MILESTONES

MILESTONE 1

Observations from a ploughman

"What is it that decides what organs shall suffer a case of disseminated cancer?" This question intrigued Stephen Paget, assistant surgeon to the West London hospital and the Metropolitan hospital, whose self-effacing paper of 1889 records his careful analyses of case histories that led to the visionary 'soil and seed' hypothesis of metastasis.

"When a plant goes to seed, its seeds are carried in all directions," he wrote. "But they can only live and grow if they fall on congenial soil." This idea was at odds with one prevalent theory of the time, which stated that cancer cells, having been spread through the body in the blood or lymph, could lodge in a tissue and persuade the surrounding cells to grow similarly. However, Paget followed the school of thought that all cancer cells could continually develop wherever they settled, but grew only in certain organs that were somehow predisposed to a secondary cancer.

Paget reasoned that if the organs where secondary tumours arose were 'passive' in the process, then these cancers would be distributed randomly. By analysing 735 case histories of fatal breast cancer, he found that metastases formed in the liver far more often than in any other organ — even those such as the spleen that could be considered to have the same exposure to the cancer cells because of similar blood flows.

This was enough to persuade Paget that sites of secondary growths are not a matter of chance, and that some organs provide a more fertile environment than others for the growth of certain metastases. "The best work in the pathology of cancer is now done by those who … are studying the nature of the seed," he noted. "They are like scientific botanists; and he who turns over the records of cases of cancer is only a ploughman, but his observation of the properties of the soil may also be useful."

This proved to be the case and, although it languished in the shadows for many years, the seed and soil hypothesis was revived fully in 1980 by Ian Hart and Isaiah Fidler. By this time, clinical observations had established that certain organs were, indeed, more susceptible to metastasis, even after specific properties of the tumour cells and other host factors had been accounted for.

So, Hart and Fidler examined whether the locations of metastases exist merely because tumour cells tend to come to rest in particular organs — for instance, because the blood capillaries are more narrow — or because the distributed cells can only grow at particular sites, in accordance with the Paget hypothesis. Using mice, they grafted kidney, ovary and lung tissue under the skin or into the muscle, and showed that the transplanted tissues established their own blood supply. They then injected the mice with melanoma cells. Metastases developed in the grafted lung and ovary tissue but not in the renal tissue, thereby showing a distinct preference.

Notably, radioactive labelling of the injected cells showed that they were equally likely to be trapped in the kidney tissue as in either of the other transplants. So, just landing in a tissue is not sufficient for cancer cells to develop a secondary tumour; rather, some property of the tissue itself must sustain the new growth. The idea that cancer cells require some 'nourishment' from their environment to develop still motivates research today, with the focus now being on unravelling the molecular mechanisms that bring seed and soil together to promote metastases.

Helen Dell, Nature, Locum Associate
News and Views Editor

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MILESTONE 2

Lack of principles

The genetic basis of cancer is a cornerstone of modern cancer research that began to unravel over a century ago.

In 1890, David von Hansemann described in detail the mitotic figures of 13 different carcinoma samples. In every case, he found examples of aberrant mitotic figures. These included multipolar mitoses and anaphase figures that showed asymmetric distribution of 'chromatin loops' (or chromosomes). He postulated that these aberrant cell divisions were responsible for the decreased or increased chromatin content found in cancer cells.

At the beginning of the twentieth century, the zoologist Theodor Boveri pursued this — largely ignored — association between aberrant mitoses and malignant tumours. One of his important innovations was to devise experimental manipulations of sea urchin eggs that allowed him to induce multipolar mitoses and, therefore, aberrant chromosome segregation. Boveri, for example, found ways to generate cells with multiple copies of the centrosome — an organelle that organizes the poles of the mitotic spindle, which he had also discovered and named. By following the fate of cells with different chromosomes, he surmised that individual chromosomes were qualitatively dissimilar and transmitted different inheritance factors. He then suggested that aberrant mitoses led to the unequal distribution of chromosomes, which, in most cases, would be detrimental. Yet, on occasion, a "particular, incorrect combination of chromosomes" would generate a malignant cell endowed with the ability of "schrankenloser Vermehrung" (unlimited growth), which would pass the defect on to its progeny. The foundations for viewing cancer as a genetic disease were laid.

Boveri applied his concept to explain disparate phenomena linked to cancer, and made a number of bold and bafflingly accurate predictions. Today, we can see that he foretold the existence of cell-cycle checkpoints ("hemmungseinrichtungen"), tumour-suppressor genes ("teilungshemmende chromosomen") and oncogenes ("teilungsfoerdernde chromosomen"). He further envisaged that 'poisons' (including nicotine), radiation, physical insults, pathogens, chronic inflammation and tissue repair might all be linked to the development of cancer by indirectly promoting aberrant mitoses or other events that cause chromosome imbalances.

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With astonishing prescience, Boveri applied his model further to explain the emergence of different tumour types within one tissue, and anticipated the clonal origin of tumours, the allelic loss of recessive chromosome elements, the heritability of cancer susceptibilities, the similarity of the steps that initiate tumorigenesis and those responsible for cancer progression, and the sensitivity of cancer cells to radiotherapy. All of these ideas have since found wide acceptance and molecular explanations.

Subsequent work by several investigators showed that known carcinogens, such as ionizing radiation, acted as mutagens, which further underscored the genetic basis of cancer. A consistent chromosomal abnormality that was found in 1960 in chronic myeloid leukaemia — the Philadelphia chromosome (see Milestone 10) — lent further support to this idea.

MILESTONE 3

Hide and seek

The immune system has an amazing ability to seek out and destroy that which is deemed foreign, and generally leaves 'self' alone. Yet, tumour cells, thanks to accumulated mutations and altered patterns of gene expression, differ from their normal counterparts. Could the same killing power that eradicates infection be harnessed to destroy cancer cells — cells that are nevertheless self?

Paul Ehrlich thought so. In 1909, he suggested that, thanks to the immune system, tumour development was usually suppressed.

Yet, attempts to target tumours by immunotherapy have been less successful than the Ehrlich hypothesis might predict. Richmond Prehn and Joan Main, in 1957, showed that tumours induced by chemical carcinogens in mice could stimulate tumour-specific responses that were able to reject those same tumours on challenge. They concluded that tumour immunity was induced by antigens unique to the chemically-induced tumour, but found that spontaneously arising tumours were not rejected when tested in the same experimental manner.

From these and subsequent studies arose the belief, summarized by Harold Hewitt and colleagues, that naturally arising tumours were not immunogenic. Moreover, Osias Stutman had reported in 1974 that athymic mice do not have an increased frequency of tumours induced by a chemical carcinogen, implying that the concept of immune surveillance providing protective immunity was incorrect. Yet, in 1982, enthusiasm for tumour immunology was rekindled by the landmark discovery by Aline van Pel and Thierry Boon that specific immunity to spontaneous tumours could be induced by vaccinating mice with mutagenized tumour cells. Their study showed that spontaneous tumours were not inherently deficient in tumour antigens, but instead failed to stimulate an effective immune response. This failure could be overcome by vaccination, a strategy that has since been adopted in numerous clinical trials.

In a technical feat by Pierre van der Bruggen and colleagues, the Boon group later reported the first identification of a tumour-specific antigen recognized by cytolytic T cells in humans, reinforcing the idea that tumour antigens can elicit a detectable tumour-specific response. Whether that response can induce, or be manipulated to induce, rejection of the tumour remains unclear. Yet Robert Schreiber and co-workers, in 2001, prompted renewed interest in immunosurveillance, showing that immunodeficient mice are more susceptible to chemically-induced, as well as spontaneous, tumours. This proves to be a 'catch 22', however, for the immunocompetent mouse: in recognizing cancer, the immune system exerts a selection pressure on a tumour cell or immunoediting, resulting in its decreased immunogenicity and eventual escape from immune-mediated eradication. More recently, Gerald Willimsky and Thomas Blankenstein suggested that sporadic tumours in mice do not lose immunogenicity, but rather induce tolerance to evade immune detection. How

Closely linked to the Boveri hypothesis is the idea of genomic instability driving the accumulation of chromosome aberrations and mutations in cancer cells. Work by Robert Schimke and colleagues showing that cancer cells tend to amplify genes involved in drug resistance, and that these changes can be unstable, was among the first evidence of genomic instability in cancer. Today, the concept has been extended by insights into the mechanisms underlying chromosome imbalances, increased mutation rates and other forms of genetic instabilities, many of which are relevant to the development of human cancer (see Milestone 22).

With the advantage of present-day knowledge, it is tempting to reinterpret the von Hansemann depiction of the "Prinziplosigkeit als Prinzip der Krebszellen" (lack of principle as the principle of cancer cells) as the common occurrence of chromosome abnormalities and genetic instability in cancer.

Barbara Marte, Senior Editor, Nature

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either model relates to tumour growth in humans remains to be determined.

While the suggestion by Ehrlich that the immune system restricts the growth of most tumours might have been optimistic, the findings that immune cells do recognize tumours have nonetheless catalysed an upswing of enthusiasm in the field of tumour immunology, and offer encouragement for immunotherapy approaches as a potential adjunct to present cancer therapy.

Alison Farrell, Senior Editor, Nature Medicine

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MILESTONE 4

From hens to eternity

The hypothesis that viruses can cause cancer has fallen in and out of fashion since the early 1900s. Former US President Richard Nixon declared war on cancer in 1971, at which time many held the view that cancer was caused by infective agents; however, it is now known that only a few cancer types can be directly attributed to viruses. Despite this, work on RNA tumour viruses (retroviruses) led to many important discoveries in cancer research — not least the discovery of some of the first cellular oncogenes.

Peyton Rous is surely the grandfather of the field. His ground breaking work in this area began in 1910, when he discovered an avian tumour that could be transplanted to other individuals — the first of its kind. The tumour was a spindle-cell sarcoma that originated in a Plymouth Rock hen. Rous inoculated bits of this tumour into the breast and peritoneal cavity of other hens, and found that they could be successfully transferred and propagated through subsequent transplants.

A year later, Rous published another paper, which took this work a giant step further. He made cell-free filtrates from the tumour using various protocols, and found that they were sufficient to induce tumour growth. So, a biological agent in the cell-free filtrate could cause tumour development; this agent was subsequently shown to be a virus, and was named after its discoverer as Rous sarcoma virus (RSV). The importance of this finding was not fully appreciated for some time, and it was only in 1966, at the age of 77, that Rous was awarded the Nobel Prize for this research.

In 1969, Robert Huebner and George Todaro reported a series of experiments that led to their proposal that "there exists a unique class of viruses present in most, and perhaps all, vertebrates that plays an important etiologic role in the development of tumours in these animals". Their hypothesis was that C-type retroviruses — of which RSV was the most famous example — could be vertically transmitted from animal to progeny animal, and from cell to progeny cell, and that their activation by host genetic factors or environmental factors results in oncogene expression and cell transformation.

They found virus particles in almost all of the vertebrate species that were examined, and showed that tumour incidence corresponded with viral expression in several murine strains. They consequently proposed that normal cells had the capacity to activate latent tumour viruses, and that the spontaneous or induced de-repression of a viral oncogene would lead to cancer.

Although their hypothesis that most cancers were caused by expression of retroviral genes was not strictly correct, their work did lead to the identification of the first retroviral oncogene *src*, the realization that these viral genes were derived from functional cellular genes or proto-oncogenes and, finally, the identification of cellular proto-oncogenes as precursors of transforming cancer genes (see Milestones 15,16 and 17). So, this collection of papers was truly ground breaking, and paved the way for other important discoveries in cancer research.

Emma Greenwood, Executive Editor, Oncogene

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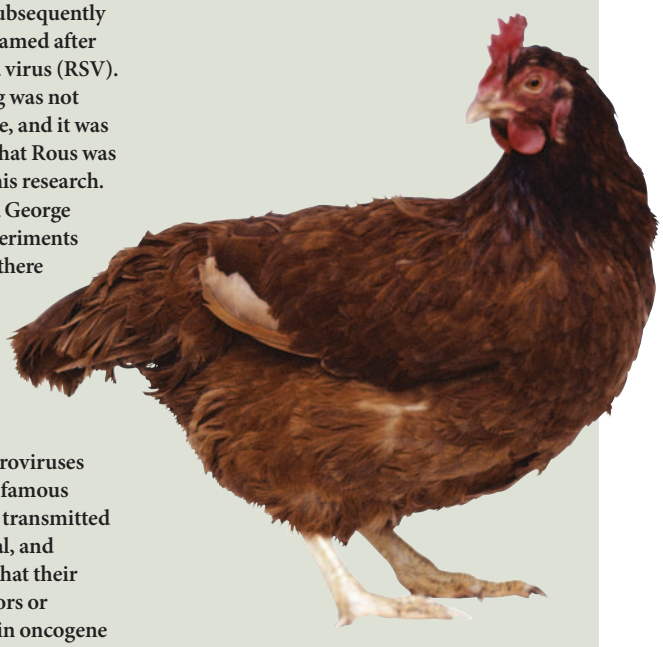
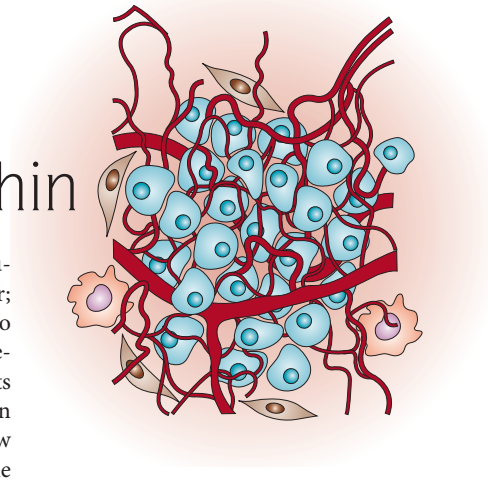


MILESTONE 5

The enemy within



It is now well accepted that hormones influence the initiation and progression of cancer; however, it took almost a century of research to move from early observations that hormone-ablative surgery benefits some cancer patients to the development of the first drug against an endocrine target. Although hormones are now implicated in several cancers, the study of the relationship between oestrogen and breast cancer has yielded the most important milestones.

Back in 1915, the first suggestion that a hormone was involved in tumorigenesis came from Abbie Lathrop and Leo Loeb, who reported the influence of internal secretions from the corpus luteum (ovarian follicles) on the development of spontaneous tumours in mice. Their small but landmark study showed that tumour incidence was delayed and reduced from 60–90% to 9% in female mice castrated before 6 months of age. As it was already known that the corpus luteum secreted an uncharacterized substance that induced growth of the breast during pregnancy, the authors speculated that this chemical might be involved in tumour formation. Eight years later, Edgar Allen and Edward Doisy identified this substance as oestrogen.

Over the next 25 years, the research of Abraham Lilienfeld, Brian McMahon, Philip Cole and others into the epidemiological relationship between female reproduction and breast cancer lent weight to the hypothesis that oestrogen was a carcinogen. However, it was the discovery of the oestrogen receptor (ER) by Elwood Jensen in 1958, and his pioneering study in 1971 on the effect of adrenalectomy on human breast cancer, that truly revolutionized this field.

Jensen studied breast cancer patients to correlate the level of ER expression on tumours with the response to hormone-ablative surgery. He found that breast tumours fell into two categories — ER-rich and ER-poor — and patients who had tumours with a high level of ER expression were more responsive to hormone-ablative therapy. This led Jensen to propose that the ER status of a tumour could predict the response to therapy.

Although this evidence for the role of the ER in breast tumours raised the possibility of developing anti-oestrogenic cancer drugs, the pharmaceutical industry was instead focusing on anti-oestrogenic compounds as contraceptives. One such candidate was ICI,46,474, which was a non-steroidal anti-oestrogen described by Michael Harper and Arthur Walpole in 1967. They published the first detailed study of the anti-oestrogenic effects of ICI,46,474 on the reproductive cycle of rats, and found it to be a safer version of known anti-oestrogens. When the development of ICI,46,474 as a contraceptive ultimately stalled, Walpole convinced Imperial Chemical Industries to market it for the treatment of breast cancer. Yet clinicians were slow to adopt the drug, and it was not until V. Craig Jordan showed that it could prevent mammary tumours in mice that they were finally persuaded to undertake the clinical studies that ultimately led to the 1973 approval of ICI,46,474 as tamoxifen.

Still, the value of tamoxifen in preventing human breast cancer was not realized until it was studied as an adjuvant to breast cancer surgery. Following a trial showing that treatment with tamoxifen after surgery reduced the incidence of contralateral breast cancer, a large-scale Breast Cancer Prevention Trial was started in 1992 by Bernard Fisher and colleagues to study the drug as a chemopreventative agent. The results were surprisingly positive — tamoxifen caused a 50% reduction in the incidence of breast cancer — which supported its use as a prophylactic drug in high-risk breast cancer patients.

Since then, tamoxifen has paved the way for research into the design of selective hormone-modulating drugs for a range of different tumour types. The successful development of these drugs might well be the first of many more milestones in this field.

Joanna Owens, Senior Editor,
Nature Reviews Drug Discovery

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MILESTONE 6

The needle in the haystack

Are all cancer cells equal or are some uniquely responsible for initiating and sustaining the growth of a tumour? Although the idea of a cancer stem cell (CSC) or tumour-initiating cell was well entrenched by the 1960s, it was not until the mid-1990s that these cells were identified and characterized.

Most of our understanding of CSCs has come from studying haematopoietic malignancies. Jacob Furth and Morton Kahn were the first to allude to CSC principles in 1937. Using cell lines, they provided the first quantitative assay for the assessment of the frequency of the malignant cell maintaining the haematopoietic tumour, at a time when the origin of leukaemia as being viral or cellular was in dispute. They showed that a single leukaemic cell was able to transmit the systemic disease when transplanted into a mouse. This was followed by the development of quantitative methods in

MILESTONE 7

Bloodlines

In 1939, Gordon Ide and colleagues adapted a technique to study the growth of blood vessels around tumour tissue transplanted into the rabbit ear. Observing robust tumour growth and induction of a complex vascular network, they made the seminal suggestion that tumours might produce a 'vessel growth-stimulating substance'. In 1945, Glenn Algire and colleagues furthered these studies by a detailed kinetic analysis of the vascular response to tumour transplants. They postulated that the growth advantage of a tumour cell over its normal counterpart might not be owing to "some hypothetical capacity for autonomous growth inherent within the [tumour] cell," but rather to its ability to continuously induce angiogenesis — that is, the formation of new blood vessels. This insightful conclusion presaged the realization that a tumour would not efficaciously grow in the absence of a blood supply and, therefore, that inhibiting development of the tumour vasculature could be exploited as a therapeutic strategy.

In 1968, Melvin Greenblatt and Philippe Shubik showed that tumour transplants stimulated the proliferation of blood vessels even when a physical barrier — a Millipore filter — was placed between the tumour

the 1960s and 1970s to measure the clonogenic potential of the cell type able to sustain tumour growth *in vivo*. Robert Bruce and Hugo Van der Gaag used the spleen colony-forming assay (CFU-S) — a tool first developed by James Till and Ernest McCulloch, and now widely used in stem-cell biology — to show that only a small subset of primary cancer tissue was able to proliferate *in vivo*. Collectively, these studies underscored the functional heterogeneity in tumours — not every cell is able to proliferate to form a colony *in vitro* or to give rise to a tumour when transplanted *in vivo* — and introduced the concept of CSCs.

However, it was not until the identification and prospective purification of CSCs by John Dick and colleagues in 1994 that concrete proof was provided for a hierarchical (or stem cell) model of cancer. Using limiting dilution analysis together with disease-initiation models, these investigators showed that when isolated from acute myeloid leukaemia (AML) patients, only a small fraction of the tumour cells with a characteristic marker signature were able to establish leukaemia in recipient mice. This provided a reproducible way of enriching cells with tumour-initiating activity and ruled out the stochastic model, which predicted that such an activity would be present in every cell fraction. AML-initiating cells were not only able to differentiate and proliferate, but also had the capacity to self-renew *in vivo* — a key attribute of stem cells.

Recently, studies in solid tumours have revealed that the CSC concept extends beyond haematopoietic malignancies. Michael Clarke and colleagues, and Peter Dirks and co-workers showed that human breast and brain tumours are not homogeneous, but rather contain a small subset of cells that can be prospectively isolated and are able to initiate phenotypically heterogeneous cancers *in vivo*.

The identification of solid tumour stem cells provided researchers with a firm basis on which to re-evaluate cancer therapies to target and eliminate not only the bulk population of tumour cells, but also the rare but potent self-renewing cells that initiate and sustain cancers. Efforts are now underway to unravel the mechanisms that regulate CSC function, and to determine whether such cells arise through mutations accrued in normal tissue stem cells or whether stem-cell properties are acquired in more differentiated progenitor cells.

Myrto Raftopoulou, Associate Editor,
Nature Cell Biology

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and the host stroma. They concluded that the vessel growth-stimulating substance of Ide and co-workers was a true diffusible substance that could, in theory, be identified. In 1971, Judah Folkman and colleagues isolated just such a 'tumour angiogenic factor (TAF)' from tumour extracts, and proposed that the growth of malignancies might be prevented if TAF activity were blocked.

Folkman expanded on this concept in his visionary synthesis of the contemporary tumour-angiogenesis literature, and proposed that tumour cells secrete a soluble factor that stimulates the proliferation of endothelial cells, that these in turn control tumour expansion and, in the absence of new vessel growth, that tumours do not increase beyond 2–3 mm in size, entering instead a state of 'dormancy'. He further speculated that anti-angiogenesis — that is, inhibiting the recruitment of new blood vessels into a tumour and thereby inducing dormancy, such as by an antibody directed against TAF — might be a powerful approach to tumour therapy.

The proposal by Folkman of targeting the vasculature rather than the tumour cell itself was a complete departure from conventional therapeutic strategies, and was not initially well received by the oncology community. Moreover, angiogenesis was no hotbed of research at the time — the number of angiogenesis papers published in 1971 could be counted on just one hand. Yet, the Folkman article rekindled interest in angiogenesis and inspired new investigators to join the field.

Nonetheless, it was another 18 years before Napoleone Ferrara and colleagues purified and subsequently identified the gene encoding vascular endothelial growth factor (VEGF), which is a secreted protein that can stimulate both vascular endothelial cell proliferation *in vitro* and angiogenesis *in vivo*. An isoform of VEGF proved to be identical to vascular permeability factor, which was cloned simultaneously by Pamela Keck and co-workers, and was originally identified by Harold Dvorak and colleagues in 1983. Soon thereafter, two groups independently showed that the cells nearest to areas of low oxygen (hypoxia) in a tumour, and therefore in most need of blood vessels, had the highest expression of VEGF, and that hypoxia could directly induce expression of VEGF in cells in culture. Gregg Semenza and colleagues would later identify hypoxia-inducing factor 1 (HIF1) as the transcription factor responsible for VEGF expression under hypoxia.

In the meantime, Ferrara and colleagues provided definitive evidence that VEGF stimulates tumour angiogenesis and growth in mice by inhibiting its function using a blocking antibody. This finding paved the way for the development and clinical application of a humanized version of the antibody, bevacizumab (Avastin). Based on early and encouraging success in cancer patients when used in conjunction with chemotherapy, bevacizumab was approved by the United States Food and Drug Administration in 2004 for the treatment of metastatic colorectal cancer, bringing validation to the Folkman hypothesis of more than 30 years earlier that targeting the tumour vasculature is a viable strategy to treat cancer.

Alison Farrell, Senior Editor,
Nature Medicine

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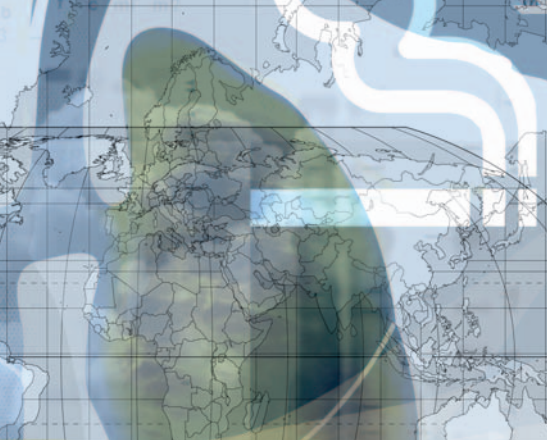

MILESTONE 8

Smoking gun

During the first half of the twentieth century, consumption of manufactured cigarettes increased greatly in the Western world. A rapid increase in lung cancer in men was also evident, and the prevailing view was that this was a result of improved diagnosis, although there was also discussion about the role of increased air pollution or cigarette smoking. More than anyone else, the research of the British epidemiologists Richard Doll and Tony Bradford Hill was responsible for the now widely accepted view that most lung cancers are caused by cigarette smoking.

In 1939, a German study indicated that non-smokers were more common in healthy populations than among lung cancer patients. There followed reports of several case–control studies associating lung cancer with cigarette smoking, including a study in early 1950 from researchers in the USA, Ernst Wynder and Evarts Graham. This study involved over 600 lung cancer cases and 600 controls. Six months later, Doll and Hill reported a larger case–control study in the *British Medical Journal*, and concluded that smoking was "a cause, and an important cause" of lung cancer.

The real milestone came when Doll and Hill designed a prospective cohort study to overcome concerns regarding bias. They sent out questionnaires to more than 34,000 male British physicians to collect details of their smoking habits, which were followed up with further questionnaires, and recorded the causes of death. The first report of this study was published in the *British Medical Journal* in 1954, with a follow-up report in 1956. Their earlier case–control findings were confirmed. They showed a higher mortality in smokers than in non-smokers, and a clear dose–response relationship between the amount smoked and the death rate from lung cancer. The data also indicated a progressive and significant reduction in mortality with the increase in the length of time over which smoking had been given up. There was remarkably little difference between the smoking habits of doctors who lived in large towns and those who lived in other districts, so the authors concluded that lung cancer could not be attributed to a differential exposure to atmospheric pollution.

"This was, I believe, the first unequivocally-established cause of cancer that affected large proportions of the general population."
Laurence N. Kolonel

These reports, along with other cohort studies published in the 1950s, formed the basis for the 1964 report of the United States Surgeon General, which concluded that "Cigarette smoking is causally related to lung cancer in men; the magnitude of the effect of cigarette smoking far outweighs all other factors."

The Doll and Hill study is unique in its regular updating of the smoking habits of the participants. The latest (and final) 50-year follow-up report was published in 2004 by Doll and colleagues, including Richard Peto, a 30-year collaborator on the study. The results showed that among men born between 1910 and 1930, prolonged cigarette smoking caused death to occur on average 10 years earlier than that of lifelong non-smokers, but cessation at age 50 halved the hazard and cessation at age 30 almost eliminated it.

Despite these indisputable data, and consequent findings identifying the carcinogens in tobacco and establishing the mechanisms of carcinogenesis, about 1 billion men worldwide are daily smokers and smoking still causes about 1.2 million deaths worldwide annually.

Ezzie Hutchinson, Chief Editor,
Nature Reviews Cancer

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MILESTONE 9

It takes (at least) two to tango

During the first half of the twentieth century, the view that genetic mutations could cause cancer was gaining firm ground (see Milestone 2). Yet, ideas about viral causes were also widespread, and the genetic model had yet to explain the age distribution of cancer incidence.

A multitude of studies in the 1950s and 1960s applied mathematical models to cancer-mortality rates as a function of age, to explore whether a series of mutations, accumulating over time, could explain the epidemiological data. If cancer was caused by successive mutations (or hits) its incidence should be associated with a certain power of age. This approach led, for instance, Carl Nordling to conclude that, indeed, around seven mutations fitted the age distribution for a range of human cancers. Peter Armitage and Richard Doll

MILESTONE 10

Cutting and pasting chromosomes

A small chromosome identified in the cancer cells of patients with chronic myelogenous leukaemia (CML) was the first genetic defect to be associated with cancer. This chromosome was subsequently found to be the product of a translocation, a breakthrough that led to the identification of chromosome translocations in other cancers and the discovery of many oncogenes.

In a paper presented at a National Academy of Sciences meeting in 1960, University of Pennsylvania researchers Peter Nowell and David Hungerford first reported the presence of a "minute chromosome" that replaced one of the four smallest autosomes in CML cells. They did not observe this defect in other types of leukaemia or lymphoma cells, and many other cells from these patients contained a normal karyotype. This unique chromosome was designated 'The Philadelphia chromosome' after the city in which the discovery was made.

For many years, scientists thought that the Philadelphia chromosome resulted simply from the loss of genetic material.

reached similar conclusions in a paper from 1954, but, in a further study in 1957, revised their model to conclude that the epidemiological data were consistent with many common forms of cancer developing in two steps, of which one or both could be somatic mutations.

Other work in the 1950s and 1960s, including that of James Neel and Philip Burch, concentrated on childhood cancers whose development in early life reduced some of the complexity of other forms of cancer; this led researchers to deduce that multiple, perhaps as few as two, inherited and/or somatic mutations had a role in retinoblastoma, neurofibromatosis and childhood leukaemias (see also Further Reading).

In a seminal paper in 1971, Alfred Knudson took the idea of multiple hits an important step further. He noted that "what is lacking is direct evidence that cancer can ever arise in as few as two steps and that each step can occur at a rate that is compatible with accepted values for mutation rates". Knudson analysed 48 cases of retinoblastoma for the occurrence of bilateral or unilateral tumours, and the presence of a family history of the disease. Using Poisson statistics, he showed that the distribution observed was consistent with retinoblastoma being caused by two mutations. In familial cases, one hit was inherited whereas the other one was acquired later; in sporadic tumours, both changes were somatic, with a similar mutation rate for both hits. The Knudson model explained why multiple tumours occurred in both eyes in inherited cases, but only unilaterally in sporadic occurrences.

Knudson and colleagues subsequently extended the two-hit model to secondary tumours in retinoblastoma patients and to other childhood cancers. The now famous two-hit hypothesis was, in later years, to merge with the concept of allelic loss of tumour-suppressor genes when it became clear that the development of retinoblastoma was associated with mutations in both alleles of the retinoblastoma gene *RB* (also known as *RB1*), and that one *RB* mutation was inherited in familial cases of the disease (see Milestone 11).

The current view of cancer has built on these findings: we now know that all human cancers display a multitude of genetic and epigenetic changes, and that a number of such alterations are required for the step-wise progression of tumour development (see Milestone 14).

Barbara Marte, Senior Editor, Nature

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In the early 1970s, the development of techniques such as quinacrine fluorescence and Giemsa banding allowed researchers to identify and track segments of chromosomes. Accelerating the burgeoning field of cytogenetics, Janet Rowley used these technologies to identify a genetic abnormality in CML cells — the addition of extra material to chromosome 9. She then noticed that the amount of additional material was approximately equal to the amount missing from chromosome 22, and proposed that there was a "hitherto undetected translocation between the long arm of 22 and the long arm of 9" that resulted in formation of the Philadelphia chromosome. In the same year, Rowley reported another translocation between chromosomes 8 and 21 in acute myeloblastic leukaemia cells. She went on to discover more than a dozen translocations that were specific to other types of cancer cells, notably t(15;17) in acute promyelocytic leukaemia and t(14;18) in lymphoma, which was also described by Carlo Croce and colleagues. Cytogenetic analysis is still one of the most reliable methods of diagnosis and of determining prognosis in patients with leukaemia or lymphoma.

So, how do these chromosome rearrangements cause cancer? In 1982, Annelies de Klein and colleagues reported that the human cell homologue (c-*ABL*; also known as *ABL1*) of the transforming sequence of Abelson murine leukaemia virus was translocated from chromosome 9 to chromosome 22q in CML cells. This finding indicated a role for c-*ABL* in the generation of human leukaemia. At the same time, Rebecca Taub and co-workers, and Riccardo Dalla-Favera and colleagues reported the translocation of c-*MYC* into the immunoglobulin heavy chain locus, through a translocation between chromosomes 8 and 14. This translocation had been identified by Laura Zech and co-workers, and is frequently observed in Burkitt lymphoma cells. Evidence that the translocation and subsequent deregulation of MYC expression is an oncogenic event was later provided by the *Eμ-Myc* mouse model generated in 1985 by Jerry Adams and colleagues.

Cloning of the breakpoints of other cancer-associated translocations would subsequently lead to the discovery of many other oncogenes, such as B-cell lymphoma 2 (*BCL2*) and tumour suppressor genes.

Kristine Novak

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MILESTONE 11

The road less travelled

Few would argue that the path to scientific discovery is short and simple. The realization that cancer could arise through the inactivation of recessive genes — tumour suppressors — is a case in point.

Throughout the 1970s and 1980s, oncogenes dominated the field of cancer research, and so the prevailing thought was that tumours were caused by activating mutations. The famous two-hit hypothesis was also finding increasing support (see Milestone 9), but lacked insights into the nature of the hits.

Perhaps the strongest impetus to pursue the unorthodox idea of tumour suppressor genes was provided by Henry Harris and colleagues, who observed that normal mouse cells were dominant to malignant cells when the two types were fused in the laboratory. This conceptually simple yet technically demanding work pierced the first hole in the theory that (dominant) oncogenes were the general rule.

While many scientists had previously presented support for a model of allelic loss (see Further Reading), it was David Comings who, in 1973, articulated a general framework for a role of tumour suppressor genes in all types of cancer: inherited tumours, he argued in a theoretical paper, were the result of a germline mutation in regulatory genes that suppressed tumorigenesis, followed by the somatic loss of the homologous allele. In non-heritable cancers, both alleles would be affected in somatic cells. However, the field had to wait 10 years to pin this hypothesis to a molecular locus.

Then, Webster Cavanee and colleagues localized the retinoblastoma gene (*RB*; also known as *RB1*) to a small region on chromosome 13; they showed that inherited and sporadic cancers had the same second hits, and that these cause homozygosity for mutations at the *RB* region, thereby confirming the allelic-hit hypothesis. By the end of the decade, the first two tumour suppressor genes — *RB* and *p53* (also known as *TP53*) — would be identified.

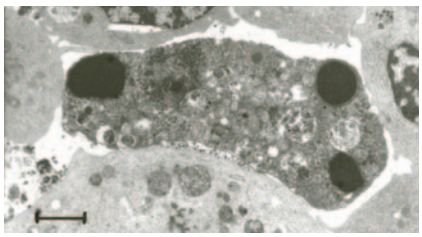
In 1986, Stephen Friend and colleagues isolated a human cDNA that mapped to the *RB* region and, importantly, was deleted at least partly in tumours. The next year, two groups — Wen-Hwa Lee and co-workers, followed by Yuen-Kai Fung and colleagues — cloned *RB* by chromosome walking their way to a cDNA fragment that hybridized to transcripts in normal tissue, but was aberrantly expressed or deleted in retinoblastomas. This pointed to the inactivation of *RB* as being causative for cancer, a conclusion that was confirmed by Huei-Jen Su Huang and colleagues, who rescued the neoplastic phenotype of *RB*-mutant retinoblastoma cells with wild-type *RB*.

The involvement of p53 in cancer was known for 10 years before its true role was identified. In 1989, Bert Vogelstein's group identified *p53* as the gene uncovered by the cancer-associated deletions on chromosome 17p, and showed that one copy was mutated and the other deleted in colorectal cancers. Similar to *RB*, the tumour suppressor function of p53 was confirmed by showing that it rescued the growth phenotype of p53-mutant carcinoma cells. If *p53* caused tumours only when both alleles were mutant, then it could not be the proto-oncogene it was widely regarded to be. Arnold Levine's group helped to dispel this misconception further, by showing that the *p53* mutations that arose in transformed cells *in vitro* were of the same type as that which occurs

MILESTONE 12

Death defying

By the late 1960s, it was recognized that the spontaneous loss of tumour cells was an important component in the growth of tumours and, although cell death was the most likely cause, little was known about the mechanisms involved. John Kerr had already shown that cells died with a morphology that was distinct from necrotic cells, but it was not until the description of apoptosis in a 1972 review by John Kerr, Andrew Wyllie and Alastair Currie that specific roles for cell death in cancer development were proposed.



An electron micrograph showing an apoptotic haematopoietic cell (Bar = 2 μm) © Nicola McCarthy.

Kerr, Wyllie and Currie suggested that, unlike necrosis, apoptosis might represent a genetically regulated cell-suicide programme, and, importantly, they stated: "We should now like to speculate that hyperplasia might sometimes result from decreased apoptosis rather than increased mitosis, although we emphasize that we know of no definitive studies to support such a hypothesis."

Importantly, in 1988, David Vaux, Suzanne Cory and Jerry Adams showed that expression of the B-cell lymphoma 2 (*BCL2*) gene, which had been identified by others as being translocated in follicular lymphoma (see Milestone 10), could promote the survival of haematopoietic cells after the removal of growth factors (Gwyn Williams and co-workers were later to show that these growth factors suppressed apoptosis). Vaux and colleagues also showed that the oncogene *Myc* cooperated with *Bcl2* to produce tumours in immunocompromised mice. They suggested that BCL2 provided a distinct survival signal that might contribute to neoplasia by allowing a clone to persist until other oncogenes, such as *Myc*, became activated. This and subsequent work provided evidence that cell survival was regulated independently of cell proliferation, and that impaired cell death, similar to enhanced proliferation, was indeed a key step in tumour development. In the same year, John Reed and colleagues found that overexpression of BCL2 in an immortalized mouse cell line did not induce proliferation or transformation *in vitro*. Although these cells did produce tumours in mice, further mutational events were required. In 1989, Tim McDonnell, Stanley Korsmeyer and colleagues reported that the expression of a BCL2–immunoglobulin fusion protein in B cells prolonged their survival — an event that this group also showed was tumorigenic.

Soon after, other oncogenes, such as the breakpoint cluster region (*BCR*)–Abelson leukaemia viral oncogene (*ABL*; also known as *ABL1*), were shown to suppress apoptosis. Conversely, several groups, including those of John Cleveland and Gerard Evan, reported that overexpression of MYC induced apoptosis. Initially, this seemed counterintuitive — why would the upregulation of an oncogene associated with increased proliferation induce cell death? It was proposed that MYC-induced apoptosis was part of a tumour suppression mechanism. Apoptosis as a mechanism to limit tumorigenesis was further supported by

in human cancers — that is, they were inactivating mutations that probably acted in a dominant–negative manner.

Tumour suppressors and oncogenes started out at opposite poles; yet, in just 15 years, the field came full circle with the realization, as Comings had predicted years earlier, that tumour suppressors oppose the action of transforming genes — a mechanistic link that has provided the basis for all subsequent models of malignancy.

Tanita Casci, Senior Editor,
Nature Reviews Genetics

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the finding that the tumour suppressor p53 induced apoptosis (see Milestone 20).

These discoveries and many others have shown that failure to induce apoptosis produces hyperplasia, whereas further mutations are required to produce overt neoplasia. Overall, the concept that the inability of a cell to die was potentially tumorigenic revolutionized the way in which tumorigenesis was viewed and greatly influenced treatment strategies.

Nicola McCarthy, Senior Editor,
Nature Reviews Cancer

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MILESTONE 13

Environmental awareness

Context is everything for cells and, in addition to the importance of finding an appropriate blood supply (see Milestone 7), tumorigenic potential will often only be realized when cells find themselves in a tissue environment that they can subvert to their advantage. This phenomenon was first clearly noted in the 1970s, although it was decades before the cellular basis was uncovered.

In 1975, Beatrice Mintz and Karl Illmensee asked what would happen if mouse tetracarcinoma cells were placed in a 'normal' environment. They took the tetracarcinoma cells from embryoid bodies *in vivo*, and injected them into developing mouse blastocysts. Surprisingly, normal mice were born with no evidence of tumours. When the authors looked more closely, they found that tumour-derived cells were present in large numbers and contributed to several unrelated tissues, the most notable being functional spermatozoa. From this, Mintz and Illmensee concluded that the tumour cells were developmentally totipotent and could revert to normal behaviour in the appropriate environment. At the time, they also speculated that the original tumorigenic state might not involve a mutation.

This study was to have a strong influence on Mina Bissell. Inspired by this mysterious behaviour of tumour cells, Bissell began to focus her own research on the influence of the microenvironment. In 1984, she published a study, together with David Dolberg, showing that the ability of Rous sarcoma virus (RSV) to induce tumours was also context dependent. The tumour-inducing behaviour of RSV when injected into the wings of newly hatched chicks was already known, and the viral gene v-*src* had been identified as the sole culprit.

What Bissell found, however, was that if RSV was injected into 4-day-old embryos, no tumours were produced, despite the spread of RSV infection throughout the embryo and active v-Src expression. Furthermore, if the infected embryonic cells were isolated and grown in culture, they now became transformed. So, something about the environment of the embryos was able to block tumorigenesis, despite the presence of v-Src. The following year, her group went on to show that wounding was one important influence on the ability of a cell to succumb to tumorigenesis. When RSV was injected into a chick wing to produce a local tumour, a second tumour would only be seen if a wound was simultaneously induced at a remote site. The Bissell group later found that the factor responsible was transforming growth factor-β (TGF-β) — an early and surprising demonstration of the dual action of this cytokine.

"These experiments form the groundwork for our current understanding that the environment in which cells are growing can influence the expression of the transformed phenotype" *Sara Courtneidge*

It is only during the past 10 years that we have begun to understand the molecular basis for how the local tissue environment, and processes such as inflammation and infection, can influence tumorigenic cells. For example, in 1997, Bissell and colleagues showed that blocking integrin function was sufficient to revert the malignant phenotype of human breast cancer cells both in culture and *in vivo*. Others, including the groups of Luis Parada and Harold Moses, were able to show in mouse models that genetic alterations in cells of the tumour microenvironment contribute to, and can even be sufficient for initiating, the development of cancer.

Alison Schuldt, Senior Editor, Nature Cell Biology

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MILESTONE 14

Step by step

The possibility that cancer could have a genetic basis was recognized in the early twentieth century (see Milestone 2). In addition, evidence for a clonal origin of tumours had emerged, as had views of cancer as a multistep process. Leslie Foulds, for example, early on described cancer as a "dynamic process advancing through stages that are qualitatively different", progressing from precancerous stages to increasingly invasive and metastatic stages.

Yet, the now prevailing concept of Darwinian evolution and the stepwise progression of tumours was perhaps most convincingly articulated by Peter Nowell in 1976. His article incorporated the idea of cancer being caused by multiple mutations or 'hits' (see Milestone 9) into a general framework of tumour development and progression, through the accumulation and selection of genetic changes.

Nowell concluded that the first step results in cell proliferation that is "unrestrained to some degree", allowing for a selective growth advantage. While also acknowledging the potential role of epigenetic alterations (see Milestone 19), he suggested that, as the result of acquired genomic instability in the expanding cell population, rare subvariants endowed with an extra selective advantage could emerge. Sequential rounds of clonal selection would produce tumour-cell populations with more aggressive phenotypes. Support for this concept came from the observation that advanced solid tumours often had a greater degree of aneuploidy than early stage lesions, and from the discovery of specific chromosomal changes that developed during the clinical progression of leukaemias.

Nowell discussed the mechanisms underlying genomic instability, such as DNA-repair defects or mitotic errors (see Milestones 2 and 22), and noted that diverse agents that cause cancer, such as ionizing radiation and viruses, can induce genetic changes and might contribute to the initial changes as well as the subsequent alterations.

Nowell wrote "it would be helpful if we could associate specific chromosomal alterations with particular aspects of tumour suppression". However, at the time, few consistent changes had been noted, with the exception of the famous Philadelphia chromosome (see Milestone 10). Although Nowell anticipated similarities between different tumours, he also

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recognized that these would be difficult to identify amongst the multitude of evolutionary steps, and that variations due to different selection pressures were likely.

Subsequent years saw the identification of a number oncogenes and tumour-suppressor genes that were altered in human cancer. In an influential paper in 1990, Eric Fearon and Bert Vogelstein amalgamated these findings together with the idea of clonal evolution into a coherent molecular model of multistep tumorigenesis.

Focusing on colorectal cancer, the authors noted the clonal nature of the disease, and the consistent occurrence of mutations in the *KRAS* oncogene and the allelic loss of known or candidate tumour-suppressor genes, including *p53* (also known as *TP53*). Although certain changes were preferentially associated with specific stages of disease progression, the authors documented a multitude of chromosomal and other changes, such as frequent DNA hypomethylation of specific

MILESTONE 15

Bad seeds

The identification by G. S. (Steve) Martin of the Rous strain of avian sarcoma virus (RSV) that was temperature sensitive for transformation implied that RSV contained an 'oncogene' that conferred its tumorigenic properties in chickens. Work by Peter Duesberg and Peter Vogt soon showed that the genome of RSV contained RNA sequences that were missing from replication-competent but transformation-defective viral variants. Using elegant techniques developed in the laboratories of Michael Bishop and Harold Varmus, Dominique Stehelin used subtraction hybridization procedures to generate a cDNA probe that specifically hybridized to the putative oncogene (or *src* sequences) of RSV. In 1976, Stehelin and colleagues found that radiolabelled 'cDNA*src*' hybridized to homologous sequences in the DNA of normal chicken cells, and to less-conserved sequences in the genomes of other avian species. So, RSV had acquired its transforming activity by recombining with, and transducing, the chicken cellular '*c-src*' oncogene.

"These were very unexpected findings that launched the whole field of oncogenes." *Julian Downward*

An analysis of the thermostability of the DNA duplexes formed between the RSV v-*src* probe and avian cellular DNAs provided an indication of the extent of relatedness, and showed that cellular c-*src* DNA sequences diverged in accordance with the phylogenetic distances between the different species. Using less-stringent hybridization conditions, Deborah Spector, Varmus and Bishop detected more distantly related c-*src* sequences in human and mouse DNA, but not in sea urchin, fruit fly or *Escherichia coli*.

Bishop, Varmus and colleagues went on to study the gene product of v-*src*, which had first been identified by Joan Brugge and Raymond Erikson. Using the same experimental approach, the Oppermann, Bishop and Varmus team (followed a few months later by the Erikson laboratory) identified the cellular form by precipitating proteins from extracts of uninfected vertebrate cells with antisera derived from rabbits with RSV-induced tumours. This led to the isolation of a 60-kDa phosphoprotein in chicken cells, and subsequently in quail, rat and human fibroblasts.

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The 60-kDa proteins shared several antigenic determinants with the viral protein, and were chemically and structurally similar, although not identical. In addition, the cellular homologues seemed to function as protein kinases in a similar way to that previously shown for the viral protein (see Milestone 16).

Together, these papers provided the first evidence of the presence of genes related to viral oncogenes in the genomes of healthy vertebrates — this proved, as Bishop famously said, that 'the seeds of cancer are within us'. Their function in the host organism remained unclear, together with the question of whether their function had been altered by the virus. These discoveries led to an explosion of

regions. They therefore considered the total accumulation of changes, rather than their sequence, as most important for tumour progression. They also concluded that five or more genetic alterations were probably required for the development of carcinomas, with fewer changes needed for benign tumorigenesis.

This model of cancer evolution through the accumulation of mutations in both oncogenes and tumour-suppressor genes, and the stepwise selection of more malignant tumour-cell populations, has since been widely adopted and generalized to all common forms of cancer. At a time when we are beginning to see the basic understanding of the molecular changes underlying tumorigenesis translated into the development of targeted therapies (see Milestone 24), it is well worth noting the foresight of Nowell in suggesting that individual differences in the genetic and biological changes in each tumour might warrant personalized therapies.

Barbara Marte, Senior Editor, Nature

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oncogene research, which resulted in the identification of more than 40 different oncogenes, and provided a framework for understanding signal-transduction pathways that control normal cellular growth (see Milestone 16). Varmus and Bishop went on to win the Nobel Prize for their discovery.

Arianne Heinrichs, Chief Editor, Nature Reviews Molecular Cell Biology

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MILESTONE 16

Phosphorylate and conquer

The mystery of the cell-transformation process and the seemingly simple principles that are involved in cancerous growth began to be unravelled with the study of retroviruses. Research into the function of the protein products that are encoded by oncogenes followed closely on the heels of the discovery of the oncogenes themselves (see Milestones 15 and 17).

In 1978, in an effort to understand the function of the *src* oncogene from the avian Rous sarcoma virus (RSV), Michael Bishop and colleagues prepared anti-sera from rabbits that were infected with RSV-induced tumours, to isolate and further characterize the putative product of *src*. They found that the anti-sera precipitated a 60-kDa phosphorylated protein product, which they consequently designated as pp60. They showed that phosphorylation of this protein was essential for its function, and that it probably possessed a kinase activity. This study, along with similar findings from the group of Raymond Erikson, indicated that protein phosphorylation might be important in the transformation process.

In late 1979, Tony Hunter and colleagues identified a phosphotyrosine kinase activity in protein immunoprecipitates of the polyoma virus middle T antigen. Soon thereafter, studies of the Abelson murine leukaemia virus by David Baltimore and colleagues, and of RSV by Hunter and Bartholomew Sefton, identified tyrosine-specific protein kinase activities associated with the product of the Abelson leukaemia viral oncogene (Abl) and Src. Both groups showed that these enzymes were essential for the malignant transformation of cells by the oncogenic proteins. As viral Src was derived from an evolutionarily conserved *c-SRC* cellular proto-oncogene (see Milestone 15), logic dictated that all vertebrate cells must contain at least one protein kinase that phosphorylates tyrosine. Yet, the functional relationship between kinase activity and oncogenesis remained unclear.

Work published by the Stanley Cohen laboratory a few months later, using cell membranes isolated from epidermal growth factor (EGF)-responsive carcinoma cells, provided further insights. They found that activation of the EGF receptor (EGFR)–protein kinase complex resulted in protein phosphorylation, mainly on tyrosine residues. The association of both virus-induced and EGF-induced cell growth with the activation of protein kinases specific for tyrosine was certainly intriguing, and indicated a functional relationship between oncoprotein activity and receptor signalling.

Importantly, shortly thereafter in 1983, Mike Waterfield and colleagues, and Harry Antoniades and colleagues identified the putative transforming protein of simian sarcoma virus as platelet-derived growth factor (PDGF). In addition, in 1984, Mike Waterfield, Yossi Schlessinger and colleagues reported that v-*erbB* from the avian erythroblastosis virus encodes a similar protein to EGFR. These three papers, along with those identifying the cellular Ras oncogenes (see Milestone 17), showed that the oncogenes found in retroviruses encode components of the normal growth-regulatory machinery of the cell.

Many other oncogenes were shown to encode proteins that bound to these kinases as upstream modulators or downstream signal transducers. Not only was it now apparent that tumours might arise owing to the de-regulation of these kinase-mediated signalling pathways, but the possibility of therapeutic intervention was also evident. The outcome of much of this early work has been the development of therapeutic agents (see Milestone 24) that target the mutated oncogenes.

Ekat Kritikou, Locum Associate Editor, Nature Reviews Molecular Cell Biology

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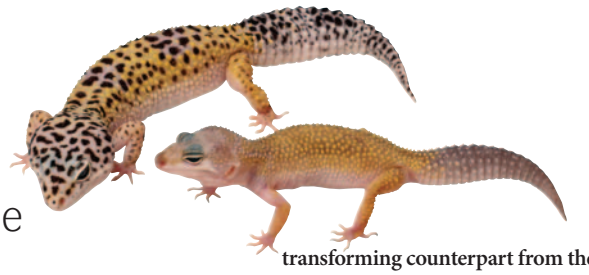
MILESTONE 17

An important difference



The idea that cancer is a disease of altered genes was widely discussed among basic scientists in the 1970s. The clinching evidence that brought it to wider attention was the discovery of mutations in the genome of tumour cells that, when transferred into other cells, were sufficient to cause transformation.

By the late 1970s, it was well known that retroviral oncogenes could rapidly transform cells, and that the viruses had acquired these genes from the genomes of the mammalian and avian cells that they infected (see Milestone 15). It was therefore proposed that mutations in the cellular homologues of these genes could transform cells in the absence of any viral involvement, and that this occurred in a substantial proportion of human cancers. Key discoveries by the Robert Weinberg and Geoffrey Cooper groups showed that such transformation could occur when the DNA of a chemically mutagenized transformed mouse cell was transferred. However, the precise identity of the transforming gene was not known, as a lot of irrelevant DNA was also transferred.

Finally, in 1982, the Weinberg, Michael Wigler and Mariano Barbacid groups all cloned the first oncogene, from bladder carcinoma lines, after closing in on the relevant DNA by numerous rounds of transfection. In each round, more of the irrelevant DNA was lost, until the actual oncogene could be cloned with the use of linked sequence tags. These cloned cellular genes had the same transforming properties as the oncogenes from retroviruses.

Having uncovered the presence of cellular oncogenes, attention turned immediately to their identity. Within a few months, the Weinberg and Barbacid groups, as well as Cooper and colleagues, had shown by restriction endonuclease mapping and Southern blotting that the oncogenes in question were the cellular homologues of the *ras* genes from the Harvey and Kirsten sarcoma viruses.

However, such analysis was not detailed enough to identify any difference between the normal cellular human c-Ha-*RAS1* gene and its transforming counterpart from the carcinoma lines. This implied that the two versions of the gene were similar and any sequence difference was subtle. Using an elegant molecular genetics strategy that has since become obsolete, the Weinberg, Barbacid and Wigler groups systematically substituted each restriction fragment from the non-transforming allele with the corresponding one from the transforming allele. In this way, they were able to hone in on the genetic lesion and, by the end of 1982, all three groups had discovered the same single amino-acid change: glycine to valine at position 12. Subsequent research has shown that this change alters the structure of the RAS protein to make it constitutively active.

During just 1 year, not only was the concept of the cellular oncogene confirmed by the cloning of cellular *RAS*, but the activating mutation was also identified. The developments of 1982 were a crucial step towards the modern understanding of cancer as a complex interplay between different types of genetic lesion.

Patrick Goymer, Assistant Editor,
Nature Reviews Cancer and Nature Reviews Genetics

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MILESTONE 18

Teaming up for transformation

In the early 1980s, evidence indicated that the oncogenic transformation of primary cells involved at least two stages: establishment (the immortalization of cells) and cellular transformation. With this in mind, Hartmut Land, Luis F. Parada and Robert Weinberg, working at the same time as Earl Ruley, investigated how oncogenes cooperate to induce tumour development.

Weinberg and colleagues examined the effect of expressing two recently identified oncogenes — an activated variant of Harvey *RAS*1, EJ *RAS* (see Milestone 17), and either a viral or a mammalian clone of *Myc* — in primary rat embryonic fibroblasts (REFs). They found that, despite its capacity to transform rodent cell lines, EJ *RAS* could not transform REFs. These cells initially proliferated, but then underwent crisis and arrest. EJ *RAS*-expressing REFs were also unable to form tumours in immunocompromised mice. However, REFs expressing *Myc* and *RAS* grew rapidly as foci that were able to establish long-term cultures when passaged *in vitro*, and could form tumours in mice. These tumours were not metastatic, implying that beyond MYC and RAS cooperation, further oncogenic events might be required to produce an invasive tumour (although we now know that tumours seeded subcutaneously in mice often do not metastasize). Similar results were found by Ruley using adenoviral E1A, polyoma virus middle T antigen and T24 Harvey *RAS* expressed in baby rat kidney cells.

MILESTONE 19

From obscurity to the clinic

In the early 1980s, the cancer field was abuzz with the first discoveries of oncogenic mutations linked to cancer. The genetic mutation responsible for the transforming properties of the *RAS* oncogenes was found in 1982, to great acclaim (see Milestone 17). In this climate, the first observations of epigenetic abnormalities in cancer were overshadowed and ignored by many in the field. However, studies in the 1980s showed that epigenetic changes can occur to both oncogenes and tumour suppressors, and have led to our present appreciation of epigenetic markers as diagnostics and therapeutic targets for cancer.

Epigenetic phenomena can be defined as heritable changes in cellular information other than the DNA sequence, which usually involve covalent modifications to DNA or histones. These modifications are involved in controlling gene expression — for example, the methylation of DNA at CpG dinucleotides in gene promoters is associated with the silencing of transcription. In 1983, Andrew Feinberg and Bert Vogelstein purified DNA from several primary human tumour tissues and, using methylation-sensitive restriction enzymes, found lowered DNA methylation of specific genes compared with DNA from adjacent normal cells. With the predominant concept at the time being that cancer is caused by activation of oncogenes, these findings implied that altered DNA methylation could underlie oncogene activation.

Later in the 1980s, the concept of tumour-suppressor genes, such as retinoblastoma, was becoming well defined (see Milestone 11). So, it was encouraging when relevant epigenetic changes were found in these tumour-suppressor genes. For example, Valerie Greger *et al*. showed that an unmethylated CpG island at the 5′ end of the retinoblastoma gene becomes hypermethylated in tumours from retinoblastoma patients, leading the authors to speculate that methylation could contribute directly to the silencing of tumour suppressors. Later studies — such as those of Naoko Ohtani-Fujita *et al.* and James Herman *et al.* — correlated the methylation of the tumour-suppressor genes with their actual silencing in cancer.

More direct evidence linking DNA hypermethylation with cancer formation came several years later from Rudolf Jaenisch's group. They used mice carrying a '*Min*' mutation in the adenomatous polyposis coli (*Apc*) gene. These mice develop intestinal polyps early in life and are a model system for the early stages of human colorectal cancer. Peter Laird *et al*. reduced DNA methylation in *Min* mice by mutating a DNA methyltransferase gene and using the methyltransferase inhibitor azacytidine. The reduced DNA methylation led to a decreased number of polyps in the animals, lending support to the idea that tumour-suppressor genes are hypermethylated and silenced in cancer, and can be reactivated by inhibiting DNA methylation.

DNA methylation inhibitors, such as azacytidine, are now approved for clinical use, although there is controversy about whether they work by reactivating tumour suppressors. Furthermore, the debate over whether altered DNA methylation has a causal role in initiating cancer remains alive today. Yet, it is remarkable that work carried out back in 1980 by Peter Jones and Shirley Taylor showing the effects of chemicals such as azacytidine on DNA methylation and cell differentiation, which attracted little attention at the time, opened the door to the idea of cancer treatment aimed at reversing DNA methylation.

Alex Eccleston, Senior Editor, Nature

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Oncogene cooperation was also studied in avian erythroid progenitor cells by Thomas Graf and colleagues. In 1986, they showed that the non-transforming viral gene *v-erbA* could cooperate both *in vitro* and *in vivo* with *v-src*, v-Ha-*ras* and *v-erbB*

The ability of different oncogenes to cooperate in producing cellular transformation has been a cornerstone of cancer research during the intervening years. With the development of further molecular techniques, it has become possible to piece together how and why specific oncogenes cooperate so effectively.

Land and colleagues went on to show that high expression levels of RAS, like those used in the experiments described above, induce G1 arrest in primary cells owing to the expression of the cell-cycle inhibitor p21. By contrast, expression of MYC was found to induce both proliferation and apoptosis (see Milestone 12). MYC and RAS cooperate because MYC can act in numerous ways to circumvent the RAS-induced G1 growth arrest, and RAS prevents MYC-induced apoptosis by activation of the anti-apoptotic kinase AKT.

B-cell lymphoma 2 (BCL2) and MYC also cooperate effectively (see Milestone 12). BCL2 is an unusual oncogene product in that, unlike RAS, it cannot transform rodent cell lines and, unlike MYC, it does not induce proliferation. Nevertheless, in 1990, Andreas Strasser and colleagues showed that BCL2 synergizes with MYC to rapidly produce tumours in mice. This is because, as shown later by several groups, MYC-induced apoptosis is suppressed by the expression of BCL2, leaving the proliferative capacity of MYC unchecked.

Identification of the growth-restrictive aspects of oncogene activation, such as cell-cycle arrest and apoptosis, allowed cancer biologists to appreciate the complexities of the molecular cross-talk in tumour cells, and to begin to understand the pathways that have evolved to limit cellular transformation (see Milestone 20).

Nicola McCarthy, Senior Editor,
Nature Reviews Cancer

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MILESTONE 20

Stop or die!

Half a century ago, epidemiologists proposed that cancers result from multiple 'hits' (see Milestone 9). Initially, the focus was on dominantly acting viral oncogenes and activating mutations in the *RAS* oncogene. Later, cell fusion and genetic experiments showed that recessive mutations cause defects in tumour suppression (see Milestone 11). Bert Vogelstein reconciled the oncogene and tumour-suppressor camps by describing how both events are necessary for colorectal carcinogenesis (see Milestone 14). Arnold Levine, David Lane and colleagues discovered the first tumour-suppressor gene, *p53* (also known as *TP53*), although it was initially described as an oncogene. Levine showed that p53 suppresses transformation, while Vogelstein reported that both *p53* alleles are mutated in colorectal cancer, a finding subsequently extended to most common human tumour types, with over 20,000 *p53* mutations now on record. The second tumour suppressor to emerge was the retinoblastoma protein RB (see *Milestones in Cell Division* Milestone 15). Both RB and p53 have been on the citation bestseller lists ever since it became apparent that the main DNA tumour viruses transform cells by inactivating both RB and p53. The RB pathway is now firmly enshrined in cell-cycle regulation, and defects in this pathway are a universal feature of cancer.

In 1989, David Livingston and Ed Harlow published an early milestone: they found that RB is phosphorylated in a cell cycle-dependent manner, as synchronized primary and immortalized cells enter the DNA-replication phase (S phase). They reported, separately, that SV40 T antigen, which can drive G1-arrested cells into the cycle, only binds unphosphorylated RB — the first indication that this is the growth-suppressive form of RB. Therefore, they surmised that unphosphorylated RB acts to block exit from G1.

p53 has emerged as a crucial guardian of the genome, and several exceptional papers first described its role in the DNA damage-checkpoint response. It was known that both p53 and DNA damage inhibit DNA replication, and cause G1 cell-cycle arrest. Michael Kastan and colleagues connected these findings in haematopoietic cells by showing that the G1-checkpoint arrest correlates with p53 protein induction, and that this response is sensitive to caffeine — later shown to block ATM kinase — and cycloheximide. Importantly, cells with mutant or no p53 did not arrest in G1 after γ-irradiation (IR), while maintaining a second checkpoint arrest in G2. In a second paper, Kastan generalized these findings and showed that re-expression of p53 in p53-null cells rescued the IR-induced G1-checkpoint arrest. Conversely, a p53 mutant was able to abrogate the G1 checkpoint in p53 wild-type cells in a dominant–negative fashion. A third paper by Kastan placed p53 in a checkpoint-signalling pathway; he noted that cells from ataxia telangiectasia (AT) patients also lacked the G1 DNA-damage checkpoint and, proposing that the defects in AT and p53 are functionally linked, he documented a decreased p53 induction in AT cells after IR. Importantly, this paper used primary embryonic fibroblasts from p53-null mice, rather than transformed cell lines. Just previously, p53 had been shown to be a sequence-specific DNA-binding protein capable of transcriptional activation. Furthermore, it was known that the radiation sensitive *GADD45* gene was not induced in AT and several tumour cell lines. Kastan showed that *GADD45* induction requires p53, and that wild-type p53 bound to a p53 consensus site in the gene promoter. Therefore, this paper not only uncovered upstream and downstream events in the p53-dependent DNA damage-signalling pathway, but also described one of the first p53 target genes. The importance of these papers is threefold: they explain how the cell cycle is arrested after DNA damage, and how p53 loss might contribute to genetic instability and tumour formation, and they show that DNA damage elicits a signal-transduction response involving the gene mutated in AT (now known to be the ATM kinase), p53 and p53 target genes.

By the mid-1990s, it became clear that apoptosis was a key tumour-suppressive pathway (see Milestone 12), and that p53 induces apoptosis and is required for DNA damage and oncogene-induced apoptosis. To investigate the role of p53-dependent apoptosis in brain tumour progression, Holly Symonds and colleagues used transgenic mice expressing a SV40 T-antigen mutant that inhibits RB, but not p53. Tumour growth relative to wild-type T antigen slowed in p53-wild type, but not in p53-null, mice; p53-heterozygous mice exhibited stochastic emergence of p53-null tumours, and this correlated with decreased apoptosis. At the same time, Sharon Morgenbesser and colleagues reported increased proliferation and apoptosis in the developing ocular lens of RB-null mice; apoptosis was suppressed in RB/p53 double-null mice, indicating p53 dependence. These papers, together, are the first to describe that inappropriate S-phase entry owing to loss of RB results in p53-dependent apoptosis, thereby linking the two central tumour-suppressor pathways in the cell.

These studies represent only a couple of the milestones in our understanding of RB and p53, and their role in cell-cycle and DNA-damage checkpoints, which have dominated cancer research for the past decade.

Bernd Pulverer, Editor, Nature Cell Biology

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MILESTONE 21

The human touch

Although we note that cancer is a complex and challenging disease that has been attacked on many research fronts, we must also be aware that there is a human side to dealing with cancer.

In the late 1980s and early 1990s, the genetic basis for cancer-predisposition syndromes rapidly unravelled with the discovery of a number of tumour-suppressor genes that were found to be inherited in mutant form in affected families. These included genes associated with the childhood cancer Wilms' tumour, Li Fraumeni syndrome, neurofibromatosis, familial adenomatous polyposis, von Hippel-Landau disease and retinoblastoma (see Milestone 11). Many of these genes are now known to have key roles in cell proliferation, cell-cycle checkpoints and cell death (see Milestone 20).

At the same time, researchers were frantically searching for, and finding, more susceptibility genes and molecular mechanisms, so that genetic counsellors and patient-care professionals around the world could begin translating these important advances into real-world advice for patients who were forced to make profound decisions. The discovery of inherited mutations leading to more common diseases, such as breast cancer and colon cancer, serves to illustrate this point.

Before 1990, we knew that 5–10% of breast and colon cancers occurred in familial patterns, but whether these were owing to shared environments, several interacting genes or single major genes was not known. A breakthrough came with the identification of genes on chromosomes 2 and 17 that were associated with major fractions of colon and breast cancers, respectively. These, and related genes discovered shortly thereafter, were *MSH2* and *MLH1* in hereditary non-polyposis colorectal cancer, and *BRCA1* and *BRCA2* in hereditary breast cancer syndromes. Interestingly, in both types of hereditary cancer, as well as in other cancer-predisposition disorders, the predisposing genes affect DNA repair rather than cell growth *per se* (see Milestone 22).

The penetrance of mutations varies in families, and most breast and colon cancers do not appear to be of hereditary origin. Yet, for families dealing with these cancers, the identification of the genes meant that testing was possible, and difficult prophylactic-care decisions could be informed by the test results.

In the decade since their discovery, testing for cancers resulting from mutations in cancer-susceptibility genes has become more common. However, the decision to be tested and knowing what to do with the information have not necessarily become any easier. For example, prenatal testing is now available for conditions such as neurofibromatosis, which is a common hereditary disease that leads to numerous benign tumours throughout the body.

Unfortunately, like *MSH2* and *BRCA1*, the neurofibromatosis gene *NF1* is large and subject to a range of mutations, so testing is difficult unless a specific mutation has been identified in another family member. Moreover, as in hereditary breast and colon cancer syndromes, the chance of developing tumours and their severity varies greatly, even when a mutation is found. Basic cancer research must therefore lead directly to the translation of important findings into information for patients. The advent of the

Internet has greatly assisted this process, and websites such as that of the National Cancer Institute in the United States are consulted by thousands of patients and their family members each day.

Chris Gunter, Senior Editor, Nature

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MILESTONE 22

Indirect but just as effective

Although many cancers result from mutations in prototype oncogenes and tumour-suppressor genes that regulate cell proliferation and apoptosis (see Milestones 10, 11, 12, 20 and 21), cancer can also arise indirectly from defects in the protective cellular mechanisms that repair DNA damage. This idea originated in the study by Theodor Boveri of chromosomal imbalances in somatic cells (see Milestone 2). The type of DNA damage can range from the subtle, such as a single unrepaired base lesion, through small deletions or insertions, to macroscopic changes that manifest as non-reciprocal chromosome translocations (see Milestone 10). Genetic instability at any of these levels can predispose to cancer by increasing the rate at which potentially oncogenic mutations and chromosomal alterations occur.

When cells are exposed to ultraviolet (UV) light, base adducts are formed that must be excised for replication to occur. This process, nucleotide-excision repair (NER), involves recognition of distortion of the DNA helix, assembly of a complex on and around the lesion, and excision of a single-strand fragment containing the modified base. Several human syndromes show UV hypersensitivity, and one, xeroderma pigmentosum (XP), displays a strong predisposition to skin cancer.

XP patients were originally classified in eight complementation groups. In 1990, two human genes with roles in NER were cloned, and both were linked to XP. The sequence of excision-repair cross-complementing 3 (*ERCC3*; also known as *XPB*), cloned by Geert Weeda *et al*., implied that it encoded a DNA helicase. Complementation studies showed that in the unique XP group B individual, a splicing mutation in *ERCC3* resulted in a frameshift. Kiyoji Tanaka *et al*. later cloned the XP group A-complementing protein (*XPA;* also known as *XPAC*) gene, the mRNA of which was reduced in cells from XP-A individuals. From its sequence, XPA was proposed to promote incision surrounding the lesion.

The association between XP and DNA repair deficiency arose from the extreme UV sensitivity of the patients, rather than specific observations of damage at the DNA level. In patients with hereditary non-polyposis colon cancer (HNPCC), however, the link to defective repair was obvious: microsatellite repeat sequences in their cells had changes similar to those found in bacterial mismatch repair (MMR)-deficient mutants.

This observation encouraged efforts to locate human genes with homology to the bacterial MMR proteins MutS, MutH and MutL. In 1993, two groups using complementary approaches identified MutS homologue 2 (*MSH2*) as an HNPCC-associated gene. Whereas Richard Fishel *et al.* went directly after homologues of MutS using a degenerate primer strategy, Frederick Leach *et al.* used markers linked to HNPCC to define the disease locus, and then isolated the candidate MMR gene. Additionally, Leach *et al.* reported that chromosome 2-linked HNPCC families had mutations in *MSH2*. A few months later, in 1994, the gene responsible for chromosome 3-linked HNPCC was cloned by Nickolas Papadopoulos *et al.* and Eric Bronner *et al.* Not surprisingly, this turned out to be the human MutL homologue, *MLH1*.

Gross chromosomal changes are consistently observed in human cancers, and their mechanistic basis is the subject of active investigation. One line of research indicates that the combination of telomerase dysfunction and p53 inactivation leads to chromosome instability. Late-passage telomerase-deficient mice were known to have shortened telomeres and chromosome instability, but cell viability was compromised. By introducing p53 deficiency into this background, Ronald DePinho and colleagues were able to show that cell survival could be promoted, allowing neoplastic transformation to occur. Furthermore, Steven Artandi *et al.* found that in telomerase- and p53-deficient epithelial cells, telomeres become progressively shortened, leading to a rise in chromosomal instability (non-reciprocal translocations and end-to-end fusions) and accelerated carcinogenesis. Another line of research implies that the maintenance of the mitotic-spindle checkpoint is essential for chromosome stability in cancer cells. Sandra Hanks *et al.* found that mutations of the spindle-checkpoint gene *BUB1B* caused a cancer-predisposition syndrome characterized by premature chromosome separation. Other cancer-predisposition syndromes caused by alterations in genes associated with chromosome-level repair are ataxia telangiectasia, Bloom syndrome and hereditary breast cancers (see Milestone 21).

These and other studies established that DNA repair defects of various forms and severity initiate genetic instability that affects cancer development. Whether genetic instability is actually mandatory for cancer development in non-familial cancers remains controversial, although these findings stress the importance of protecting the integrity of the genome as a tumour-suppression mechanism.

Angela K. Eggleston, Senior Editor, Nature

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MILESTONE 23

Profiling cancer expression

Tailoring cancer therapy to specific tumour types maximizes efficacy while minimizing toxicity. Historically, cancer classification has been based on morphology, but cancers with seemingly identical morphological and histopathological features can progress and respond to therapy in radically different ways. A better method of classifying cancers was needed to help predict clinical outcome and make the most of the available therapy — the possible solution came from microarray technology.

The first evidence that gene-expression profiling could distinguish between cancer types came in 1999, from Todd Golub, Donna Slonim and colleagues. They chose two types of leukaemia as a test case: acute myeloid and acute lymphoblastic. The approach involved identifying a 'predictor class' of genes, based on their non-random expression patterns, and evaluating the prediction strength. In addition to distinguishing between the two types of leukaemia on the basis of expression-profile differences, the method could also predict their responsiveness to chemotherapy. The paper laid out a general analytical approach to cancer classification based on gene expression, which could be adapted to assign cancers to hitherto unknown classes.

A year later, Ash Alizadeh, Michael Eisen and colleagues used a similar approach to uncover gene-expression heterogeneity in diffuse

MILESTONE 24

Precision weapons

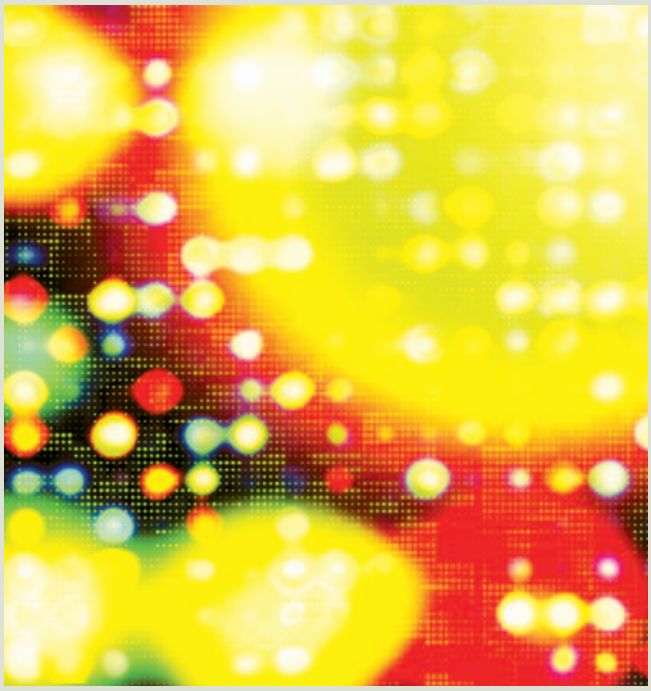
The phrase 'the war against cancer' might have become clichéd over the decades, but it does help to portray how much we have relied on advances in weaponry to score numerous victories against the disease. Tamoxifen proved that cancer treatments can behave like 'magic bullets' (see Milestone 5) and avoid the toxic effects of traditional chemotherapy treatments. Yet, the discovery

large B-cell lymphoma (DLBCL), which is the most common type of non-Hodgkin's lymphoma. The expression profiles identified two distinct forms of DLBCL and correlated with the responsiveness of the tumours to treatment.

The next landmark example of how expression profiling can help to predict clinical outcomes came from the breast cancer field. In this case, specific molecular signatures (of genes involved in the cell cycle, invasion, metastasis and angiogenesis) were shown to accurately predict high likelihood of metastases and, therefore, poor overall prognosis, in the absence of other indicators. This study was the first to show that metastatic potential can be gleaned from the gene-expression data of the primary tumours. After further refinement, related breast cancer-profiling diagnostics have since become commercially available.

Although it might still be too early to see the effect of this technology in the clinic, an important feature of microarray analysis is its lack of bias, which allows microarray-based cancer classification to be systematic and not limited by our previous biological knowledge.

Magdalena Skipper, Chief Editor,
Nature Reviews Genetics



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of oncogenes (see Milestones 4, 15 and 17) offered the possibility of creating 'laser-guided' treatments — drugs that strike at the heart of tumours by zeroing in on the genetic abnormalities that make cells grow uncontrollably.

The first of these molecular-targeted treatments was a monoclonal antibody called trastuzumab (Herceptin; Genentech). Trastuzumab blocks the human epidermal growth factor receptor 2 (HER2) protein that is overexpressed by gene amplification in around 25% of breast cancer cases. Patients with this form of breast cancer have a worse prognosis; however, in the first trial carried out with trastuzumab, Dennis Slamon and colleagues found that women with advanced breast cancer who received the new drug as well as the usual chemotherapy fared better than those who received chemotherapy alone.

If trastuzumab proved that molecular-targeted treatments could effectively treat cancer, then a drug for chronic myeloid leukaemia (CML) called imatinib mesylate (Glivec; Novartis) changed our thinking about the power of designing such therapies. CML is a rare cancer that is characterized by the union of chromosomes 9 and 22, which fuses two genes called breakpoint cluster region (*BCR*) and Abelson murine leukaemia viral oncogene homologue 1 (*ABL*; also known as *ABL1*), to form a tyrosine kinase that signals myeloid cells to grow and proliferate continuously (see Milestone 10). Imatinib mesylate was rationally designed to block the BCR–ABL active site, and when Brian Druker and colleagues carried out the first trial with the drug they found that almost all patients (98%) with therapy-resistant CML saw their blood counts return to normal. Yet, it turns out that imatinib mesylate is not as selective as first thought, and this promiscuity could help to treat other cancers. George Demetri and colleagues were the first to show that imatinib mesylate could treat patients with advanced gastrointestinal stromal tumours by blocking c-KIT.

Designing targeted drugs for more common and complex cancers, however, presents added challenges, as illustrated by the story of gefitinib (Iressa; AstraZeneca). Gefitinib blocks the activity of a tyrosine kinase called epidermal growth factor receptor (EGFR) that is overexpressed in 40–80% of lung cancers. Yet, gefitinib turns out to be effective in only 10–19% of lung cancer patients. Thomas Lynch, Daniel Haber and colleagues explained how the target protein governs whether the drug will work. Patients who respond to gefitinib have specific mutations clustered around the ATP-binding pocket of the EGFR protein where the drug binds, whereas patients who do not respond tend not to carry these mutations.

Equally important as knowing who will respond to treatments is knowing who will develop resistance, and Charles Sawyers and colleagues showed that this is also determined by the target protein. Six out of the nine patients studied, who had relapsed after imatinib mesylate treatment, acquired the same amino-acid substitution in the ABL kinase domain, which affects the interaction of the drug with the kinase; the other three showed *BCR–ABL* gene amplification.

Understanding the molecular underpinnings of response and resistance to these, and other molecular-targeted treatments, is helping to create a new wave of drugs that can harness or circumvent these mechanisms — some of which are already beginning to enter the clinic. The war against cancer might be far from being won, but the era of molecular-targeted treatments could prove to be one of the most important turning points in determining the outcome.

Simon Frantz, News Editor,
Nature Reviews Drug Discovery

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