Genes and cancer: a clinical perspective

MATTHEW ELLIS, MB, MRCP(UK)
Medical Registrar

KAROL SIKORA, PhD, MRCP(UK), FRCR*
Professor of Clinical Oncology

Departments of Medicine and Clinical Oncology, Royal Postgraduate Medical School, Hammersmith Hospital, London

Oncogenes are a family of unique sequences of DNA whose abnormal expression is associated with the development of malignant cell behaviour. They were first demonstrated in rapidly transforming RNA viruses. Their significance lies in the discovery that they are not viral in origin, but are derived from normal cellular DNA and appear in the retrovirus as a consequence of recombinant events between the virus and host's genetic information. The presence of a retrovirus acquired oncogene leads to uncontrolled cell proliferation and differentiation arrest, the hallmarks of malignancy. The exact mechanism by which this is achieved remains unclear, but sequence homology with growth factors and their receptors, together with functional characteristics pointing to a role in cell cycle control, provide intriguing leads in the study of the signalling mechanisms that regulate cell growth. The exact relationship between viral oncogenes and their normal cellular parents, the proto-oncogenes, remains controversial. It is likely, however, that proto-oncogenes do have malignant potential and, when activated by the processes of amplification, mutation, translocation and deletion, can promote tumour formation. Recent techniques in oligopeptide immunisation have been used to develop sets of monoclonal antibodies against oncogene products. These novel reagents can be used to investigate oncogene function in normal and neoplastic tissue and have already demonstrated their potential as tumour markers with prognostic capability. Furthermore, by purifying and analysing oncoproteins, their function can be explored and possibly open new avenues for therapy.

There are three conventional approaches to the treatment of cancer—surgery, radiotherapy and chemotherapy. The first two are effective in dealing with local disease in a wide variety of malignancies. Despite its widespread use, chemotherapy is only effective in prolonging survival of certain relatively rare tumour types. For common cancers such as lung, colon and breast it has little efficacy in prolonging life [1]. The problem in devising strategies for selective tumour cell destruction is the similarity of the cancer cell to its normal counterpart.

Observations on the mutagenicity of carcinogens, the presence of damaged and translocated chromosomes in malignant cells and the recognition of families with an inherited predisposition to the development of cancer, led to the belief that the key to understanding the differences between normal and malignant cells lay in the study of the genome [2]. It was predicted that the genetic changes took place in small discrete sequences of DNA termed oncogenes. The problem lies in the complexity of the human genome. With 50,000 functional genes buried within thousands of kilobases of non-coding sequences, where should the search begin?

Viral oncogenes

Although techniques were not initially available to search the human genome for putative oncogenes, the study of the genetics of tumour viruses became feasible by virtue of their relative simplicity. Segments of viral genome could be readily cloned and sequenced by the new recombinant DNA techniques. Tumour viruses are categorised into two groups, the DNA and the RNA viruses. DNA viruses showed early promise with the demonstration of a conditional mutation that affected the ability of polyoma virus to transform cells in culture. Unfortunately, their cytopathic effect and complex overlapping coding sequences made their study difficult. The RNA tumour viruses, on the other hand, were found to possess only three or four non-overlapping genes, replicated freely without destroying the host cell, and of particular interest, some were capable of rapidly inducing malignancy within days after infection, making them the most potent carcinogens known [3].

The first RNA tumour virus was described by Peyton Rous, in 1911, as a filterable agent inducing sarcomas in chickens. Subsequently, over 30 have been characterised, producing several tumour types in a variety of species including rats, mice, chickens and cats. Although they can cause tumours in primates, they are not a major cause of human tumours—with the leukaemia induced by HTLV1 being the only well defined example. The struc-

ture of these viruses is remarkably similar. There are three genes coding for proteins essential for viral replication, flanked by sequences acting as transcription promoters. Pol, the reverse transcriptase gene, converts viral genomic RNA into DNA which then integrates into the host's genome. The other two genes, gag and env, code for proteins that package nascent viral RNA. The hosts' cellular machinery is subverted by the virus to manufacture fresh virus particles which are then released to infect further cells. When fully sequenced, RNA viruses capable of rapid induction of tumours are found to have extra coding sequences spliced into their genome. This often destroys their ability to replicate independently; 'helper viruses' are necessary to provide the machinery for replication.

The element of the virus genome that was found to be responsible for the rapid transforming capability was termed the viral oncogene (v-onc). Each of these genes has been given a three letter code, depending on the species and/or tumour it caused. The necessity of this gene for transformation was demonstrated by deleting it. This causes the virus to lose its transforming capacity. Furthermore, temperature sensitive mutants were shown to maintain the transformed phenotype only at the permissive temperature. Mild heating returns infected cells to their non-malignant form. This phenomenon could be mapped to changes in the v-onc gene [4].

Cellular oncogenes

A knowledge of viral oncogene sequences allowed the generation of gene probes that could recognise similar sequences in the cellular genome by DNA hybridisation (Table 1). The discovery of homologous genes in cells that had not been infected by virus was at first a great surprise. The significance of this observation was underlined by the detection of these sequences in species as diverse as yeasts, fruit flies and man, with remarkable evolutionary conservation [5]. This suggested that they were essential to basic cellular functions.

These genes have been termed proto-oncogenes to emphasise that they are only potentially oncogenic. There are a number of important differences between viral oncogenes and their cellular homologues. There are small changes in nucleotide sequences. Intervening non-coding

Table 1. Examples of viral oncogenes and their cellular homologues

Oncogene	Origin	Tumour	Human gene
v-src	chicken	sarcoma	c-src
v-ras	rat	sarcoma	c-ras
v-myc	chicken	leukaemia	c-myc
v-fes	cat	sarcoma	c-fes
v-sis	monkey	sarcoma	c-fes
v-erb B	chicken	erythroblastosis	c-erb B
v-myb	chicken	myeloblastosis	c-myb
v-fms	cat	sarcoma	c-fms
v-abl	mouse	leukaemia	c-abl
v-fos	mouse	osteosarcoma	c-fos

sequences (introns) divide proto-oncogenes into several pieces that are later spliced together by post-transcriptional processing.
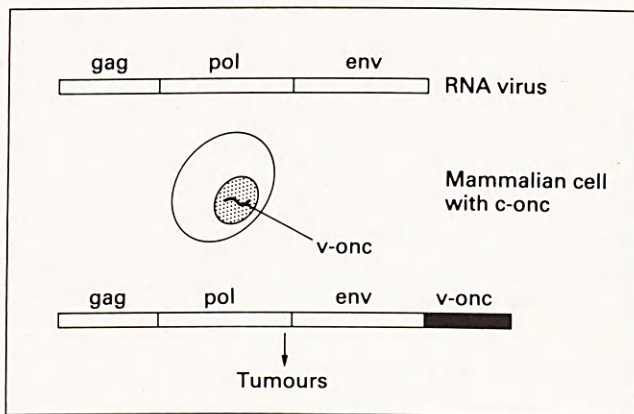

Fig. 1. *Origin of viral oncogenes: a retrovirus infects a mammalian cell and hijacks the cellular oncogene after some modificiation.*

It would appear then that, far from being viral, oncogenes are cellular in origin (Fig. 1). The illegitimate recombinant events that must occur to transduce a cellular oncogene into a RNA virus are rare. The presence of a v-onc is a survival disadvantage to the virus as these viruses may be replication defective. For this reason, epidemics of acute retrovirus-transformed animal tumours have never been seen. They are best understood as rare events which, although a poor model for human carcinogenesis, have led us to begin to detect cellular genes that are involved in cancer through other mechanisms.

Transfection

Most proto-oncogenes so far identified have been found through their homology to a v-onc. A very different approach has been to use transfection assays to detect genes that can directly convey the malignant phenotype. DNA extracted from tumour cell lines or fresh tumour biopsies can be cleaved with restriction enzymes and precipitated with calcium phosphate [6]. In this form, certain mouse fibroblasts (NIH 3T3 cells) can take up foreign DNA and incorporate a proportion of it into its genome and subsequently express the genes involved—a process termed transfection. As a consequence, some cells develop the characteristics of malignancy with loss of contact inhibition causing colonies to pile up and then grow as tumours in nude mice. The analysis of these transformed cells shows mainly mouse, but some human DNA. By successive rounds of transfection, the amount of human DNA can be whittled down to the gene responsible for transformation.

Experiments of this type first demonstrated that DNA from a human bladder carcinoma, responsible for transformation in the transfection assay, was an oncogene of

the c-ras family. Indeed, most of the oncogenes detected by this assay are of the c-ras type [7]. This is probably due to the constraints of the system rather than the c-ras gene being the gene most frequently responsible for human cancer. However, the transfection of NIH 3T3 cells by c-ras from a variety of tumours has yielded fascinating information about mutations at defined sites within the gene that are tumourigenic. Most of the mutated genes isolated so far contain single base-pair changes either at positions corresponding to the 12th or 61st amino acid from the N-terminus. This implies that these two sites are vital in maintaining the proper control of the molecule. The switch of a single amino acid can clearly result in changes that subvert the cell to malignancy. Further analysis has shown that about one-fifth of all human tumours contain point mutations in the c-ras gene.

Chromosome translocation

Chromosome abnormalities are a common feature of malignancy. Some tumours exhibit specific translocations. An example is Burkitt's lymphoma which almost invariably carries a translocation involving chromosome 8. Reciprocal exchange occurs most commonly with the long arm of chromosome 14, but also occasionally with chromosomes 2 and 22. The break-point in chromosome 8 is at the site of the c-myc oncogene [8]. Translocation places this gene near the immunoglobulin gene loci on chromosomes 14 (G heavy chain), 2 (k-light chain) and 22 (λ-light chain).

The Philadelphia chromosome in chronic granulocytic leukaemia (CGL) is a chromosome 9:22 translocation. Here c-abl—the proto-oncogene related to the v-onc of the murine Abelson leukaemia virus—is moved from chromosome 9 into a small fragmented segment of chromosome 22 [9]. Examination of the break-point on chromosome 22 shows that it always occurs within a 5.8 kb segment. This region is designated bcr or break-point cluster region. An mRNA transcript has been detected in CGL cells that is a fusion product of the abl and bcr regions [10]. The protein product of this mRNA may well be instrumental in triggering myeloid cell proliferation, possibly through increases in tyrosine kinase activity. The construction of antibodies against this protein may well provide diagnostic and possibly therapeutic reagents of remarkable specificity.

Gene amplification

Other types of chromosome abnormality seen in tumours are the development of homogenous staining regions, and the formation of double minute chromosomes—additional mini-chromosomes lacking centromeres. Both are associated with the phenomenon of proto-oncogene amplification—as many as 100 copies of each gene per cell in some cases. A gene probe to v-myc has demonstrated c-myc amplification in several human colon carcinoma cell lines. The same probe also showed amplification of genes with sequence homology with c-myc, but with non-homologous regions. Two new but related oncogenes, N-myc in neuroblastoma and L-myc

in small cell lung cancer were revealed in this way [11]. Oncogene amplification may well be a feature of tumour progression. Indeed, there is a good correlation between advanced clinical stage at presentation of a neuroblastoma and the presence of amplified N-myc in the tumour genome [12]. Furthermore, N-myc copy number may have prognostic value in that high copy number tumours are less sensitive to chemotherapy.

Mechanisms of oncogene activation

There are two models for the activation of a proto-oncogene. The first is the quantitative model where transformation occurs as a result of abnormally high levels of expression of an intact gene. There is also a qualitative model where a gene undergoes mutation to make a product with a cellular activity that differs from its normal counterpart. Almost certainly, both mechanisms operate in the production of human tumours.

To assess these possibilities, functionally modified or unmodified oncogenes have been tested in the NIH 3T3 transfection assay. With few exceptions, molecularly cloned cellular oncogenes fail to transform cells. Furthermore, phage or plasmid vectors, carrying copies of the c-src gene, fail to transform normal diploid cells. The enhanced transcription of c-myc in retroviral lymphomas in chickens is an argument advanced for the quantitative model, but this does not explain the 20 per cent of retroviral lymphomas where myc is not activated. Translocation of c-myc sequences in Burkitt's lymphoma was thought to cause enhanced transcription due to promoter sequences associated with the immunoglobulin loci. But even after exhaustive study there is no consensus as to whether c-myc expression is enhanced in Burkitt's lymphoma when compared with normal cells [13]. Efforts to relate elevated expression of some oncogenes with neoplastic transformation has failed to show positive correlations. Finally, oncogenes are frequently active in normal untransformed cells and particularly so during embryogenesis, liver regeneration or wound repair. Genes such as c-raf, c-ras and c-myc are expressed at different levels in various tissues throughout embryogenesis [14].

Evidence that qualitative changes in oncogenes can generate malignancy is persuasive. Most v-oncs identified are altered by point mutation and deletion when compared with their cellular precursors. Furthermore, the gene product is frequently a fusion protein derived from both viral and cellular sequences.

Comparison of the src gene of Rous sarcoma virus (RSV) and its cellular prototype demonstrates scattered point mutations amounting to 1–2 per cent of the sequence. Both ends of the v-src gene include small regions that are not related to essential retroviral genes and are not contiguous with c-src sequences. In an attempt to identify which changes in the src gene are crucial to its transforming potential, a number of variants of the virus have been developed. If RSV is constructed to express c-src rather than v-src, such viruses cannot induce transformation in chicken cells. Even when c-src is expressed at very high levels by the insertion of an active promoter sequence, the morphological transformation of the cells

does not extend to growth in nude mice [15]. Experiments with a variety of constructed c-src genes suggest that loss of the C-terminal amino acids leads to oncogenic activation.

Oncoproteins

Genes exert their activity through the production of proteins. These are the executive molecules of the cell through their structural, enzymatic and regulatory functions. The isolation and characterisation of oncoproteins is in its infancy. The conservation of oncogene sequences throughout vertebrate evolution points to a pivotal role for their resulting proteins in normal cell development. Cancer genes are clearly not unwanted guests but essential constituents of the cell's genetic apparatus. They betray the cell only when their structure or control is disturbed.

Table 2. Evidence that oncogenes are involved in human cancer.

1. Existence of c-onc in humans
2. Transfection studies
3. Chromosomal aberations
4. Amplification and mutation of c-oncs
5. Oncogene product function
   c-sis — platelet derived growth factor
   c-erb B — epidermal growth factor receptor
   c-fms — colony stimulating factor receptor
   c-erb A — steroid receptor
   c-myc — nuclear acting

The evidence implicating oncoproteins in growth control is strengthened by the determination of the function of several oncogene products [16] (Table 2). The c-sis gene product, p28 c-sis, has been shown to have homology to 109 amino acids of platelet-derived growth factor [17]. This important protein is involved in wound healing and the control of growth. It binds to a receptor on the cell surface and subsequently activates a biochemical cascade culminating in increased cellular activity. A second oncogene, c-erbB, has been found to have homology to epidermal growth factor receptor [18]. This large trans-membrane structure consists of two components. The receptor itself is on the outside of the cell membrane and is associated with an internal component, a 70,000 molecular weight structure with protein kinase activity. When epidermal growth factor binds to its receptor it activates the protein kinase on the inside of the cell, so culminating in growth potentiation. Presumably aberrant production of the internal portion in a 'locked-on' position could result in growth without the requirement for exogeneous epidermal growth factor. The c-erbB gene indeed codes for this internal portion. A third oncogene implicated in growth control is c-myc [19]. This 62,000 molecular weight protein binds in a curious way to the matrix of cell nuclei. It has a very short half-life of 20 minutes and has been implicated in cell-cycle control. The level of c-myc mRNA increases when cells are stimulated into division.

In order to examine the relevance of oncoproteins, molecular flags for them have been constructed by making monoclonal antibodies. As the genes have been cloned, their DNA sequence can be determined. The recently devised technique of peptide immunisation has been used to construct such antibodies [20]. Using a genetic dictionary, the DNA sequence of the gene is converted into the amino-acid structure of the oncoprotein. Peptides of between 10 and 20 amino acids long are are then synthesised. The regions chosen for synthesis and immunisation are those predicted to be exposed within the intact molecule. This prediction is made using computer plots of the relative hydrophilicity and hydrophobocity of different parts of the amino-acid sequence. Mice are then immunised to produce polyclonal and monoclonal antibodies (Fig. 2). In this way, antibodies suitable for a variety of clinical uses can be developed.
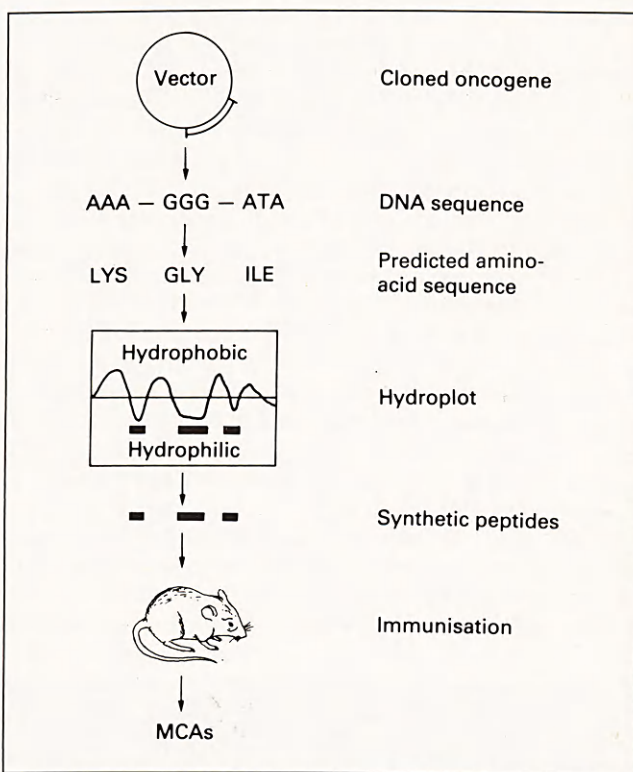

Fig. 2. *Production of monoclonal antibodies to synthetic peptides predicted to be immunogenic by their hydrophilicity.*

Clinical use

Qualitative or quantitative alteration in oncogene expression is clearly important in the production of tumours. With current technology, such changes could be used as markers for the detection and monitoring of cancer in a patient. DNA and RNA can be extracted from tumour cells, and studied by hybridisation techniques to evaluate oncogene amplification and rearrangements, and to assess their level of transcription. Monoclonal antibodies can be used to determine the level of translation by directly measuring oncoprotein products. Table 3 lists

Table 3. The clinical potential of oncogenes.

1. Diagnosis — Tumour markers
           — Immunohistology
           — Tumour localisation
2. Prognosis — Biological behaviour
3. Cancer risk prediction
4. Prevention
5. Therapy

various areas where such information may be useful in the management of patients.

RNA transcript levels of oncogenes have been assayed extensively, using specific gene probes in Northern blotting. Using this technique, several studies have showed clearly elevated levels of oncogene transcript in many tumours. A serious problem with the detection of transcripts in biopsy material is the rapid degradation of cellular RNA which necessitates stringent techniques for collection and preparation. However, several clear-cut associations between clinical behaviour and gene expression have now been made. In prostatic cancer, a significantly higher level of c-myc transcript was observed in patients with invasive adenocarcinoma [21]. In colorectal cancer, dot blot hybridisation of total biopsy RNA showed up to 40-fold elevation in tumour samples when compared with normal colon [22]. C-myb expression was found to be detectable only at low levels in normal colon, whereas increases of up to four-fold were found in tumour samples [23]. In a large study of carcinomas of the head and neck region, a significant elevation in expression of H-ras, K-ras and c-myc was found in both pre-malignant and malignant tumours when compared with normal tissues. Similar observations have also been made in breast cancer [24].

Over the last two years, monoclonal antibodies (MCAs) have been raised against several synthetic peptides or recombinant fusion proteins from oncogenes. The major advantage of utilising oncoproteins as tumour
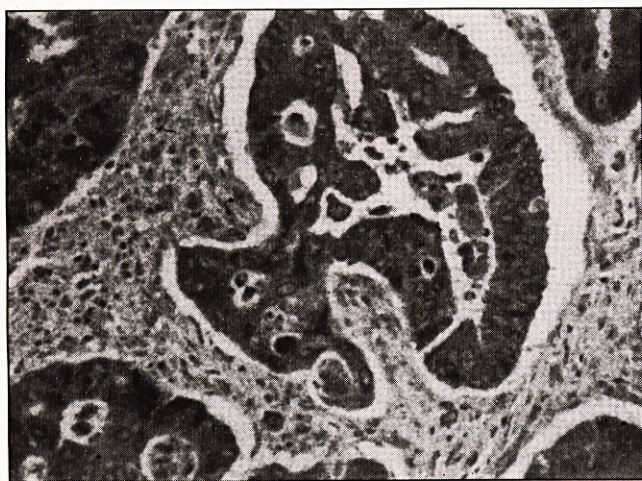
markers, is that, unlike the analysis of DNA and RNA which requires fresh samples, protein may be detected by immunohistology, often using formalin-fixed wax block material. Such procedures for storing surgical specimens have been routine for many decades and most pathology departments have acquired a huge collection over the years. An example of a tumour stained with an antibody for p62 c-myc is shown in Fig. 3.

There are two major problems associated with immunohistology using MCAs. First, quantitation is difficult. Second, the specificity of the MCAs in these conditions has to be proven. Immunhistological fixation and the staining reagents used may alter the protein molecules. For this reason, considerable effort in searching for an appropriate antibody is an essential pre-requisite for any study.
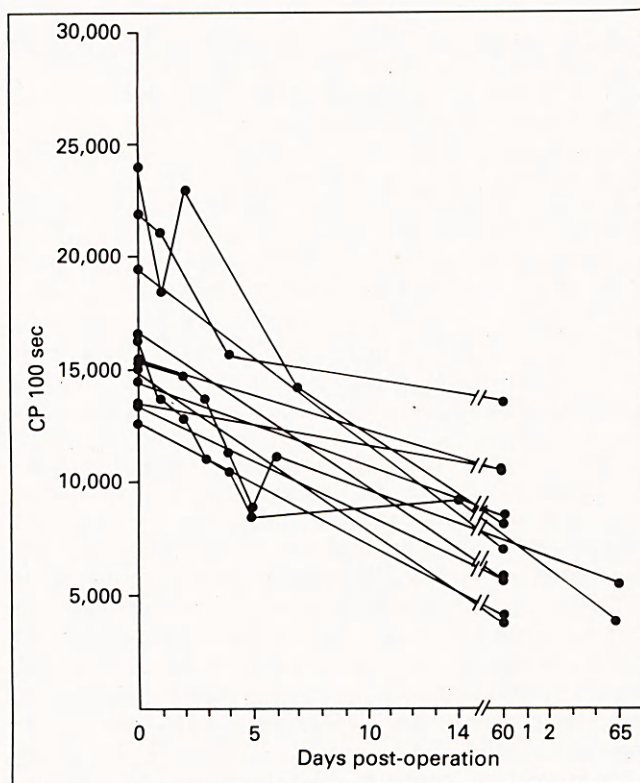

Fig. 4. *Fall in level of circulating c-myc protein after surgery for colorectal carcinoma.*

Although histology is good for giving geographical information about the distribution of oncoproteins in normal and malignant tissues, it is bad for quantitation. A sensitive flow cytometric assay has been developed to precisely quantitate oncoproteins in nuclei isolated from wax-embedded tumours [25]. Correlations can now be made with differentiation state and clinical outcome with levels of myc and other proteins in lung, colonic and testicular cancer. Furthermore, the sera from patients with these diseases have been found to contain circulating oncoprotein which relates clearly to tumour load [26] (Fig. 4). Analysis using an immuno-blotting technique demonstrated a three-fold increase in the titre of c-myc
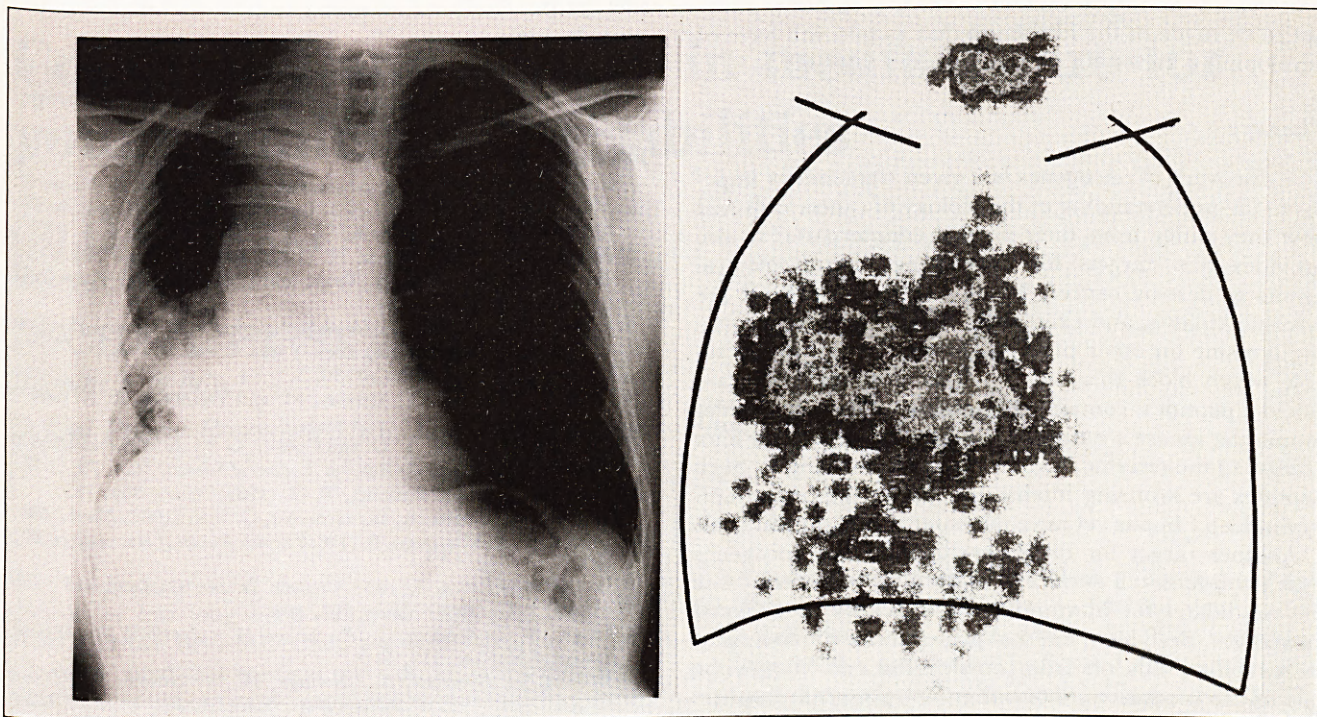

Fig. 3. *Immunoperoxidase staining of colorectal carcinoma with anti p62 c-myc monoclonal antibodies showing binding to tumour chords.*

Fig. 5. *Immunolocalisation of small cell carcinoma of the bronchus by radiolabelled anti p62 c-myc antibodies. The chest x-ray shows tumour in the right mid and lower zones. The scan shows update of ^{131}I over the same areas. A small amount of free ^{131}I is being concentrated in the thyroid, despite blocking with cold iodide.*

related proteins in the sera of patients with a wide range of solid tumours, when compared with both healthy controls and patients with non-malignant diseases. Oncogene products may therefore provide novel markers of great clinical value.

Another diagnostic possibility is the use of anti-oncogene product monoclonal antibodies for immunoscintigraphy. Tumour localisation by radiolabelled monoclonal antibodies has been extensively investigated over the last few years. The major problem has been the lack of specificity of most of the antibodies which makes tumour definition difficult in patients after scanning. Antibodies against epidermal growth factor receptor and p62 c-myc have been labelled with ^{131}I and shown to localise a variety of tumour types [27]. An example is shown in Fig. 5. At the moment, there is little evidence that such scans are any more sensitive than conventional radiological procedures. Such techniques may be of value in finding out more about the tumour's response to drugs or radiation and thus have clinical utility.

The most immediate likely clinical use of oncogenes is the sub-classification of tumours on the basis of their biological and clinical behaviour. Thus, if oncoprotein expression can be shown to be related to invasion and metastasis, then accurate prediction of the course of the tumour in an individual patient may be made. A pathologist already does this to some extent by determining the differentiation state, but this is often only of limited value. In breast cancer, for example, little prognostic information is obtained by classical histology.

Cancer risk prediction

Certain relatively rare cancers have well-defined inheritance patterns. Retinoblastoma, for example, is autosomal dominant. Rare cancer families have been recognised with a high incidence of common tumours including breast and colorectal disease. There are now hints that the genetic make-up of an individual can predispose to the risk of carcinogenesis by physical and other agents. The DNA sequence in and around some oncogenes show considerable variation. Such sequence variations can be detected by restriction fragment length polymorphism (RFLP). This technology has been used successfully for the diagnosis of genetic disorder from trophoblast samples in the early stages of pregnancy.

Briefly, it involves taking a sample of DNA from a patient, peripheral blood lymphocytes being the usual source, cleaving it with restriction enzymes at defined sites, running the DNA on an electrophoretic gel and probing with an oncogene. Sequence differences will produce different patterns on binding. The recognition of polymorphic patterns in populations after restriction enzyme digestion is the basis of RFLP techniques. RFLPs have been observed to occur around the c-ras gene locus [28]. Certain patterns are found to be more likely to be associated with leukaemias, lung and colon carcinoma.

Although such techniques of risk prediction are currently imprecise, it is likely that, as we understand more about growth control genes, the DNA sequences which predispose to malignancy will be identified. It is conceiv-

able that in the future such analyses will enable a print-out to be made of the likelihood that a child will have of developing a variety of tumour types in later life.

Therapy

The discovery of oncogenes has given tremendous impetus to the understanding of the biology of cancer cells and how they differ from their normal counterparts. It also provides new targets for developing pharmacological agents to destroy cancer. Many oncogene products are tyrosine kinases, and exert their effects by phosphorylating tyrosine on other proteins. Several agents are available which block this activity. The most intriguing are suicide peptides containing tyrosine. These molecules mimic the kinase's natural substrate and bind with high affinity to the enzyme, so irreversibly destroying it. Such peptides are arousing intense interest in the biotechnology industry but as yet have not entered any clinical trial.

Another target for drug development are oncogenes which produce cell surface receptors. C-erbB and c-fms are candidates for immunological attack by monoclonal antibodies. Both have internal protein kinase activity and by switching this off from outside the cell, it may be possible to modulate abnormal growth patterns. Furthermore, it may be possible to derive peptides which can act as gene down-regulators. An example of this effect has come from recent studies on LHRH agonists [29]. Receptors for LHRH are found not only in the anterior pituitary, but also in certain tumours. It may well be that the endocrine manipulation of breast and prostatic cancer may be acting by the down-regulation of the tumours LHRH receptor with subsequent slowing in tumour growth. It may be possible to develop similar down-regulating substrates for oncogene products.

Conclusion

The remarkable conservation of the DNA sequence of oncogenes across wide reaches of evolutionary time, points to an essential role for their products in normal development. The control of cell division and differentiation are complex requiring the interaction of many different molecular mechanisms. The study of oncogenes allows dissection of the growth control apparatus of the cell. It is in this molecular keyboard that cancer and several other diseases must begin. By understanding more about these molecules, tools of diagnostic and therapeutic relevance are likely to be uncovered over the next decade.

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