

## REVIEW

# Methods of molecular analysis: assessing losses and gains in tumours

R Roylance

The study of chromosomal aberrations has facilitated the understanding of tumorigenesis. By applying molecular genetic techniques to regions highlighted by cytogenetic study, many genes important in tumorigenesis have been identified. This review will describe the cytogenetic and molecular cytogenetic techniques used to identify these changes. The clinical information that they can provide, including diagnostic and prognostic information, will also briefly be discussed.

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Historically, the first cytogenetic method available was the karyotypic analysis of tumours, which examines the number and size of chromosomes. In 1960, a small morphologically distinct chromosome was found in chronic myeloid leukaemia.<sup>1</sup> It was termed the Philadelphia chromosome, and was the first tumour specific chromosomal abnormality to be identified. This was heralded as a huge scientific breakthrough and it was anticipated that this would be the first of many such specific chromosomal aberrations. However, technical difficulties meant that no other consistent chromosomal changes were found for over a decade. The introduction of chromosomal banding in 1970 revolutionised the field of cancer cytogenetics because it meant that chromosomes could be identified precisely and reliably. Rapid progress was made in revealing consistent chromosomal aberrations in leukaemias, lymphomas, and sarcomas. The Philadelphia chromosome was shown to be derived not just from chromosome 22 material but to result from a translocation,<sup>2</sup> although it would take another decade before it was found to be a reciprocal translocation t(9;22)(q34;q11), with the genes at the breakpoint identified and the molecular consequences of the translocation established. Other examples of tumour specific translocations include the following: t(15;17)(q22;q21), found in acute promyelocytic leukaemia, subtype M3; t(11;22)(q24;q12), found in Ewing's sarcoma; and t(2;13)(q35–37;q14), found in alveolar rhabdomyosarcoma. The translocations found in small round cell tumours of childhood, of which Ewing's sarcoma is an example, have proved extremely useful diagnostically. Morphologically, these tumours are often almost identical, but can now be distinguished on the basis of their specific genetic aberrations.

However, despite rapid expansion in knowledge regarding haematological malignancies there has remained a relative paucity of data regarding carcinomas. This reflects the difficulty in obtaining chromosomes from solid tumours and the complexity of the changes found. Chromosomes are obtained from cells either by direct preparation or by short term culture. Direct preparations, made shortly after removal of the tumour, although thought to be representative of the *in vivo* situation, are of limited usefulness because they contain only a few metaphases, which tend to be poor in quality. There is a further selection bias in that only those cells about to divide immediately before removal of the tumour are subject to analysis. Short term culture yields more metaphases that are of higher quality than direct preparations. However, it too has limitations, principally the difficulty of identifying those cells that should be studied; that is, which cells are from the surrounding benign stroma and which are tumour cells. Furthermore, after short term culture karyotypes of carcinomas are often diploid. This may be because changes are below the resolution limits of karyotyping, with any changes present being submicroscopic.<sup>3</sup> Alternatively, there may be outgrowth of normal cells or selection *in vitro* against aneuploid cells. Evidence for the latter has come from studies where fluorescence *in situ* hybridisation (FISH) performed on direct preparations revealed clonal aberrations and aneuploidy, which were lost following culture.<sup>4,5</sup> Another difficulty with analysing metaphases from solid tumours is that the karyotypes are much more complex, with many more rearrangements and marker chromosomes. Furthermore, DNA amplification is often seen. Cytogenetically, it appears as one of two forms; either as double minutes—small, spherical usually paired chromosome-like structures that lack a centromere and may contain circular DNA in chromatin form<sup>6</sup>—or, alternatively, it may appear within chromosomes as homogeneously staining regions. These stain with intermediate intensity throughout their length, rather than with the normal light and dark banding pattern seen in trypsin-Giemsa stained preparations. However, from karyotypic analysis there is no way of identifying where the amplified DNA has originated from.

Further advances in identifying genetic changes in tumorigenesis have resulted from the introduction of molecular cytogenetic techniques. FISH

Correspondence to:  
R Roylance, Molecular and  
Population Genetics  
Laboratory, Imperial  
Cancer Research Fund,  
Lincoln's Inn Fields, London  
WC2A 3PX, UK;  
r.roylance@icrf.icnet.uk

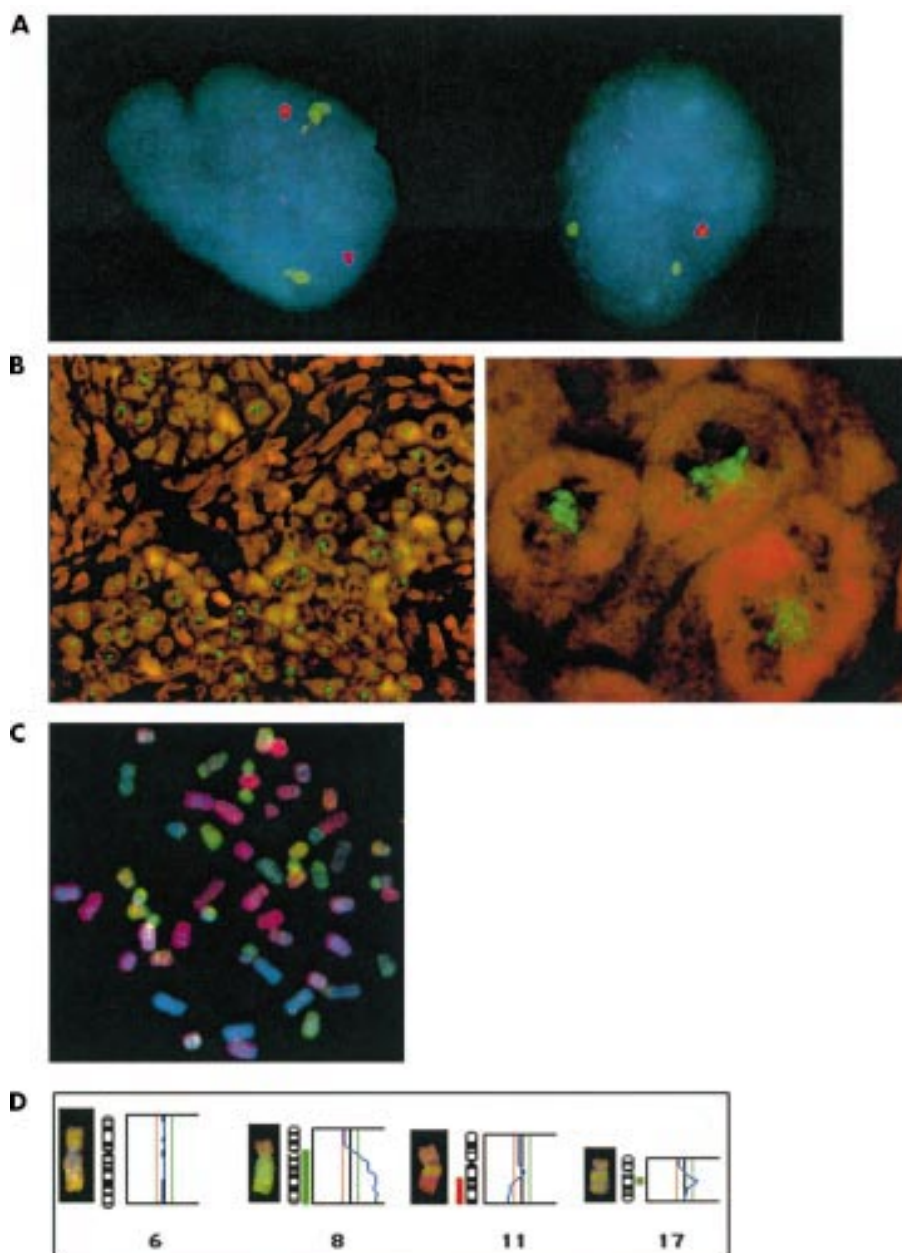
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"The introduction of chromosomal banding in 1970 revolutionised the field of cancer cytogenetics"

**Abbreviations:** CGH, comparative genomic hybridisation; FISH, fluorescence *in situ* hybridisation

uses fluorescently labelled probes for the visualisation of DNA sequences on metaphase spreads or interphase nuclei (fig 1A, B). Both numerical and structural aberrations can be determined. Probes can be for the whole chromosome, centromere, or locus specific. Interphase nuclei can be obtained from a range of clinical specimens including touch preparations, fine needle aspirates, bone marrow smears, and archival material. FISH has proved useful in several clinical settings to determine prognosis. For example, in neuroblastomas, several chromosomal changes

have been identified that correlate with a poor prognosis, namely: MYCN amplification, loss of 1p36, gain of 17q, and the absence of aneuploidy.<sup>7</sup> Patients with breast cancer with ERBB2 overexpressing tumours have a lower overall survival rate and shorter time to relapse compared with patients whose tumours do not overexpress this protein.<sup>8</sup> The assessment of overexpressing tumours is clinically relevant because targeted treatment using a recombinant humanised monoclonal antibody directed against HER2/neu, Herceptin, is now available.<sup>9</sup>



**Figure 1** (A) Two prostate nuclei isolated from frozen tumour material, counterstained with DAPI. The nucleus on the left is normal with two copies of the 10 centromere (green) and two copies of PTEN (red); the nucleus on the right shows loss of PTEN, with retention of the centromere. (B) Fluorescence in situ hybridisation (FISH) analysis with ERBB2 (green) on a tissue section of paraffin wax embedded grade III invasive ductal breast carcinoma. The nuclei are counterstained with propidium iodide. The left hand panel shows the section at low power and the tissue architecture is clearly shown with only some cells demonstrating amplification of ERBB2. The panel on the right shows nuclei from the left panel at higher magnification; high level amplification of ERBB2 can be clearly seen appearing as a "cluster". (C) This is a karyotype of a colorectal cell line, labelled using multicolour FISH. (D) This panel shows representative comparative genomic hybridisation results from an invasive ductal breast carcinoma. The chromosomes are shown to the left of the chromosomal ideograms. The profiles represent the composite results from the analysis of 10 metaphase spreads. The central blue line represents a fluorescence ratio profile of 1.0. The green line to the right and the red line to the left of this central line represent gains (ratios greater than 1.15) and losses (ratios less than 0.85), respectively. Chromosome 6 shows neither gains nor losses, and has a fluorescence ratio of 1.0. Chromosome 8 shows an excessively green long arm, which is confirmed as gain by the fluorescence ratio; similarly, chromosome 17 shows discrete amplification of the band 17q12. Chromosome 11 demonstrates loss of the long arm.

Because FISH can be performed on tissue sections it is particularly useful for assessing the amount of genetic heterogeneity within tumours and for establishing genotype–phenotype correlations (fig 1B). However, studying multiple genomic regions is time consuming. A recent advance is the use of tissue arrays to screen a large number of tumours rapidly.<sup>10</sup> Each array is constructed in a paraffin wax block from which ~ 200 sections can be obtained. Each section contains 1000 tumour samples, which are 0.6 mm in diameter and 0.1 mm apart. Multiple sections of the array provide targets for parallel in situ detection of DNA, RNA, and protein in each specimen. The advantages of this technique are that multiple samples can be analysed very rapidly, the importance of novel genes can be confirmed, and it is possible to correlate genetic findings with clinical information. However, at present the use of FISH on tissue arrays is confined to assessing increased copy number or amplification; it is not possible to assess the loss of genetic material. There is also a debate over how representative 0.6 mm diameter sections are of an overall tumour.

“Fluorescence in situ hybridisation has proved useful in several clinical settings to determine prognosis”

Multicolour FISH (M-FISH) or spectral karyotyping (SKY) are similar techniques, which differ only in their analysis procedure.<sup>11–12</sup> By using a combination of fluorochromes both techniques simultaneously label all the chromosomes in different colours, therefore enabling visualisation of every chromosome in a single experiment (fig 1C). Cryptic rearrangements, marker chromosomes, and double minutes are easily identified, which would not have been possible using simple karyotypic analysis alone, although small deletions and intrachromosomal rearrangements will still go undetected. However, although useful in the analysis of haematological malignancies and sarcomas, the biggest limitation of these techniques for the analysis of carcinomas remains that of obtaining chromosome preparations.

Comparative genomic hybridisation (CGH) is a molecular cytogenetic technique that is particularly useful in the study of solid tumours (fig 1D).<sup>13–14</sup> Only tumour DNA is required and DNA from archival material can be used. By using an adaptation to the standard protocol CGH can be performed using very small amounts (ng) of DNA. The entire genome is screened for gains and losses of genetic material in a single experiment, and the method is essentially a modified in situ hybridisation. Differentially labelled test (green) and reference (red) DNA are co-hybridised to normal metaphase spreads. Gains of genetic material in the test DNA are seen as an increase in the green : red fluorescence ratio and losses are seen as a decrease in the green : red fluorescence ratio. Fluorescence ratios are measured using digital image analysis. There is a limit to the resolution of this technique; losses are detectable when the region affected exceeds 10 Mb<sup>15</sup>—smaller regions of gain are detected if there is high level amplification—for example, a 2 Mb region that is amplified five times will be visualised.<sup>16</sup> Balanced rearrangements cannot be detected. Once regions of gain or loss have been identified, these regions can be defined further using FISH or molecular genetic techniques.

There are now many established examples of the usefulness of CGH in identifying genetic regions worthy of further investigation. Positional cloning of regions found amplified in breast cancer has led to the identification of STK15<sup>17</sup> and AIB1<sup>18</sup> on the long arm of chromosome 20, and PS6K on the long arm of chromosome 17.<sup>19</sup> In other malignancies, the amplification target gene has been identified based on candidate genes previously cloned and localised to the region of gain indicated by CGH—for example, the telomerase gene on 3q26 in cervical and other cancers<sup>20</sup> and the androgen receptor gene on Xq11–q12 in hormone refractory prostate

## Take home messages

- Fluorescence in situ hybridisation (FISH) has proved useful in determining prognosis in several diseases (for example, in neuroblastomas and breast cancer)
- A recent advance in FISH has been the use of tissue arrays to screen a large number of tumours rapidly
- Comparative genomic hybridisation (CGH) has been particularly useful in the study of solid tumours (such as breast and cervical cancer) and has identified a tumour suppressor gene in Peutz-Jeghers disease (LKB1)
- In the future these techniques will probably be used increasingly in the clinical setting

carcinoma.<sup>21</sup> To date, only one tumour suppressor gene has been identified as a consequence of CGH analysis. By analysing polyps from patients with Peutz-Jeghers disease, Hemminki and colleagues<sup>22</sup> found loss of 19p by CGH. Linkage analysis also confirmed the presence of a susceptibility marker on 19p. Subsequent mutational analysis found truncating germline mutations in a known but previously unmapped gene, LKB1, a serine/threonine protein kinase, in several patients with Peutz-Jeghers disease.<sup>23</sup>

Although some of these techniques already have clinical applications, some remain in the research setting. It can be anticipated that with advances both in our knowledge of the genetic profiling of tumours and the advances in array technology, these techniques will increasingly be used in the clinical setting.

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