epiphenomenon and not of essential importance. Only with the advent of chromosome banding and subsequently molecular

techniques that made it possible to identify a wide variety of specific somatic changes in essentially all human tumors, were these concepts reinforced.

Meanwhile, we were able to make limited progress in other areas. On the basis of clinical progression in leukemia and preleukemia, as well as on earlier collaborative work on radiation carcinogenesis with Leonard Cole at the NRDL (where we also stumbled upon xenogeneic bone marrow transplantation), we extended our views on tumor development. In a 1965 review co-authored with Cole[5], we suggested that clinical tumor progression could result from the sequential acquisition of multiple genetic changes to the neoplastic clone, and the potential importance of genetic instability in this population, facilitating this process. I began using these ideas, ultimately dating back to Boveri, in my introductory lectures on cancer to medical students and eventually published a more detailed view of this 'clonal evolution' concept a decade later. Again, it was not until the era of molecular cancer genetics that this was substantiated in a number of human neoplasms, and particularly colon cancers[6], and became more widely accepted.

Also in the 1960s, we were able to use another fortuitous observation—that the bean extract phytohemagglutinin (PHA) was a lymphocyte mitogen[7]. This finding, and further technical developments in tissue culture and chromosome procedures, provided a new and easier approach to studying normal human chromosomes using peripheral blood lymphocytes. This was of little benefit in tumor cytogenetics, in which spontaneously dividing cells were plentiful, but it did provide a standard method for constitutional cytogenetics as well as a basis for a variety of *in vitro* immunological studies.

After these early developments in tumor cytogenetics and related areas, the next two decades finally provided the exciting linkages between various types of cancer and specific cytogenetic and molecular genetic alterations.

JANET ROWLEY

While I was working in a clinic for retarded children, in 1959, Down Syndrome was discovered to be due to trisomy for chromosome 21. I was moving to Oxford with my family, and decided to learn cytogenetics, which I did with Marco Fracarro at the Oxford MRC Unit. I also studied the pattern of DNA replication of human chromosomes with Laslo Lajtha and, in 1962, with the support of Leon Jacobson, I continued my research on DNA replication in the Section of Hematology/ Oncology at the University of Chicago.

I began studying patients with pre-leukemia using unbanded chromosomes; these cells often had clonal gains or losses of chromosomes 6–12 (the so-called 'C group' chromosomes), but it was impossible to determine whether the changes were consistent. As noted by Peter Nowell, most scientists thought the chromosome changes in leukemias were an epiphenomenon with no relevance to the development of the disease. I took a second sabbatical in Oxford in the early 1970s, just as banding was being developed; I worked in the laboratory of Walter Bodmer. I studied banding with Peter Pearson at the MRC Unit, and applied it to the patients I had previously studied in Chicago.

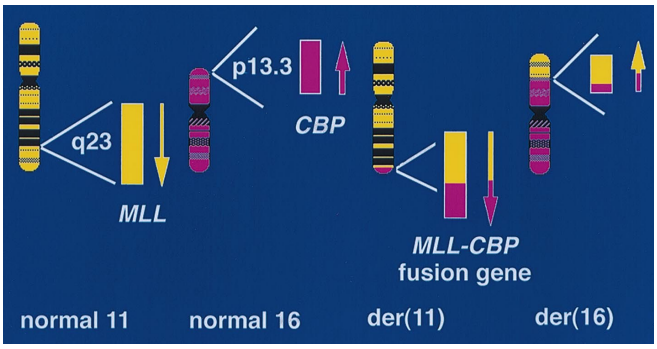
Banding was the breakthrough that allowed us to prove that the chromosome gains and losses were not random events, and in 1972 I discovered the first translocation, one involving chromosomes 8 and 21 in patients with acute myeloblastic leukemia. Later that same year, I studied cells from CML patients in blast crisis, because they have additional chromosome changes, often extra C group chromosomes, and I confirmed the Philadelphia chromosome as a chromosome 22. However, I noticed that one chromosome 9 always had an extra band at the end of the long arm; thus I went back to examine material from the same patients in the chronic phase when they had only the Ph chromosome. To my great surprise, it was obvious that a chromosome 9 in each of these patients was also abnormal. This soon led to the conclusion that, in fact, the Philadelphia chromosome was the result of a translocation with a small part of chromosome 22 being deposited on chromosome 9, an observation that was subsequently published in *Nature*[8].

Mapping the genes involved (Reprinted with permission from *Blood*, **90**, 535 (1997).

At this point, I was very perplexed because I now had two consistent rearrangements, the 8;21 and 9;22 translocations, each associated with a different type of leukemia. There was no precedent for such specific translocations, and the mechanisms behind such translocations were mostly a mystery.

In 1977, we discovered the 15;17 translocation found in acute promyelocytic leukemia (APL). The 15;17 translocation was seen exclusively in APL, providing convincing evidence of the specificity of translocations. This third example of a translocation, and particularly one with such specificity, convinced me that the chromosome changes were an essential component of the leukemogenic process and I soon became a 'missionary', attending hematology meetings in the 1970s and early 1980s carrying the 'gospel' that chromosome abnormalities were an essential component of hematologic malignant diseases to which the clinical community should pay attention. Many other recurring translocations were identified not only in leukemia but also in lymphoma and sarcoma, reinforcing the message[9]. Moreover, by the early 1980s it became clear that chromosome abnormalities, particularly in the acute leukemias, had prognostic implications—some translocations were associated with a better response to treatment—and gradually the karyotype of the patients with leukemia became a factor in the type of treatment that they received.

A real revolution took place in October 1982, when it was announced independently by Carlo Croce collaborating with Robert Gallo[10] and by Phillip Leder[11] that they had cloned the translocation breakpoint of the 8;14 translocation previously described by Laura Zech. It is impossible to exaggerate the importance of this discovery. It pro-



Formation of a new fusion gene by the balanced translocation t(11;16)(q23;p13.3).

vided information about the genes that were involved and, moreover, some new and quite astonishing information about the genetic consequences of these translocations.

The 9;22 translocation was cloned in 1984 by Gerard Grosveld and his colleagues and was shown to involve the Abelson (*ABL*) leukemia gene on chromosome 9 and a gene that was newly discovered as a result of cloning the breakpoint—that is, the breakpoint cluster region (*BCR*) gene on chromosome 22. In the 9;22 translocation, the *BCR* gene provided the 5' portion and the *ABL* gene provided the 3' portion including all of the essential domains of *ABL* that are involved in leukemogenesis in experimental animal leukemias. This was the first indication that translocations could lead to in-frame fusion and the formation of chimeric genes that encode a fusion mRNA and protein. Most translocations in acute leukemia and in sarcomas lead to in-frame fusion genes that are unique tumor specific markers[12,13] with considerable diagnostic importance.

One of the essential unanswered questions now is the cause of chromosome translocations. For some translocations in lymphoid tumors the involvement of the recombinase enzyme seems fairly clear. For myeloid disorders, however, there is little evidence that recombinase has a role, and thus the focus is on other DNA sequences that might predispose to breaks, such as ALU and ku sequences, translin and topoisomerase II (topo II) sites.

There is an unfortunate group of patients who have an initial solid tumor or leukemia treated with drugs that inhibit the religation function of topoisomerase II (topo II), and some of these patients develop treatment-related acute leukemia. Many of these patients have translocations involving a particular gene that our own laboratory has studied extensively: the *MLL* gene[13] (Fig.1). We and others have shown that there is a specific topo II cleavage site in the vicinity of many of the breaks in *MLL* that occur in these treatment-related leukemias. In addition, we have recently shown that there is a DNase I hypersensitive site in the same location. The unanswered question is whether there are similar topo II cleavage sites or DNase I hypersensitive sites within the breakpoint regions of the other chromosomes involved in translocations.

ALFRED KNUDSON

Having arrived in medicine after early fascination with genetics and embryology, I not surprisingly found myself attracted to pediatrics and, after an 'awakening' experience on the pediatric unit at the Memorial-Sloan Kettering Cancer Center, to pediatric oncology in particular. As exciting as the early successes with treatment were, my interest moved towards the etiology of the array of neoplastic conditions that affect children. At first I was intrigued by the possibility that childhood acute lymphocytic leukemia might be due to a virus, but could find no evidence for it.

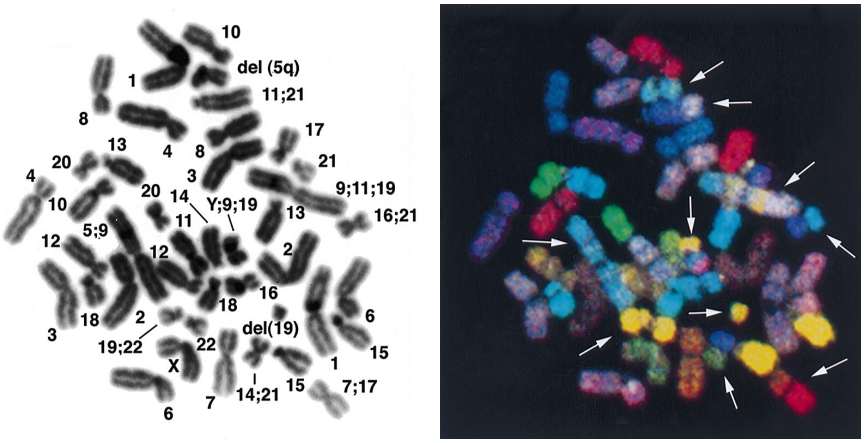
Turning my attention to the solid tumors, I became interested in their possible genetic origin. One case of neuroblastoma occurred in a child with neurofibromatosis type 1 who had no affected relatives and seemed therefore to represent a case of a new germline mutation in what we know as the *NF1* gene. This in turn set off the idea that neuroblastoma itself might, in some cases, be due to a new germline mutation and, like retinoblastoma, might sometimes be hereditary and other times not. What, then, might be the relation between the hereditary and non-hereditary forms? At first it seemed a daunting problem, as only a small percentage of cases of retinoblastoma had a positive family history of the disease. However, by 1970 it was apparent that the offspring of bilaterally affected individuals with no family history of the disease were at risk of developing the tumor—the 'hereditary' fraction included patients with new mutations and was obviously much greater than had been suspected; perhaps as much as 40% of all cases. Most unilateral cases are not heritable, although some are. Most hereditary cases had multiple retinoblastomas and were bilaterally affected, but some had only one tumor and a few percent of obligatory carriers had none.

It was obvious that a germline mutation is not sufficient to produce a tumor. Indeed, at the cellular level, tumor formation is very rare, with a Poisson mean of just three tumors per patient, yet millions of target cells. However, the fact that some cases are diagnosed as early as birth indicated that not many events are necessary for tumor development. The simplest hypothesis, in fact, was that just two events were necessary for tumor formation, that both events are mutational, and that heritable and non-heritable cases both involve somatic mutation for the second mutation. Supporting this conclusion was the observation that a semilog plot of yet-undiagnosed cases versus age fitted a one-hit expected relationship for bilateral (that is, heritable) cases and a two-hit relationship for unilateral (mostly non-heritable) cases[14]. The most attractive interpretation of the two mutations was that they involved the two copies of a single gene (a gene we now call *RB1*). Although inheritance of susceptibility is dominant, oncogenesis is recessive. My favorite candidate in 1973 was a gene that coded for a cell-surface tissue-recognition molecule that signaled the turn-off of cell replication[15]. I later applied the term 'anti-oncogene' to such recessive genes. The preferred term today is tumor suppressor gene.

The location of the *RB1* gene was revealed by cytogenetic analysis. A few cases of retinoblastoma are associated with a constitutional deletion of chromosome 13. We, and Francke and Kung, independently localized the shared deletion region to 13q14 (refs. 16,17). I proposed that although the first event could be either an intragenic mutation or a gene deletion, the second event, on the homologous chromosome, might be a new intragenic mutation, a new gene deletion, non-disjunction, or somatic recombination[18]. Other investigators provided

the means for testing these ideas. Definitive molecular proof of the recessive mechanism came with the cloning of the gene by Friend *et al*. in 1986: *RB1* was the first tumor suppressor gene to be cloned[19].

Louise Strong and I had proposed that the two other embryonal tumors, neuroblastoma and Wilms tumor, might also be caused by the same two-hit mechanism. We still do not have a gene that is responsible for heritable neuroblastoma, but one gene, *WT1*, does account for a minority of heritable and non-heritable cases of Wilms tumor and does follow the two-hit pattern. With David Anderson, a pioneer of the study of hereditary colon and breast cancers, we surveyed all hereditary forms of cancer and proposed that a two-hit model mechanism might be operating in all cases[20]. Now, with about thirty responsible genes cloned, it seems that this theory was correct for nearly all the earliest apparent tumors, such as adenomatous polyps and small carcinomas, whereas other genetic changes characterize the related truly malignant tumors.



Spectral karyotyping (Reprinted with permission from *Leukemia*, **12**, 1119 (1998).

I have concluded that embryonal tumors require fewer events because they arise in tissues whose stem cells are rapidly proliferating, whereas the common carcinomas generally arise in renewal tissues whose stem cells must undergo mutations that increase their mitotic rate[21]. For one condition, hereditary non-polyposis colon cancer, the five genes that can be mutated in the germline are not tumor suppressor genes but, rather, are DNA mismatch repair genes. However, the tumors do show mutation or loss of the wild-type homologue, and these cells in turn show a great increase in specific locus mutations, thus expediting passage through remaining necessary steps to cancer. For a few hereditary cancers, the responsible gene is an oncogene.

Meanwhile, the cloning of *RB1* has led to functional studies of its role in regulating the cell cycle; mutations in it increases the rate of cell birth. Another tumor suppressor gene, *TP3*, responsible for Li-Fraumeni syndrome (a predisposition to multiple tumors; chiefly breast cancer and sarcomas) has been shown to regulate the process of apoptosis; mutation in it decrease cell death. It is not surprising, then, that these two genes (and others that directly affect their function) are the most commonly mutated in human cancers. It is also no coincidence that three 'smart' DNA tumor viruses, SV40, adenovirus and human papilloma virus, produce proteins that interact with and interfere with the function of RB1 and TP53.

Conclusions

The remarkable discoveries that have translated the initial findings of recurring cytogenetic abnormalities into a molecular understanding of the genes involved and how they are altered has had a major impact on biology and medicine. Many of the translocations have prognostic importance—the presence of these translocations is important for clinicians as they plan the treatment for patients and their presence can alert physicians to an impending relapse in a patient. Many of the genes identified at translocation breakpoints and at sites of deletions are involved in cell growth and differentiation. Moreover, analysis of some of the genes involved in translocations, such as *BCL2*, which regulates apoptosis, opened up a new area of mammalian cell biology. Some are highly conserved in organisms from yeast to *Drosophila* to mice, emphasizing the unity of biology and providing model organisms in which to conduct experiments.

Early in our careers, the two dominant theories of carcinogenesis concerned viruses and somatic mutations. Since then, virologists found that transforming genes, or oncogenes, of RNA tumor viruses are related to host proto-oncogenes, that can become mutated in the absence of any virus, for example by the process of translocation that underlies the formation of the Philadelphia chromosome, the first cancer-specific somatic genetic change to be discovered. On the other hand, the transforming genes of DNA viruses do not have host counterparts; they produce proteins that interact with at least two important suppressor genes associated with certain hereditary cancers, with the non-hereditary forms of those same cancers, and indeed with many human cancers. In a sense, both the viral and the somatic mutational hypotheses were correct and led to the synthesis of a new view of cancer.

The challenge for the future is to match our molecular genetic understanding with a functional understanding of the genes involved in translocations, the other oncogenes and tumor suppressor genes in normal cells, of the genes that regulate them, and of their downstream targets. This will provide a far more complete and robust understanding of the role that these genes play in growth and differentiation in normal and malignant cells.

What has been remarkable over the past 15 years is the collaborative role of cytogeneticists, of molecular geneticists and cell biologists and of physicians, including clinicians, pathologists and epidemiologists, in providing new insights to enhance our knowledge of malignant transformation. This partnership is likely to expand in the future with benefits to the basic biology community, as well as to physicians treating patients. One of the essential goals of physicians is to translate the genetic understanding that we are achieving into more accurate diagnosis; this is fairly well advanced. The fusion genes and the mutated tumor suppressor genes are unique tumor specific markers. With further understanding of the alterations in the functions of these genes and proteins, it should be possible to target cells with these altered genes/proteins specifically and

to spare the other normal cells in the patient. A model would be patients with APL and the 15;17 translocation who respond to treatment with all-transretinoic acid (ATRA), which has now become specific genotypic therapy for APL patients. It is hoped that similar strategies will be applicable to other tumor specific alterations in the future. Thus, physicians will be able to provide more effective and less toxic therapy that will be tolerated much better by patients.

In summary, research has resulted in a much more sophisticated model of the multiple complex genetic changes leading to cancer. Viewed realistically, these studies reveal that there is no single simple answer to the cause, cure or prevention of cancer. Although progress in the foreseeable future is likely to occur in incremental stages rather than in major breakthroughs, we hope that the rapid pace of current discoveries will be matched by an acceleration in the development of ever more successful therapy.

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