Reviews in Medicine

Molecular biology in medicine

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Introduction

Molecular biology has become an essential component in many branches of medical research. Recent technological advances promise a revolution in the way in which disease is detected, monitored and controlled in the patient. The purpose of this review is to highlight some of the areas in which this new technology has had the greatest impact. It is expected that this rapid pace of advance will continue as the analysis of the human genome gathers momentum. The immediate focus of genome research is to map individual DNA fragments along the length of chromosomes but the ultimate goal is to determine the complete genetic organization of the human genome. The information provided by this world-wide program is expected to have a major impact on many aspects of medical research. For example, there are many disorders for which the genetic basis is unknown or uncertain. The provision of a complete map of the human genome will provide the basis on which these disorders can be investigated.

Molecular cytogenetics

The first consistent chromosomal abnormality to be described in a tumour cell was the Philadelphia chromosome.¹ However, it was not until the introduction of high resolution chromosome banding^{2,3} that the occurrence of other abnormalities could be fully investigated. Since then, much detailed information has been derived concerning the incidence and nature of chromosomal abnormalities in human genetic disease and malignancies. The definition of chromosomal abnormalities associated with a clinical condition has often represented a valuable starting point for the cloning of the appropriate disease gene. The advent of molecular cloning techniques has provided the possibility of both analysing these events at the molecular level and exploiting them as specific markers useful in disease management. In this respect, the recent

development⁴ of the polymerase chain reaction (PCR) is providing valuable new tools for the diagnosis and monitoring of disease.

Recently the technique of non-radioactive hybridization has been developed to the point where it offers a new form of karyotype analysis based on DNA sequence. By labelling DNA probes with biotin⁵ or digoxigenin⁶ the resultant signal on hybridized chromosomes can be visualized by fluorescence. Probes can consist of plasmid, phage or cosmid clones and, although the more repetitive the probe is the greater the resultant signal, it is now feasible to detect single copy probes down to several kilobases (kb) in length. At the other extreme, yeast artificial chromosomes (YACS) can be used to provide probes of 100–1000 kb.⁷ These are particularly useful for the long-range analysis of chromosome translocations by *in situ* hybridization.⁸ This approach has been used successfully to demonstrate that the leukaemia-associated t(4;11) translocation breaks within 300 kb of the CD3 gene cluster on chromosome 11.⁹ Probes specific to the repetitive alphoid sequences present at centromeres of chromosomes can be used to examine complex karyotypes.¹⁰ A particularly useful application of this technology is the 'painting' of chromosomes by hybridizing with a mixture of probes obtained from chromosome-specific libraries.¹¹ Such chromosome-specific 'paints' have been prepared for each chromosome and are finding application in the analysis of complex karyotypes such as those found for solid tumours. A further refinement of this approach is the detection of signals in the interface nucleus, thus obviating the need for dividing cells. This has been used for the detection of trisomy 21.¹¹ An interesting extension of this approach is the derivation of chromosome paints from abnormal chromosomes. This has been achieved by flow sorting small amounts of individual translocated chromosomes and the direct preparation of paints by PCR technology. This approach allows the direct analysis of marker chromosomes which are until now unidentifiable. In addition to the analysis of

chromosomal abnormalities, non-radioactive *in situ* hybridization also has considerable potential for the detection of viral sequences. Epstein–Barr virus viral sequence has been detected in nasopharyngeal carcinoma patients¹² and cytomegalovirus sequence in biopsies from patients with acquired immunodeficiency syndrome (AIDS).¹³

Molecular genetics of inherited disorders

Use of linkage for disease gene mapping

Linkage analysis has been used extensively to establish the chromosomal location of genes for a series of inherited disorders. In this approach, the inheritance of polymorphic alleles is traced through an affected family. An allele is said to be linked to the disease gene if it is present in affected individuals at a frequency greater than chance. The stronger the association the more 'tightly' the polymorphic marker is said to be linked to the disease gene. The chance of being mistaken in this analysis is a measure of how far the disease locus is from the marker allele. By following the inheritance of such an allele it is possible to predict who is going to be affected in a family. It should be emphasized that the marker allele is not, in itself, the cause of the disease. Rather it can give vital information about the chromosomal location of the disease gene. Thus, linkage analysis is often the prelude to a search for the disease gene and provides a useful starting point. An important limitation of linkage analysis is that it can be performed only when several individuals are available from an affected family and when there are polymorphisms available at an appropriate locus.

The distance between two loci is measured genetically in units of centiMorgans (cM). One cM represents a 1% chance of recombination during meiosis. It is possible to use this measure to determine distance along a chromosome. The entire human genome which consists of 3×10^9 base pairs has been estimated to contain 3,300 cM and therefore 1 cM is roughly equivalent to 10^6 base pairs. Thus, a linkage between a polymorphic allele and a disease gene of several cM would predict that a molecular distance of several million base pairs would have to be bridged.

In linkage studies the marker locus can be anything polymorphic, including chromosomal features, proteins and DNA molecules. Recently a large number of restriction fragment length polymorphisms (RFLPs) have been defined in the genome and are being used effectively to establish linkage with disease genes.¹⁴ RFLPs are based on the occurrence of nucleotide differences (approximately one base every 200 bases) which either create or remove restriction enzyme digestion sites.

A probe to such a region can detect the polymorphic variation by Southern analysis and thus the inheritance pattern of each allele can be readily determined. RFLPs in general represent neutral changes which have no functional consequences and are inherited in a Mendelian fashion. In addition to establishing linkage to disease genes, RFLP analysis has been used conversely to exclude the involvement of candidate genes.¹⁵ A valuable clinical application of this approach is the detection of the carrier status for diseases such as haemophilia B.¹⁶

A rarer type of DNA polymorphism is represented by the 'variable number of tandem repeats' (VNTR) or minisatellite DNA sequences,¹⁷ which consist of short sequences arranged head-to-tail and repeated multiple times. The high degree of variability of the number of repeats creates a high level of polymorphism in the population. Since some probes detect many of these VNTR loci simultaneously it is possible to create a complex Southern blot pattern which is totally individual specific. This is often referred to as 'DNA fingerprinting' and has important applications in paternity testing¹⁸ and in forensic science.¹⁹ An interesting clinical application of DNA fingerprinting has been the identification of the origin of engrafted bone marrow following transplantation.²⁰ This approach has also been used to search for mutations in tumours and cell lines.²¹

The cloning of the Duchenne muscular dystrophy gene

The isolation of the gene for Duchenne muscular dystrophy (DMD) represents a successful application of a range of molecular genetic techniques to a clinical problem. This disease was known to be an X-linked condition affecting young males. The gene was mapped more precisely to Xp21.2 by the observation in affected females of chromosomal translocations between the X chromosome and autosomes. Additionally, RFLP analysis linked the gene to a polymorphic marker at Xp21. Thus, a knowledge of the probable position of the disease gene provided a starting point in attempts at its isolation. One approach²² involved the use of a subtraction cloning technique to isolate a DNA fragment which was present on a normal X chromosome but absent from the X chromosome with a small Xp21 deletion in an affected individual. This clone, referred to as pERT87, was also found to be missing from some affected boys with no visible chromosomal deletion. Subsequently, surrounding fragments of DNA were found to be missing in other patients strongly suggesting that this region was part of the DMD gene. An alternative approach which used the occurrence of chromosomal translocations in female patients

successfully led to the isolation of part of the same gene. It has emerged that the DMD gene is spread over 2 million base pairs²³ and encodes a 16 kB mRNA muscle specific transcript for a protein named 'dystrophin', which has been shown to be absent from DMD patients, as expected.

The molecular analysis of this condition has yielded an explanation²⁴ for the distinction between Duchenne muscular dystrophy and a milder form known as Becker muscular dystrophy (BMD). In DMD the deletions are such that no protein is produced, whereas in BMD reduced amounts of dystrophin or a protein of an abnormal size is produced.

The cloning of the cystic fibrosis gene

Cystic fibrosis (CF) is an autosomal recessive disorder affecting approximately 1 in 2,500 Caucasian individuals. It follows that about 1 in 25 individuals will be heterozygous carriers. Its frequency and clear inheritance pattern made CF an ideal target for linkage analysis using RFLPs. Eventually a linkage was established with polymorphic markers on chromosome 7. In particular the gene was found to lie between the markers *met* and J3.11. The distance between these markers was approximately 1.6×10^6 base pairs, which is large enough to contain many genes and it was therefore necessary to refine the linkage map by identifying extra polymorphic markers in this region. This was achieved with the KM-19 marker which effectively reduced the region for the CF gene to approximately 5×10^5 base pairs. Since this remained a large region it was necessary to examine genes from this region as possible candidates for the CF gene. A problem in this analysis was that there was no chromosomal translocation to help pinpoint the CF gene as there had been for the DMD gene. Eventually a careful analysis of this region identified a gene²⁵⁻²⁷ whose expression matched that expected for the CF gene (i.e. lung, pancreas, liver, intestine and sweat glands). In order to prove that this gene was the CF gene it was necessary to identify the molecular nature of the alterations in carriers and patients. The cloning and sequencing of the mRNA from a patient revealed a consistent difference from the normal gene in the form of a 3 bp deletion resulting in the loss of a phenylalanine amino acid.²⁶ Subsequently this deletion has been found in about 75% of carriers and patients, effectively proving that the CF gene had been correctly identified.

An important consequence of the cloning of the CF gene is that carrier detection is now possible and couples at risk of having affected children can be informed of their status. A further important consequence is that an understanding of the nature of the protein involved is likely to lead to better

approaches to treatment, including drug design and gene therapy.

Molecular analysis of Huntington disease

Huntingdon disease (HD) is an autosomal dominant disorder which affects approximately 1 in 20,000 individuals. A particular feature is its late onset, starting at an average age of about 37. It was anticipated that it should be possible to establish linkage using DNA probes for RFLPs although it was expected that many probes would have to be tested. By chance, early in this analysis, a probe called G8 was shown to be tightly linked to the Huntingdon gene.²⁸ This linkage analysis was greatly aided by the availability of a large Venezuelan pedigree in which Huntington disease was common. It was possible to estimate that the G8 marker was about 3 cM from the HD gene. Furthermore, *in situ* hybridization has placed the G8 marker at the tip of the short arm of chromosome 4.²⁹ Other markers have subsequently been derived and been shown to lie even closer to the HD gene. These markers have thus defined the area of the genome in which the HD gene must reside and candidate genes have to be identified and examined for the HD lesion.

Although the disease gene has not yet been identified, it is possible to use RFLP markers to determine the likely status of an individual within a family in which Huntington disease is occurring.³⁰ This analysis is only possible if other family members are available, so that the affected chromosome can be identified.

Molecular diagnostics of cancer

In malignancy, some chromosomal changes seem to be highly correlated with particular tumours, whilst others are more general in their incidence. An individual malignancy can often have more than one alteration, with secondary changes superimposed on an original primary event. Many chromosomal translocations, in which genetic material is exchanged between chromosomes, have been documented. Other types of chromosomal alterations can include interstitial deletions, monosomy, trisomy, aneuploidy and the appearance of chromosomes so rearranged as to be unrecognizable.

Although karyotype analysis can yield important information about the disease state there are important limitations. A single chromosome band can be reckoned to consist of about 10^7 base pairs and therefore any alteration which involves less DNA than this may be difficult to observe. Additional problems when dealing with tumour tissue can include a low yield of mitoses and poorly

banded chromosomes. Usually a minimum of 20 metaphases will be examined with a higher number being required in difficult cases. A particular advantage of karyotype analysis over Southern analysis is that the whole cell is observed and that multiple events can be documented in a single analysis.

Detection of chromosomal translocations by Southern analysis

In Southern analysis³¹ genomic DNA is first digested with a restriction enzyme and size fractionated by electrophoresis on an agarose gel. After transfer to a nylon membrane the DNA is usually probed with a radiolabelled DNA fragment thus revealing the pattern of restriction sites on the corresponding genomic DNA sequence. Under standard conditions of agarose gel electrophoresis DNA fragments in the range 10³ to 20 × 10³ base pairs can be resolved. A reciprocal translocation results in 2 new junction regions with an abnormal pattern of restriction enzyme sites. Hence if a DNA probe corresponds to a sequence close to such a junction. Southern analysis of the genomic DNA will reveal an abnormal hybridization pattern. In principle a suitable DNA probe can be used to detect translocation in tumour cells for which karyotype data is lacking. This approach can only be successful if the breakpoints are known to be clustered within a limited range. Some breakpoints have been shown to occur over a wide range (100 kb) and it would therefore be difficult to use a single probe to detect all such translocations.³² This problem can potentially be solved by using pulsed field gel electrophoresis^{33,34} to provide a much larger range of analysis (150–1000 kb). A further problem is that deletions are known to occur around junction regions^{35,36} and this could result in loss of sequence homologous to the probe and lack of detection of the rearranged allele. Provided such difficulties are taken into account, this approach to detection of translocation can be used to obtain information which cannot readily be acquired by conventional cytogenetics. In particular, a rearrangement can be detected without the need for dividing cells.

Using such approaches the involvement of certain genes in chromosomal translocations has been well established and these are listed in Table I. The translocation t(9;22) which generates the Philadelphia chromosome is one of the best studied at both the cytogenetic and molecular level.^{37–39} The majority of patients with chronic myeloid leukaemia (CML) have the characteristic Philadelphia translocation t(9;22) (q34;q11) in their leukaemic cells. The oncogene *c-abl* which is normally present on chromosome 9 is translocated to chromosome 22 where it comes into juxtaposition with the 5'

Table I The molecular analysis of chromosomal translocations has revealed the involvement of the above genes

Disease	Translocation	Genes	Reference
CML	t(9;22)	C-ABL/BCR	37, 38
AML	t(15;17)	PML/RAR	107
AML	t(6;9)	CAN/DEK	108
ALL	t(9;22)	C-ABL/BCR	39
Burkitt's lymphoma	t(8;14)	C-MYC/IGH	40
	t(2;8)	IGK/C-MYC	41
	t(8;22)	C-MYC/IGL	42
Follicular lymphoma	t(14;18)	IGH/BCL-2	43, 44
B-CLL	t(11;14)	BCL-1/IGH	45
B-CLL	t(14;19)	IGH/BCL-3	46
T-cell lymphoma	inv(14)	IGH/TCRA	47, 48
	t(7;9)	TCRB/?	49

C(A)ML = chronic (acute) myeloid leukaemia;
C(A)LL = chronic (acute) lymphatic leukaemia.

portion of a gene known as the *bcr* or *phl* gene, whose product has an unknown function in normal cells. The molecular consequence of this translocation is the transcription of chimaeric mRNA and the expression of a chimaeric *bcr-abl* protein with enhanced *in vitro* tyrosine kinase activity.³⁸ Although the breakpoints on chromosome 9 can occur over a 200 kb range the breakpoints on chromosome 22 are clustered within a 5 kb sequence rendering this translocation suitable for conventional Southern analysis. In some Philadelphia-positive acute lymphoblastic leukaemias (ALL) the break in the *bcr* gene has been shown to lie further 5' such that only the first exon of *bcr* is included in the chimaeric mRNA.³⁹

In B cell leukaemias and lymphomas the immunoglobulin genes have been found to be directly involved in certain chromosomal translocations (Table I). It was shown that the *c-myc* oncogene was translocated into the heavy chain locus⁴⁰ or, more rarely, into either of the light chain loci in Burkitt's lymphomas.^{41,42} More recently, the J_H region of the IGH locus has been shown to be involved in the t(14;18) translocation which is a common feature of follicular lymphoma. This has led to the identification of a gene on chromosome 18 (*bcl-2*) which is directly affected by the translocation.^{43,44} Similarly the t(11;14) and the t(14;19) found in B-cell CLL have been shown to involve the IGH locus and molecular analysis has led to the cloning of DNA from the breakpoint of the partner chromosomes.^{45,46} In T-cell lymphomas an analogous involvement of the T cell receptor genes has been demonstrated for both the chromosomal inversion inv(14) and the translocation t(7;9). The inversion inv(14) is thought^{47,48} to be a recombination between the TCRA and the IGH loci, whereas the t(7;9) involved a recombination

between the TCRB locus and a region on chromosome 9 which is close to, but does not involve, the oncogene *c-abl*.⁴⁹

PCR analysis

The polymerase chain reaction (PCR)⁴ has had a dramatic impact on the analysis of genetic disorders of all types. The molecular diagnostics of cancer, in particular, is undergoing a revolution in that relatively simple diagnostic techniques may now be applied to small tissue samples.⁵⁰ In this technique, repeated cycles of specifically primed DNA synthesis are used to amplify the target sequence up to one million-fold or greater. The great fidelity of this reaction means that the product DNA fragments are accurate representations of the original starting sequence. Another key feature of this approach is that the amplification starts only from the sites determined by synthetic oligonucleotide primers chosen by the user. Thus it is possible to target a short DNA fragment of several hundred base pairs long (from the complete human genome of 3×10^9 base pairs) and amplify it to almost complete purity. The amplified fragment can then be examined by a variety of means including a direct reading of its sequence.

PCR has been used for the examination of nucleotide sequence variations,^{51,52} chromosomal rearrangements,⁵³ for high efficiency cloning of genomic sequences,⁵⁴ for direct sequencing of mitochondrial⁵⁵ and genomic DNA⁵⁶ and for the detection of viral pathogens.⁵⁷

Detection of the *t*(14;18) chromosome translocation by PCR

The clustering within short regions of the majority of breakpoints on *bcl-2* facilitates the use of PCR for amplification and analysis of *t*(14;18) breakpoints. By contrast the variation in the breakpoints around *c-myc* in the *t*(18;14) make this translocation less suitable for PCR analysis. By positioning oligonucleotide primers on each chromosome adjacent to and oriented towards the expected breakpoint position it has been possible to amplify specifically only the junction sequences. Since amplification depends on both primers being present on a single DNA fragment only the recombinant fragment can be amplified. Thus DNA from normal cells is not able to act as a template for this reaction. *Bcl-2* oligonucleotide primers (either mbr or mcr) flanking the translocation have been used, with a consensus *J_H* sequence found at the 3' end of each *J_H* exon, to amplify the 14q+ junctions.^{53,58-61} This approach has been extended to the 18q- junctions using a primer based on part of the recombination signal sequences known to flank germline *D_H* sequences.⁶¹

It has thus been possible to amplify either the 14q+ or 18q- junctions directly from tumour biopsies, marrow samples or peripheral blood. Since normal cells make no contribution to this reaction, the PCR can be used as a very sensitive test for the presence of cells carrying the *t*(14;18) translocation. Control experiments indicate that this approach can detect one tumour cell in 10^5 normal cells. The very sensitivity of this assay, however, requires that great care must be taken to avoid contamination with other samples. There is sufficient variability in the *bcl-2* breakpoint, the putative N regions and the involvement of the *J_H* gene to render each recombinant fragment essentially unique. Thus the problem of contamination can be catered for by sequence analysis of the PCR products. This approach avoids the cloning of PCR products and means that a junctional sequence can be read within a few days of receiving a tumour biopsy. The variability is useful in that the junctional sequences act as unique clonal markers for each follicular lymphoma. In a recent study⁶² of patients in long-term remission from follicular lymphoma this approach was used to examine peripheral blood for the presence of residual lymphoma cells. A proportion of patients with no overt signs of disease were found to have a low percentage of circulating lymphoma cells. Sequence analysis was used to demonstrate that the cells in the peripheral blood were derived from the original tumour mass cryopreserved years previously. The significance of low numbers of cells carrying the *t*(14;18) translocation in otherwise healthy patients remains uncertain, but has a parallel in the PCR studies of residual cells in patients in remission from B-ALL.⁶³

Detection of the *t*(9;22) translocation by PCR

The positions of the breakpoints in the *t*(9;22) translocation are less clustered than those of the *t*(14;18) and therefore analysis by PCR requires that the fusion mRNA is first used as a template for cDNA synthesis.⁶⁴⁻⁶⁷ There are 3 known possible *bcr* junctions with *abl* and therefore oligonucleotides for each *bcr-abl* combination have to be designed. An example of such an analysis is shown in Figure 1 for all 3 junctions. It has been variously estimated that one leukaemic cell per 10^3 non-leukaemic cells⁶⁴ or 10^6 non-leukaemic cells⁶⁷ can be readily detected by PCR amplification from mRNA. This approach lends itself to the monitoring of leukaemic cells following bone marrow transplantation⁶⁶ or after interferon treatment.⁶⁸ In both instances residual leukaemic cells were detected in samples from some patients. This approach has also been used to demonstrate that even chronic myeloid leukaemia without the translocation expresses the *bcr-abl* fusion transcript.⁶⁹

Figure 1 Detection of BCR-ABL mRNA in acute lymphoblastic leukaemias. A series of cell lines (lanes A, B, C and D) and patients' blood samples (lanes 1-7) have been analysed by PCR for the 3 possible *bcr-abl* junctions. The products are analysed on a 2% agarose gel.

The extreme sensitivity of this technique renders it susceptible to the problem of contamination especially in a laboratory where many such reactions are being performed. This problem can be resolved for the t(14;18) translocation by direct sequencing of the PCR products, since each translocation generates unique fusion sequence. This, however, is not possible for PCR products of the *bcr-abl* fusion amplified from the mRNA and therefore extra care needs to be taken in such experiments.

Analysis of clonality by PCR

Although many lymphomas do not have suitable chromosomal translocations on which to perform

PCR analysis, about 80% of B-cell malignancies carry only 1 or 2 immunoglobulin heavy-chain gene rearrangements indicating their clonal origin. The rearrangements of the heavy-chain gene segments during B-cell commitment result in a region called the complementarity-determining region III (CDR-III) which lies between the V_H and J_H regions. This region, which encompasses the diversity region of the heavy-chain segment, because of extensive somatic mutations, provides a DNA-encoded signature specific for each B-cell clone. Suitable V_H and J_H consensus primers flanking this region can be used to amplify by PCR CDR-III sequences from DNA of B-cell population.^{70,71} An analogous approach has been developed using the rearrangements to T-cell receptor genes to monitor

residual cells in T-cell leukaemias.^{72,73} In a recent study,⁶³ the sequences amplified from leukaemias were used to generate diagnostic probes that hybridized only to the amplified CDR-III of leukaemic cells from which the sequences were derived. With these probes, leukaemic cells could be detected when diluted 1:10,000 with other cells. By cloning the amplified CDR-III into recombinant libraries, residual leukemic cells were accurately quantified in bone-marrow samples from repeated relapses and remissions in one case of acute lymphoblastic leukaemia.⁷⁰ During a clinical remission lasting greater than 7 months, malignant cells were present in marrow at greater than 1 per 1,000 cells. This approach has been used for accurate quantification of malignant cells in acute lymphoblastic leukaemia patients in clinical remission⁶³ and will allow investigation of the biological significance of low or high numbers of residual leukaemic cells in evolution of that disease. In principle this approach could be used to generate unique clonal markers for any B-cell or T-cell malignancy for which there was not a suitable chromosomal translocation.

Detection of point mutations

In contrast to the disease specificity of the chromosomal translocations discussed above, about 10% of all human tumours are thought to have acquired mutations to members of the *ras* gene family. These changes have been found to occur at certain positions within the coding sequence, resulting in critical changes to the *ras* products. The 3 members of the *ras* gene family, *H-ras*, *K-ras* and *N-ras* map to chromosomes 11, 12 and 1 respectively. The homologous p21 proteins encoded by this family can bind guanine nucleotides, have intrinsic guanosine triphosphatase (GTPase) activity and are localized at the inner surface of the plasma membrane.⁷⁴ They are thought to have a role in the transduction of receptor-mediated external signals into the cell, although the precise biochemical pathway remains to be elucidated. The transforming potential of oncogenic versions of the *ras* genes has been shown to be due to single-base substitutions which alter the corresponding amino acid and result in reduced GTPase activity.^{75,76} These point mutations have been found in either codon 12, 13 or 61 of members of the *ras* gene family⁷⁷ in tumour cells and were not found in normal cells from the same patients. In contrast to some of the specific chromosomal rearrangements discussed above, mutations to *ras* genes have been found in a wide variety of human tumours with varying frequency. One of the highest incidences (25–50%) has been reported in acute myelocytic leukaemia (AML).⁷⁷

It is clear that although the majority of mutations in haemopoietic malignancies have occurred

in the *N-ras* gene, both *K-ras* and *H-ras* can be affected. Some of the mutations have been found in cell lines and therefore could have arisen in culture. However there are clear examples of leukaemias in which the mutation was present in the primary tumour material. The high frequency of activation of *N-ras* in AML has not been matched by a similar frequency in other myeloid or lymphoid malignancies.⁷⁸ For example, none of 14 chronic myeloid leukaemia (CML) blast crises were found to have mutated *ras* genes.⁷⁹ It is also apparent that there is no obvious correlation between *ras* mutation and either AML subtype (FAB classification) or karyotypic alteration. It is therefore difficult to establish the role of *ras* mutation in the origin and progression of these tumours. It is of interest that *N-ras* mutations have been demonstrated in 3 out of 8 patients with the myelodysplastic syndrome.⁸⁰ Since it is difficult to predict when this condition will evolve into overt leukaemia it would be important to show whether the presence of a *ras* mutation could predict a leukaemic transformation.

The detection of mutations using single-strand conformational polymorphisms (SSCP)

A mobility shift analysis of single-stranded DNAs on neutral polyacrylamide gel electrophoresis has been developed⁸¹ initially to detect DNA polymorphisms. This method involves digestion of genomic DNA with restriction endonucleases, denaturation in alkaline solution, and electrophoresis on a neutral polyacrylamide gel.

Mobility shifts caused by nucleotide substitutions can be readily observed and are thought to be due to conformational changes of single-stranded DNAs, that is, single-strand conformation polymorphisms (SSCPs). An adaptation has been described for amplifying individual alleles in a mixture of 2 or more alleles by the polymerase chain reaction (PCR) to determine their nucleotide sequence.⁸² This technique involves amplifying and separating target sequences by the PCR-mediated single-strand conformation polymorphism (PCR–SSCP) method, isolating each polymorphic DNA strand, and amplifying it by a second-stage PCR for its sequence determination. By this technique, the sequence of a minor constituent (approximately 3%) can be determined accurately.

This approach can be readily adapted for the detection of mutated *ras* genes in tumour samples. Of a total of 129 tumours analysed in a particular study,⁸³ 22 contained a mutated *ras* gene. Of 66 adenocarcinomas analysed, 14 contained an activated *c-Ki-ras2* gene, one contained a *c-Has-ras1* gene with a mutation in codon 61 and 3 contained *N-ras* genes with mutations (in codon 12 in 1 and in codon 61 in 2). Mutated *ras* genes were also found in 2 of 36 squamous cell carcinomas and 2 of 14

large cell carcinomas. No mutation of the *ras* gene was detected in 8 small cell carcinomas and 5 adenosquamous cell carcinomas.

Detection of tumour viruses by PCR

The human papilloma viruses (HPV-16 and HPV-18) have been reported to be present at a high frequency in invasive squamous cell cancers of the cervix and other genital cancers.⁸⁴ PCR assays have been used to detect levels of HPV sequences at less than one genome per cell.⁸⁵ This assay can be modified to distinguish between the various HPV subtypes, by first amplifying with consensus primers and then probing with oligonucleotides particular to each subtype. A variant of HPV-16 (designated HPV-16b) has recently been identified by PCR as having a 21 bp deletion.⁸⁶ Similar PCR-based assays have been developed for the detection of other viruses implicated in human cancer. They include the hepatitis B virus (hepatocarcinomas), Epstein-Barr virus and the human T-cell lymphotropic virus (T-cell lymphomas).⁸⁷⁻⁸⁹

Detection of gene amplification

Many types of human cancer have been reported to have amplified copies of particular genes. Two examples in which the amplification appears to correlate with a poor prognosis are the *N-myc* gene in neuroblastoma^{90,91} and the *c-erb-B-2* gene in breast cancer.⁹² In addition the *c-myc* gene has been found to be amplified in cell lines derived from small cell lung cancers⁹³ and in other cancers.^{94,95} The epidermal growth factor receptor gene has been found to be amplified in brain tumours of glial origin.⁹⁶

In most of these experiments Southern analysis has been used, with appropriate controls, to quantify gene copy number. The overexpression of a gene can result not only from gene amplification but from deregulation of an unamplified gene. In this case the use of Northern blotting with appropriate controls is necessary. PCR technology may offer new possibilities for the detection of both gene amplification and overexpression. A PCR-based assay was used to demonstrate the overexpression of thymidylate synthetase mRNA.⁹⁷ It is possible that similar assays could be developed for overexpression of other genes. Currently the detection of low levels of gene amplification by PCR remains questionable, due to the difficulties in performing quantitative PCR assays.

Detection of minimal residual disease (MRD)

Induction therapy in leukaemia and lymphoma is administered in order to obtain a complete remission. However, residual disease cells may still

remain and have the ability to regenerate a new tumour mass.⁹⁸ The detection of a marker such as the Philadelphia chromosome following allogeneic transplantation for CML does not necessarily herald relapse⁹⁹ but may be a transient marker representing the presence of residual tumour cells with limited capacity for division due to prior treatment. Possibly a failure of an immunological control mechanism or a second promotional event¹⁰⁰ may be necessary for further multiplication of the remaining residual tumour cells to occur. Investigation of patients with different stages of lymphoma or leukaemia using flow cytometric analysis for κ or λ light chain expression,⁹⁹ or gene rearrangement analysis suggests that clonal evidence of disease may be found in the absence of clinical or morphological findings.^{101,102} Studies of the peripheral blood of patients in long-term follow-up of malignant lymphoma using restriction fragment length polymorphisms or PCR for the t(14;18) translocation show persistent abnormalities in a proportion despite continuing clinical remission.^{53,103,104} If the abnormalities are present for many years without the evidence of recurrence, their clinical relevance must be queried.

Quantification of the disease remaining or determination of increased disease bulk at an early sub-clinical stage may help delineate those patients requiring further therapy due to early progression while the disease bulk remains small. Those with quiescent disease markers may require no further treatment until evidence of progression is observed.^{98,99} Southern analysis does allow relative quantification of the percentage of clonal tumour cells present, although the sensitivity is poor in comparison to PCR and may only precede overt clinical relapse by a short period of time.¹⁰¹ Adaptation of PCR to quantification¹⁰⁵ will improve the early detection of clonal proliferation of MRD. However, extreme care is essential with the PCR in the detection of MRD as the sensitivity of the techniques may allow contamination to contribute to false positive results. Direct sequencing of the PCR products will provide unique clonal markers down to the base sequence level for an individual tumour and help reduce the possibility of a false positive result.

Ultimately defining the gene defect and mechanism of disease may permit the use of therapy directly targeted at the molecular changes. Lymphoid malignancies particularly demonstrating translocations involving Ig or TCR genes such as the t(8;14) of Burkitt's lymphoma, t(14;18) of follicular lymphoma and t(8;14) of T-cell neoplasms where deregulation of gene transcription has been associated with malignant transformation may be amenable to treatment by 'gene therapy'. *In vitro* experiments with antisense oligodeoxynucleotides have successfully demonstrated the ability

to decrease *c-myc* protein levels and thus malignant proliferation in cell lines containing the abnormal *c-myc* transcripts while leaving the normal *c-myc* protein expression and cell growth unaltered in control normal cells.¹⁰⁶ If *in vivo* trials of this form of 'gene therapy' are successful it will offer great potential for possible curative treatment in an often bad prognostic group of patients, particularly those with MRD destined to relapse. It will become essential to define precisely at a molecular level the

disease-associated gene rearrangements if this form of treatment is to be considered.

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References

Introduction

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