Techniques in molecular biology

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Various molecular abnormalities have been identified which correlate with the grade or stage of bone malignancies and in some cases to their morphological type. The situation is dissimilar to that of the soft tissue tumours, where so many of the tumour types are associated with unique translocations that generate unique and distinct fusion transcripts (Weiss and Brooks 1996).

Cytogenetics can provide the molecular biologist with a large scale map of the genome to "home in" on areas of interest in a particular tumour. The cytogenetic analysis of bone tumours is still incomplete due to the scarcity of these tumours, the technical problems associated with harvesting and culturing the cells, and the difficulties associated with diagnosis and classification of bone tumours.

The classification of bone tumours is almost entirely morphologically based. As such, any particular morphological class of tumour may contain a number of subgroups based on their molecular abnormalities, or that two morphologically related but separate classes may share common molecular abnormalities. For example PNET and Ewings tumours share a common genetic abnormality (namely the EWS-FLI1 fusion transcript or one of its variants), but are morphologically distinct. These factors make it difficult to generalise about the molecular features of any particular bone tumour group, when that group may be molecularly heterogeneous. Nevertheless the molecular analysis of bone tumours is essential if we are to improve our understanding and management of these disorders.

Genetic alterations in bone tumours

A small number of oncogenes and tumour suppressor genes have been assessed for abnormalities in bone tumours. This has been largely confined to the genes commonly mutated in a wide range of malignancies. A brief summary of the findings of these analyses is given below.

Oncogenes

ras: Mutations or rearrangements affecting C-HA-RAS have not been frequently encountered in either malignant bone or cartilagenous tumours (Castresana et al. 1992; Barrios et al. 1993; Ozaki et al. 1993; Dahir et al. 1994; Antillon-Klussmann et al. 1995).

myc: The amplification of C-MYC has been identified in a variety of bone and soft tissue tumours. It is associated with high grade tumours with a worse prognosis, but it is unclear whether the amplification of C-MYC is an independent prognosticator (Castresana et al. 1992; Barrios et al. 1993; Ladanyi et al. 1993; Ozaki et al. 1993; Isfort et al. 1995).

Mdr: Overexpression of the human multidrug resistance (MDR) gene correlates with poor prognosis in osteosarcoma. This correlation with poor prognosis may be due to the association of the expression of this gene with chemotherapeutic drug resistance of the tumour (Nagata et al. 1992; Imanishi et al. 1994).

mdm2: Overexpression of the MDM2 gene has been detected in a small proportion of osteosarcomas. It is more frequently overexpressed in recurrent or metastatic tumours than in primary osteosarcomas. Overexpression of the product of the MDM2 gene is believed to act by binding to and reducing the activity of p53 protein (Ladanyi et al. 1993).

Tumour suppressor genes

TP53: The association of TP53 mutations with osteosarcoma (and other sarcomas) is best seen in the Li Fraumeni syndrome (Srivastava et al. 1990). This inherited syndrome is associated with an inherited heterozygous mutation in the TP53 gene and results in the predisposition of the affected individuals to the development of various sarcomas (including osteosarcomas) and breast carcinoma. In addition to TP53 abnormalities present in osteosarcomas which occur in the context of familial syndromes, mutations and abnormalities of expression of TP53 have been described in between 21 and 37% of sporadic osteosarcomas (Porter et al. 1992; Scholz et al. 1992; Toguchida et al. 1992; Smith-Sorensen et al. 1993; Wadayama et al. 1993). Loss of heterozygosity affecting the TP53 region (17p13) is present in up to 75% of sporadic osteosarcomas (Toguchida et al. 1992; Yamaguchi et al. 1992; Hoogerwerf et al. 1994; Grundmann et al. 1995).

Molecular biological techniques for analysis of bone tumours

Detection methods	Applications				
	Chromosome copy number	Gene amplification	Chromosome translocation	Gene point mutation	Gene expression
in situ					
FISH	+	+	+	–	–
PRINS	+	+	+	–	–
in situ RT-PCR	–	–	+	+/–	+/–
in vitro					
Southern Blot	–	+	+	-	-
PCR	+/–	+	+	+/-	-
RT-PCR	–	–	+	+/-	+/–
Mutational analysis	–	–	–	+	–

FISH = fluorescence in situ hybridisation; PRINS = primed in situ labelling; in situ RT-PCR = in situ reverse transcriptase polymerase chain reaction; PCR = polymerase chain reaction; RT-PCR = reverse transcriptase polymerase chain reaction.
+ and – indicate applicability and lack thereof, respectively; +/– indicates that a method has potential application but has been little tested or that the method is less straightforward than others.
Table adapted from Weiss and Brooks (1996).

Rb: Similar to TP53, the association between the presence of inherited mutations affecting the RB gene and predisposition to the development of osteosarcoma is best seen in the inherited retinoblastoma syndrome (Draper et al. 1986; Benedict et al. 1988, Issing et al. 1993). In the retinoblastoma syndrome, the affected individuals inherit a mutation of one RB allele, and are subsequently predisposed not only to the development of retinoblastoma but also to osteosarcoma. Loss of heterozygosity affecting the RB locus (chromosome 13q14) has been identified in approximately two thirds of sporadic osteosarcomas (Araki et al. 1991; Hansen 1991; Scholz et al. 1992; Yamaguchi et al. 1992; Issing et al. 1993; Ozaki et al. 1993; Ozisik et al. 1993; Wadayama et al. 1994).

Inherent technical difficulties

Bone tumours are difficult tumours to study from a molecular viewpoint. Often the tissue is calcified and requires treatment with acid to remove the calcium before the tissue can be sectioned. This often destroys nucleic acids, making analysis of these molecules impossible. Avoiding decalcification procedures makes sectioning with preservation of histological structure virtually impossible in many tumours. Many of the in vitro techniques listed below, although made more difficult by the hard nature of the tissue, are generally technically feasible even in calcified tissue.

In situ techniques

Many of the recent molecular biological techniques have been developed for in situ use. This reflects their development largely as adjuncts to regular cytogenetic analysis. These origins also account for their largely restricted use on metaphase spreads or bare nuclear preparations, although developments which extend their use to routine histological sections is underway.

FISH

Principles and use: Fluorescence In Situ Hybridisation (FISH) is a technique that obtains specific cytogenetic information from either metaphase or interphase cells. The technique utilises the principles of nucleic acid in situ hybridisation (Gall and Pardue 1969; John et al. 1969), in that a specific nucleic acid sequence is labelled and then hybridised to the denatured DNA of the chromosomes, in order to identify and localise a specific sequence within a chromosome (Cremer et al. 1986; Cremer et al. 1988; Devilee et al. 1988; Hopman et al. 1989).

The technique has been used to identify human chromosomes in somatic cell hybrids (Pinkel et al. 1986; Collins et al. 1991), to detect numerical and structural chromosomal abnormalities (Selleri et al. 1991), to map genes to specific chromosomal locations (Trask 1991), and to detect specific gene amplification abnormalities (Shapiro et al. 1993; Misra et al. 1995).

FISH can be carried out using either metaphase or interphase chromosome preparations. Generally either routine cytogenetic preparations of metaphase chromosomes or spreads of bare interphase nuclei have been used for FISH analysis.

Strengths: FISH allows the identification of specific numerical or structural chromosomal abnormalities without the absolute requirement for cultured cells and metaphase chromosome spreads. This represents

a distinct advantage over standard cytogenetic analysis and means that the technique is possible even when only routine histologically processed material is available.

Because FISH can be undertaken even on sections processed for routine histological examination, the histology is preserved and available for analysis and correlation with the results of the FISH analysis. This is not possible with routine cytogenetics, where the metaphase spreads examined could represent stromal cells rather than tumour cells, for example.

FISH analysis can be undertaken overnight with a result available the following day. This again contrasts with routine cytogenetics, which often requires several days to generate a result.

FISH is more sensitive than routine cytogenetic analysis in the detection of specific abnormalities, in that for cytogenetic detection the abnormality has to be large enough to cause a change in the metaphase chromosome morphology which is visible in a G-banded preparation, whereas the resolution of FISH can be as high as 40kb.

Weaknesses: The nature of FISH analysis means that only a "yes or no" answer to a clearly defined question can be generated. On the other hand, cytogenetics can detect most rearrangements, even those not previously characterised.

FISH analysis requires specific DNA probes, usually cosmid or YAC probes. Many of these probes are not commercially available and so may be difficult to obtain.

In the case of chromosomal rearrangements, several examples exist where the breakpoint may be quite variable. If this variation exceeds the size of the probes used, then the abnormality may not be detected.

PRINS

Principles and use: Primed in situ labelling (PRINS) was developed as a rapid alternative to FISH (Koch et al. 1989). The technique is based upon the annealing of short oligonucleotide sequences to the target DNA in the chromosome. These oligonucleotides can then function as primers for chain elongation using labelled nucleotides, a DNA polymerase such as Taq, and the target DNA as a template. The annealing and extension steps may be repeated several times with intervening denaturing steps (cycling PRINS), to amplify the signal produced.

Strengths: PRINS offers an alternative method to FISH and has several advantages:

- the technique is rapid (approximately 2 hours as opposed to overnight),
- cycling PRINS offers increased sensitivity over FISH,
- the use of an unlabelled primer reduces background non-specific signal due to binding to structures in the cell which are not nucleic acids. Binding of the primer to these structures would not result in labelled chain-lengthening, which requires a nucleic acid template.

Weaknesses: PRINS has not been well established for the detection of single copy sequences within the genome. Its major application to date has been in the analysis of repeat sequences.

Similar to FISH, the use of PRINS on metaphase spreads and cell suspensions or bare nuclei preparations is quite well established. The use of PRINS on formalin-fixed, paraffin-embedded histological sections is not well established.

In situ RT-PCR

Principles and use: In situ gene amplification is related to the in situ hybridisation technique, but with the advantage of increased sensitivity. This increase in sensitivity is achieved by the amplification of specific sequences within the cell prior to hybridisation with a labelled probe. In common with conventional PCR, specific oligonucleotide primers and a DNA thermostable polymerase such as Taq are used to amplify the target DNA, using cycled denaturing, annealing, and extension steps (Komminoth and Long 1993).

Strengths: The major advantage of in situ RT-PCR over conventional in situ mRNA hybridisation is greatly increased sensitivity.

Another advantage when dealing with a specific fusion transcript resulting from a chromosomal translocation, is specificity. If the primers used for the PCR straddle the breakpoint, then a product will be produced. If no fusion transcript is present, then no product is produced.

Weaknesses: The technique of in situ RT-PCR is relatively new and has not been routinely applied to diagnostics, except for the detection of repetitive viral sequences. Good protocols for the diagnostic use of the technique on routinely processed histological sections do not yet exist.

A significant problem associated with in situ RT-PCR, is that the amplification products tend to diffuse away from the target cell, resulting in nonspecific signal over adjacent cells. No satisfactory solution to this problem is yet available.

In situ RT-PCR is a multistep process, where each of the major steps requires optimisation for each particular application. Failure of optimisation at any of these steps can result in either obscuration of the signal by unacceptable background staining or the loss of signal all together.

In vitro techniques

The in vitro molecular biological techniques have largely been developed by molecular biologists rather than cytogeneticists. They require extraction of the nucleic acids from the tissue prior to analysis, which makes correlation between a tumour's molecular abnormalities and its histological features difficult.

Southern Blot

Principles and use: In the technique of Southern blotting, extracted DNA is digested with one or more restriction enzymes, separated according to size and hybridised with specific, labelled nucleic acid probes. The pattern produced is compared between individuals. A large structural abnormality, or a mutation affecting a specific restriction enzyme recognition site, will result in the production of a variant pattern for that individual, when compared to control DNA (Southern 1975, Stellwag and Dahlberg 1980). Amplification of a particular segment of DNA will result in an increase in the intensity of the bands which correspond to the affected region of DNA. This change can be measured to give an assessment of the degree of amplification which has occurred. Similarly the loss of genetic material, for example the loss of one allele, will result in a reduction of the intensity of the band(s) corresponding to that region of DNA.

Strengths: Quantitation is both possible and straightforward. The ability to readily obtain quantitative data is particularly valuable when examining for gene amplification.

A single Southern blot can be repetitively probed with different DNA sequences. This means that several possible abnormalities can be examined using a single blot. In addition, archival blots can be re-examined for abnormalities that may be discovered to be important in the future.

Relatively large abnormalities can be examined, such as large structural abnormalities affecting genes or chromosomal regions. The actual nucleic sequence of the area to be examined need not be known in detail prior to the test, nor does the nature of the abnormality need to be known (i.e. Southern blot does not necessarily provide simply a "yes or no" answer).

Weaknesses: Southern blot requires a large quantity of good quality DNA. In effect this means that fresh or frozen tissue is required, in quantities of at least 10-100mg. Southern blot is not practically possible using routinely processed histological material.

Southern blot will not reveal the exact location (or usually the exact nature) of the abnormality detected. The method involves the digestion of the DNA into a range of discrete fragments, the size of which determines the resolution of the technique. Further analysis using other methods is usually required to elucidate the exact cause of the detected change in fragment size.

The process of Southern blot analysis takes three to four days to complete. In contrast to Southern blot, PCR may take a few hours to obtain a result.

PCR

Principles and use: The polymerase chain reaction (PCR) is a technique devised by Mullis and Faloona (1987) to amplify a specific segment of DNA. The method involves the annealing of two oligonucleotide primers to opposite strands at either end of a particular DNA region, and the subsequent extension of the primers by a thermostable DNA polymerase such as Taq. The process is repeated a number of times using repetitive steps of denaturation, annealing and chain elongation. The primer extension products function as a template for the next cycle, allowing exponential amplification of the fragment. The amplification of specific DNA fragments using this technology allows DNA analysis on very small quantities of tissue. This makes possible the length analysis of microsatellite markers from individual alleles, sequence or mutational analysis on a small quantity of DNA from an individual or tumour, and even the identification of individuals from tiny quantities of forensic material (Erlich 1989).

Strengths: PCR on DNA extracted from either fresh, frozen or fixed and embedded tissue provides a straightforward method for the analysis of microsatellites (for loss of heterozygosity (LOH) studies), or to amplify DNA for subsequent mutation analysis.

When combined with microdissection techniques, correlation with histology is possible.

Several PCR reactions may be undertaken on a sample of DNA at the same time (multiplexing), allowing many "yes or no" questions to be answered at once.

Weaknesses: PCR does not examine the entire genome. The technique, similar to others described above, will in general allow only a "yes or no" answer to a defined question.

PCR using specific primer pairs can only amplify quite a small length of DNA (up to about 12kb using a good quality DNA template). The presence of introns means, in some cases, that RT-PCR must be used for the amplification of the sequence of a transcribed gene.

RT-PCR

Principles and use: Reverse transcriptase polymerase chain reaction (RT-PCR) is a modification of PCR

which allows the amplification and subsequent analysis of mRNA transcripts (Dicker et al. 1989). The method involves an initial step where the mRNA transcript is used as a template for the generation of cDNA by reverse transcriptase. The cDNA is then used as a template for conventional PCR as described above. The technique is useful in the detection of fusion transcripts generated by chromosomal translocations. It is also useful in the analysis of transcribed genes where either the genomic structure (i.e., the positions of the introns in the sequence) is not known, or where the presence of alternative transcripts or other RNA-specific features make the examination of the transcript desirable, rather than the DNA.

Strengths: The technique is well established, rapid and relatively straightforward on fresh or frozen tissue.

RT-PCR is specific and highly sensitive, in the detection of well-characterised abnormalities.

When combined with microdissection techniques, correlation with histology is possible.

Weaknesses: The technique of RT-PCR is not well developed in paraffin embedded-routine histological material. In this material, RNA integrity is such that RT-PCR can be used to reliably generate fragments of up to approximately 600bp. This is because RT-PCR is dependent upon the availability of good quality, intact RNA. Because of these limits, only a small degree of variability is required in the position of the translocation breakpoint or other abnormality to render it undetectable by this technique. In addition, the moderate degradation of the transcripts in the paraffin-embedded tissue frequently means that nested PCR is required to actually detect an abnormal transcript. The extreme sensitivity of nested PCR raises the probability of contamination to levels that may be unacceptable for a diagnostic procedure.

RNA is inherently unstable in its nature. The analysis of DNA (because of its robust nature) is always easier and thus preferable to using RNA as a starting material.

Mutational analysis

Principles and use: Multiple methods are available for the analysis of mutations in DNA either directly extracted from tumour or other tissue or amplified using PCR technology. The "gold standard" of these methods remains sequencing of the DNA (or cDNA) (Sanger et al. 1977). However, sequencing is labour intensive and slow, so other techniques have been developed (and continue to be developed) which exploit the change in physical properties resulting from a change in sequence on either one DNA strand (resulting in a mismatch) or on both strands (when the affected fragment must be compared to a control fragment). Some examples of the latter techniques include single-strand conformation polymorphism (SSCP) (Orita et al. 1989), chemical cleavage mismatch (CCM) (Cotton et al. 1988), and constant denaturing gradient electrophoresis (CDGE) (Myers et al. 1987). The protein truncation test (PTT) allows the detection of nonsense or frameshift mutations which result in the generation of a stop codon thus effectively truncating the mutant protein (Roest et al. 1993, Hogervorst et al. 1995).

Strengths: Mutational analysis on DNA extracted from normal tissue from individuals whose family history suggests that they are at high risk of developing a particular malignancy or group of malignancies, allows for the screening of individuals or populations for inherited mutations that predispose to the development of malignancy. This allows those individuals who are found to carry one of these defects to be identified within a high risk group for close follow-up. It also allows the study of environmental influences in the variability of phenotypic expression of a particular mutation, resulting in greater understanding of the disease process.

Weaknesses: The study of mutations affecting specific genes in bone tumours is not well advanced. Even the characterisation of cytogenetics and even morphological diagnostics is difficult and not without controversy. Only a small number of inherited bone tumours (such as retinoblastoma-associated osteosarcoma and Li-Fraumeni syndrome) have been identified to date. These syndromes probably comprise only a tiny proportion of bone tumours diagnosed. So far, then, mutational analysis in bone tumours has largely been confined to the well known oncogenes (such as ras) and tumour suppressor genes (such as TP53).

Conclusion

The molecular biology of bone tumours is in its infancy. The complexity of the karyotypic profile of several of the malignant bone tumours is in sharp contrast to the well defined and characteristic translocations seen in many of the soft tissue tumours. A great asset to the identification of important molecular biological events in various bone tumours will be the identification of specific cytogenetic abnormalities which are common to one specific type or group of tumours.

A major problem is the difficulty in confidently diagnosing and categorising many bone tumours on the basis of their morphological appearance. Often this process requires a panel of experienced practitioners who even then may not agree. This problem of mor-

phologically categorising bone tumours, which makes the study of their molecular biology more difficult, may be assisted by the discovery of characteristic molecular features.

Several molecular biological tools have been devised which may be of value in the examination of these diseases. Many of these have only appeared in the last few years and their reliability and usefulness in diagnostic pathology has not been established. Nevertheless the use of PCR in diagnostic pathology is increasing and has proved its value, particularly in the management of the haematological malignancies. The use of this technology in all areas of diagnostic pathology and clinical management will only increase as the methods become more routine and the value of the data produced more valued.

References

Antillon-Klussmann F, Garcia-Delgado M, Villa-Elizaga I, Sierrasesumaga L (1995). Mutational activation of ras genes is absent in pediatric osteosarcoma. Cancer Genet Cytogenet 79(1): 49-53.

Araki N, Uchida A, Kimura T, et al. (1991). Involvement of the retinoblastoma gene in primary osteosarcomas and other bone and soft-tissue tumors. Clin Orthop (270): 271-7.

Barrios C, Castresana JS, Ruiz J, Kreicbergs A (1993). Amplification of c-myc oncogene and absence of c-Ha-ras point mutation in human bone sarcoma. J Orthop Res 11(4): 556-63.

Benedict WF, Fung YK, Murphree AL (1998). The gene responsible for the development of retinoblastoma and osteosarcoma. Cancer 62: 1691-4.

Castresana JS, Barrios C, Gomez L, Kreicbergs A (1992). Amplification of the c-myc proto-oncogene in human chondrosarcoma. Diagn Mol Pathol 1(4): 235-8.

Collins C, Lin Kuo W, Segraves R, et al. (1991). Construction and characterisation of plasmid libraries enriched in sequences from single human chromosomes. Genomics 11: 997-1006.

Cotton RGH, Rodrigues NR, Campbell DR (1988). Reactivity of cytosine and thymine in single base pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc Natl Acad Sci USA 85: 4397-401.

Cremer T, Landegent J, Bruckner A, et al. (1986). Detection of chromosome aberrations in the human interphase nucleus by visualization of specific target DNA with radioactive and non-radioactive in situ hybridization technique: diagnosis of trisomy 18 with probe LI84. Genetics 74: 346-52.

Cremer T, Tesin D, Hopman AHN, Manuelidis L (1988). Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumour cells by In Situ Hybridisation with chemically modified DNA probes. Exp Cell Res 176: 199-220.

Dahir GA, Vnencak-Jones CL, Schwartz HS, Butler MG (1994). Dosage and allelic restriction fragment studies and PCR analysis of the H-ras locus in giant cell tumor of bone. Cancer Genet Cytogenet 74(2): 95-8.

Devilee P, Thierry RF, Kievits T, et al. (1998). Detection of chromosome aneuploidy in interphase nuclei from human breast tumours using chromosome specific repetitive DNA probes. Cancer Res 48: 5825-30.

Dicker AP, Volkenandt M, Bertino JR (1989). Detection of a single base mutation in the human dihydrfolate reductase gene from a methotrexate-resistant cell line using the polymerase chain reaction. Cancer Commun. 1(1): 7-12.

Draper GJ, Sanders BM, Kingston JE (1986). Second primary neoplasms in patients with retinoblastoma. Br J Cancer 53: 661-71.

Erlich HA, ed (1989). PCR technology: principles and applications for DNA amplification. New York: Stockton Press.

Gall JG, Pardue ML (1969). Formulation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci USA 63: 378-383.

Grundmann E, Ueda Y, Schneider-Stock R, Roessner A (1995). New aspects of cell biology in osteosarcoma. Pathol Res Pract 191(6): 563-70.

Hansen MF (1991). Molecular genetic considerations in osteosarcoma. Clin Orthop (270): 237-46.

Hogervorst FBL, Cornelis RS, Bout M, et al. (1995). Rapid detection of BRCA1 mutations by the protein truncation test. Nature Genetics 10: 208-212.

Hoogerwerf WA, Hawkins AL, Perlman EJ, Griffin CA (1994). Chromosome analysis of nine osteosarcomas. Genes Chromosomes Cancer 9(2): 88-92.

Hopman AHN, Poddighe PJ, Smeets GB, et al. (1989). Detection of numerical chromosomal aberrations in bladder cancer by in situ hybridisation. Am J Pathol 135: 1105-1116.

Imanishi T, Abe Y, Suto R, et al. (1994). Expression of the human multidrug resistance gene (MDR1) and prognostic correlation in human osteogenic sarcoma. Tokai J Exp Clin Med 19(1-2): 39-46.

Isfort RJ, Cody DB, Lovell G, Doersen CJ (1995). Analysis of oncogenes, tumor suppressor genes, autocrine growth-factor production, and differentiation state of human osteosarcoma cell lines. Mol Carcinog 14(3): 170-8.

Issing WJ, Wustrow TP, Oeckler R, et al. (1993). An association of the RB gene with osteosarcoma: molecular genetic evaluation of a case of hereditary retinoblastoma. Eur Arch Otorhinolaryngol 250(5): 277-80.

John HA, Birnstiel ML, Jones KW (1969). RNA-DNA hybrids at the cytological level. Nature 223: 582-7.

Koch JE, Kolvraa S, Petersen KB, et al. (1989). Oligonucleotide-priming methods for the chromosome-specific labelling of alpha satellite DNA in situ. Chromosoma 98: 259-65.

Komminoth P, Long AA (1993). In situ polymerase chain reaction. An overview of methods, applications and limitations of a new molecular technique. Virchows Arch B Cell Pathol 64: 67-73.

Ladanyi M, Cha C, Lewis R, et al. (1993). MDM2 gene amplification in metastatic osteosarcoma. Cancer Res 53(1): 16-8.

Ladanyi M, Park CK, Lewis R, et al. (1993). Sporadic amplification of the MYC gene in human osteosarcomas. Diagn Mol Pathol 2(3): 163-7.

Misra DN, Dickman PS, Yunis EJ (1995). Fluorescence in situ hybridization (FISH) detection of MYCN oncogene amplification in neuroblastoma using paraffin-embedded tissues. Diagnostic Molecular Pathology 4(2): 128-35.

Myers RM, Maniatis T, Lerman I (1987). Detection and localization of single base changes by denaturing gradient gel electrophoresis. In: Methods in Enzymology (Ed. Wu R). San Diego: Academic Press, vol 155, pp 501-527.

Nagata Y, Yamasaki H, Fukuda K, et al. (1992). Expression of the multidrug-resistant gene in human musculoskeletal tumors. J Environ Pathol Toxicol Oncol 11(3): 131-7.

Orita M, Suzuki Y, Sekiya T, Hayashi K. (1989). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5: 874-9.

Ozaki T, Ikeda S, Kawai A, et al. (1993). Alterations of retinoblastoma susceptible gene accompanied by c-myc amplification in human bone and soft tissue tumors. Cell Mol Biol (Noisy-Le-Grand) 39(2): 235-42.

Ozisik YY, Meloni AM, Zalupski MM, et al. (1993). Deletion of chromosome 13 in osteosarcoma secondary to irradiation. Cancer Genet Cytogenet 69(1): 35-7.

Pinkel D, Straume T, Gray JW (1986). Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 83: 2934-8.

Porter DE, Holden ST, Steel CM, et al. (1992). A significant proportion of patients with osteosarcoma may belong to Li-Fraumeni cancer families. J Bone Joint Surg Br 74(6): 883-6.

Sanger FS, Nicklen S, Coulsen AR (1977). DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-7.

Scholz RB, Kabisch H, Weber B, et al. (1992). Studies of the RB1 gene and the p53 gene in human osteosarcomas [see comments]. Pediatr Hematol Oncol 9(2): 125-37.

Selleri L, Hermanson GG, Eubanks JH, et al. (1991). Molecular localisation of the t(11;22)(q24;q12) translocation of Ewing sarcoma by chromosomal in situ suppression hybridization. Proc Natl Acad Sci USA 88: 887-9.

Shapiro DW, Valentine MB, Rowe ST, et al. (1993). Detection of N-myc gene amplification by fluorescence in situ hybridization. Am J Pathol 142(5): 1339-49.

Smith-Sorensen B, Gebhardt MC, Kloen P, et al. (1993). Screening for TP53 mutations in osteosarcomas using constant denaturant gel electrophoresis (CDGE). Hum Mutat 2(4): 274-85.

Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 503.

Srivastava S, Zou Z, Pirollo K, et al. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 348: 747-9.

Stellwag EJ, Dahlberg AE (1980). Electrophoretic transfer of DNA, RNA and protein onto diazobenzyloxymethyl (DBM) paper. Nucleic Acids Research 8: 299.

Toguchida J, Yamaguchi T, Ritchie B, et al. (1992). Mutation spectrum of the p53 gene in bone and soft tissue sarcomas. Cancer Res 52(22): 6194-9.

Trask BJ (1991). Fluorescence in situ hybridisation. Trends in Genetics 7: 149-54.

Wadayama B, Toguchida J, Shimizu T, et al. (1994). Mutation spectrum of the retinoblastoma gene in osteosarcomas. Cancer Res 54(11): 3042-8.

Wadayama B, Toguchida J, Yamaguchi T, et al. (1993). p53 expression and its relationship to Dna alterations in bone and soft tissue sarcomas. Br J Cancer 68(6): 1134-9.

Weiss SA, Brooks JSJ, eds. (1996). Soft tissue tumors. In: Monographs in pathology (Ed. Vogel FS). Baltimore: Williams and Wilkins, 1996.

Yamaguchi T, Toguchida J, Yamamuro T, et al. (1992). Allelotype analysis in osteosarcomas: frequent allele loss on 3q, 13q, 17p, and 18q. Cancer Res 52(9): 2419-23.