Assessment of Clonality in Human Tumors: A Review

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Abstract

The various methods of determination of the clonality of human tumors are described. There are three major approaches based on X-chromosome inactivation analysis, lymphocyte analysis, and somatic mutation analysis. For each of these approaches there are established methods and more recent methods based on DNA analysis. The increasing number of methods available increases the scope of clonality determination to most tumors. All the methods have inherent advantages and disadvantages, and these are discussed in relation to their clinical application.

The clonality of human tumors has direct relevance to theories of carcinogenesis and has practical implications for both diagnosis and subsequent studies of disease progression. The purpose of this review is to discuss the different methods of clonality determination, in particular those based on DNA analysis, and to illustrate their use in human tumors.

A clonal population of cells is defined as those cells arising from the mitotic division of a single somatic cell (1). Although this definition seems straightforward, it must be recognized that the assessment of clonality may be dependent on the technique used. For example, the investigation of two cell populations may suggest two independent clones by immunoglobulin gene analysis but a single clone by X-linked DNA polymorphism analysis, reflecting the earlier occurrence in development of X-chromosome inactivation. A further consideration which complicates the analysis of clonality is that the cells constituting a single clone are not necessarily genetically identical since clonal evolution may occur within such populations of cells (2).

It is now widely agreed that most tumors have a monoclonal composition consistent with the somatic mutation theory of carcinogenesis which assumes that a tumor results from the progeny of a single cell having acquired one or more somatic mutations (3). The methods used to determine clonality of human tumors have been most readily applicable to leukemias and lymphomas, although some techniques, for example, G6PD isoenzyme analysis, have been used on solid tumors. However, several methods have been developed over recent years which enable a higher proportion of a wide variety of tumors to be analyzed for clonality. The analysis of clonality in tumors can provide clonal markers which are valuable in assessing disease progression and in providing insight into the nature of disease remission.

The methods of clonality determination can be broadly categorized into the following groups: X-chromosome inactivation, lymphocyte analysis, detection of somatic mutations, and viral integration analysis. Table 1 divides these approaches into “traditional” methods and those based on various types of DNA analysis.

Both DNA and traditional methods depend upon the demonstration that a cell population is homogeneous with respect to a particular marker. Since at the time of presentation tumors may have undergone extensive genetic change and continuing selection of particular subclones may have occurred, clonality as assessed by any of the currently available methods may still not necessarily reflect the earliest events in tumorigenesis. A tumor may have originated from several cells, the progeny of one of these cells (bearing the marker) eventually having outgrown all the others (4).

X-Chromosome Inactivation

In females inactivation of one X chromosome occurs in each somatic cell in early embryonic development and is passed onto the progeny of the cell in a stable fashion (5–7). Females heterozygous for polymorphic X-chromosome genes are therefore mosaics with respect to X-chromosome activity (8, 9). There is evidence that X-chromosome inactivation is related to differential methylation of cytosine in the DNA of X-chromosome genes (10). The exact role of gene methylation in the various aspects of X-chromosome inactivation has been extensively reviewed elsewhere and a detailed discussion of the mechanisms of X-chromosome inactivation is beyond the scope of this review (5, 7, 11–13). From the point of view of clonality assessment in tumors it is sufficient to recognize that the inactivation or methylation patterns of X-chromosome genes can be used for the detection of clonality of tumors in females heterozygous for a particular X-linked polymorphism.

G6PD Isoenzyme Analysis. The first studies of human tumor clonality using the G6PD isoenzyme system were reported by Linder and Gartler (14, 15) and Beutler et al. (9) in uterine leiomyomas and malignant tumors, respectively. This form of clonality assessment was extended to the analysis of a large variety of human tumors in the now classic work of Fialkow (16). This form of analysis is based upon the fact that a female patient heterozygous for a polymorphism of the X-linked enzyme G6PD will express both the normal type of Gdα and a variant type of Gdβ or Gdα in her normal tissues but only a single G6PD isoenzyme in each individual cell. Therefore, a neoplasm arising from a single cell will show a single G6PD isoenzyme phenotype, whereas a polyclonal neoplasm will have a double G6PD enzyme phenotype (17).

The majority of human cancers analyzed by this technique have been shown to be monoclonal (including breast cancer, carcinoma of the colon, carcinoma of the uterine cervix, ovarian teratomas, and many hematological neoplasms (16, 18–20). G6PD studies have been particularly useful in the investigation of chronic myeloid leukemia since RBCs and platelets cannot be investigated by cytogenetic or DNA analysis as they have no nuclei. A few exceptional cases of malignant tumors with double enzyme phenotypes including colonic carcinomas, breast cancers, and a hepatoma have been reported. These rare cases may
be explained by an admixture of normal cells in the tumor sample analyzed (17, 21). This might be a possible explanation for conflicting results of clonality studies in parathyroid adenoma; the demonstration of a polyclonal origin of parathyroid adenomas by G6PD isoenzyme studies (22) has not been confirmed by more recent work using molecular analysis with DNA markers (23). Nevertheless some hereditary tumors such as trichoepitheliomas (24) and neurofibromas (25) do have a double enzyme phenotype. The polyclonal composition of these tumors is presumably related to the different time course and mechanism of tumorigenesis. Similarly a polyclonal origin of colorectal adenomas in Gardner’s syndrome (familial adenomatous polyposis syndrome) has been proposed based on the analysis of G6PD mosaicism (26). However, more recent data on the clonality of colorectal adenomas collected by DNA polymorphism analysis suggest that at least some adenomas are clonal tumors (27). These divergent results may be due to the fact that in the study using recombinant techniques contaminating nonneoplastic cells were removed from the tumor by histological analysis of cryostat sections.

The G6PD approach has until recently been limited to the Gd+ and Gd− variants in blacks which are easily distinguishable from the normal B enzyme by starch gel electrophoresis. More recently, it has been possible to extend this approach to individuals heterozygous for the Mediterranean variant of G6PD by virtue of its differential utilization of 2-deoxyglucose 6-phosphate as compared to the normal isoenzyme (28–30).

X-linked RFLP Analysis. Molecular probes for X-linked polymorphic genes now make it possible to detect clonal markers in tumor cells in a manner analogous to the G6PD isoenzyme studies described above (31, 32). Essentially active and inactive copies of polymorphic X-chromosome genes are differentiated through differences of gene methylation patterns. This approach greatly widens the scope of clonality studies to include all females in whom a suitable X-linked DNA polymorphism is present.

Both normal and tumor DNA are first digested with the appropriate restriction endonuclease to distinguish the maternal and paternal copies of the gene through an X-linked RFLP. A second endonuclease sensitive to methylation of cytosine residues in its recognition sequence distinguishes active from inactive copies of the gene through changes of the DNA methylation pattern (33, 34). In a polyclonal cell population where X-chromosome inactivation occurs randomly the paternal and maternal alleles are cleaved to some extent by this enzyme so that two fragments of reduced intensity remain visible on autoradiography. In DNA extracted from a tumor with a mono-

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**Table 1 Methods of clonality determination**

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**Viral integration analysis**

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**Fig. 1. Principle of a Southern blot X-linked RFLP clonality study.** Boxes, alleles; shaded areas, methylated fraction. **Left,** DNA from a polyclonal cell population of a female heterozygous for an X-linked RFLP. Both the paternal and the maternal alleles of the gene are methylated to some extent. The RFLP is detected by BamHI; the nonmethylated parts of each allele are cut by the enzyme HpaII and, therefore, the intensity of both fragments is reduced on autoradiography. **Right,** DNA from a monoclonal cell population of a female heterozygous for the same X-linked RFLP. In this particular case only the paternal allele of the gene is methylated and is, therefore, not cleaved by HpaII. However, the nonmethylated maternal allele is digested completely by this enzyme and hence is not detectable. The fact that in this cell population only one of the two alleles is methylated is evidence of clonality.

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Bone Marrow Reconstitution after Treatment for Leukemias. Some of the most interesting reports recently published using both G6PD isoenzyme studies and X-linked RFLP concern the nature of clinical remission in ANLL. There is good evidence for a monoclonal origin of leukemic cells from both karyotypic and G6PD isoenzyme studies. It would be reasonable to assume that when a patient enters a clinical remission this would be accompanied by a return to a normal (i.e., polyclonal) pattern of hematopoiesis. However, both G6PD and X-linked RFLP studies have provided evidence suggesting that this is not always the case. Using the X-linked RFLP approach, Fearon et al. (35) found that 3 of 13 patients with ANLL in remission had mature granulocytes of a monoclonal origin, presumably (although not proven) of the same clone as their original blasts. Fialkow (36) using G6PD isoenzyme studies showed 5 of 13 patients with ANLL in remission to have

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partially or completely clonal marrow stem cells. Experiments in cats have shown that, following chemotherapy with dimitubusulfan, hematopoiesis may return with a monoclonal pattern, presumably reflecting damage to or depletion of normal stem cells (37). In two studies of allogeneic bone marrow transplant recipients treated for leukemia, the majority of the patients showed no evidence of clonal reconstitution of granulocytes and mononuclear cells from their grafts as assessed by the X-linked RFLP method (38, 39). However, two patients had monoclonal or oligoclonal donor-derived hematopoiesis after allogeneic transplantation suggesting that in these cases a single or a very limited numbers of stem cells were sufficient to dominate the hematopoietic system (39). Further studies of ANLL are required to confirm that clonal granulocyte populations in in patients in remission are indeed part of the same leukemic clone as seen in the presentation marrow.

Lymphocyte Analysis

Immunoglobulin Light Chain Analysis. One of the standard methods for defining monoclonality in B-cell neoplasms is the demonstration of a single light chain isotype, either \( \kappa \) or \( \lambda \), on the cell surface of neoplastic lymphoid cells; this technique is readily applicable to B-cell chronic lymphocytic leukemia and some cases of B-cell non-Hodgkin's lymphomas (40, 41). There is no analogous system of surface markers indicating clonality in T-cell tumors. However, monoclonal antibodies which are specific for families of related variable regions which use the same variable region genes can act as indicators of the clonality of some T-cell populations (42).

Immunoglobulin and TCR Gene Analysis. The more recent use of molecular probes to detect immunoglobulin and TCR gene rearrangements overcomes many of the limitations of immunoglobulin light chain analysis (43–45). Essentially all types of lymphoid malignancies are amenable to this type of analysis. Immunoglobulin and TCR gene rearrangements give rise to DNA markers unique to each individual lymphoid cell and its progeny. In a polyclonal lymphoid cell proliferation these rearrangements are not detectable by Southern blotting since they are well below the threshold of the method's sensitivity. However, all cells in a monoclonal population have the same genetic rearrangement which is readily detected by Southern blotting. Immunoglobulin and TCR gene rearrangements may also provide information about the cell lineage of lymphoid neoplasms. In general, immunoglobulin gene rearrangements are indicative of B-cell neoplasms, whereas TCR gene rearrangements are regularly found in T-cell lymphomas and leukemias. Using these techniques it has been possible to prove, for example, a clonal B-cell origin of hairy cell leukemia and a clonal T-cell origin of mycosis fungoides.

A significant limitation of the conventional Southern blotting technique is its inability to detect gene rearrangements at much below the 5% level. The polymerase chain reaction technique now offers the possibility of detecting clonal markers at much lower levels. There are major problems in the amplification of immunoglobulin or TCR\( \alpha \beta \) rearrangements because of the very large number of variable and joining segments which can undergo rearrangement. However, the TCR\( \gamma \) genes have a more limited germline repertoire and a method has been described which detects TCR\( \gamma \) rearrangements by polymerase chain reaction in leukemic samples (46). It is hoped that generally applicable methods will be developed to detect immunoglobulin and TCR rearrangements present at very low levels since these would be extremely useful clonal markers in the study of minimal residual disease in lymphoma and leukemia.

It should be noted that immunoglobulin gene rearrangements are not consistently stable clonal markers since they are subject to variability as a result of ongoing somatic mutation. Differences in immunoglobulin gene rearrangement patterns among various specimens from a given case do not necessarily reflect true bi- or multiclonality in lymphoid tumors. This has been clearly shown in two studies of follicular lymphoma bearing the characteristic chromosomal translocation t(14;18). In the study of Raffeld et al. (47) 6 of 16 cases showed variation in immunoglobulin patterns over time, although the t(14;18) breakpoint was conserved in all individuals. Similarly the study by Cleary et al. (48) of five bigenotypic and biphenoypic follicular lymphomas showed that four of these cases had identical t(14;18) rearrangements confirming the single cell origin for these neoplasms. The single exceptional case was further studied by nucleotide sequence analysis of cloned breakpoint DNA which showed identical t(14;18) crossovers in the two subpopulations. The translocated chromosome 18 DNA rearrangements are more reliable clonal markers, although these may also be affected by somatic mutation.

Somatic Mutations

Cytogenetics. Many human tumors have now been found to have consistent, nonrandom chromosome abnormalities which behave as clonal markers (49). The classic example is the Philadelphia chromosome in chronic myeloid leukemia (50). Increasingly, chromosome studies are being used not only for the detection of clonal markers but also to provide information relevant for diagnosis, classification, and prognosis (51–53). The limitations of cytogenetics are that only cells in mitosis can be studied and that these cells cannot be identified morphologically. This latter problem has been at least partly overcome in a recent study of clonal chromosomal abnormalities in acute leukemia by a new technique using the detection of lineage-specific antigens in dividing leukemic cells by monoclonal antibodies (54). In solid tumors cytogenetic studies are difficult as cell cultures are often unsuccessful. DNA analysis now provides a complementary approach for the detection of clonal chromosome abnormalities in solid tumors.

Chromosome Losses Detected by Molecular Probes. The loss of chromosomal material is one of the most common abnormalities in malignant cells. DNA analysis depends on the ability to distinguish the two chromosomal homologues by the detection of RFLPs. The analysis is informative if constitutional DNA displays heterozygosity for a particular RFLP; loss of one of these alleles in tumor DNA indicates chromosomal loss. This approach has demonstrated chromosomal loss in many tumors. The use of probes detecting polymorphisms due to VNTRs makes this method feasible in most cases on account of their high heterozygosity rates (55–57). This approach has led to the discovery of tumor-suppressing genes or "antioncogenes" as demonstrated by the example of the retinoblastoma gene and its protein product (58–60). However, it should be noted that DNA analysis is a relatively crude technique to detect chromosomal loss in a mixed population of normal and abnormal cells as compared to cytogenetics.

Chromosome Translocations Detected by Breakpoint Cluster Region Probes. Chromosomal translocations can now also be detected in human neoplasms by DNA analysis. The principle of this analysis is that a DNA probe is used which hybridizes to the region of the breakpoint on one of the chromosomes involved in the translocation. Breakpoints must be clustered within a defined DNA sequence otherwise a large number of probes would have to be used for the detection of translocated...
sequences. Studies of the Philadelphia chromosome illustrate this approach; the breakpoints on chromosome 9 are variable but those on chromosome 22 are clustered in most cases over a region of 5.8 kilobases, known as the breakpoint cluster region (61, 62). DNA probes which hybridize to the breakpoint cluster region on chromosome 22 will detect most cases of Ph′-positive chronic myeloid leukemia.

Other translocations which may be detected directly by Southern blotting include the t(11;14) found in some B-cell neoplasms (63), the (14;18) characteristic of follicular lymphomas (64–66), and the t(8;14) as found in sporadic Burkitt’s lymphoma (67). In principle these translocations may now be detectable at very low cell concentrations by the polymerase chain reaction technique, provided the breakpoints on the chromosomes are clustered within defined regions (68, 69).

Point Mutations. An exciting recent discovery in cancer research is that specific point mutations occur in the oncogenes of some human tumors. The mutations best characterized are those in the ras oncogenes occurring predominantly at codons 12, 13, or 61; efficient screening methods based on gene amplification have now been developed for their detection (70–72). There is interest in the use of such mutations in the clonal analysis of ANLL. Unfortunately the present data indicate a disparity in the incidence of these mutations at presentation and relapse (73). It has been reported that ras gene mutations occur in over one-third of human colorectal cancers and that most of the mutations are at codon 12 of the c-Ki-ras gene (74–76). Furthermore it was shown that in five of six cancers that the same ras mutation was present in the adenomatous (benign) regions of the tumor, suggesting that the mutation preceded the development of the malignancy (74).

A further example of the use of a ras gene mutation is the report of the pluripotent stem cell origin of idiopathic myelofibrosis (77). The peripheral blood cells of one patient showed a point mutation at codon 12 of the N-ras oncogene. This mutation was then used as a marker to investigate the clonal relationship of different cell lineages by cell separation analysis. The presence of this specific mutation in granulocytes, monocytes, erythroblasts, B-lymphocytes, and T-lymphocytes indicated a pluripotent stem cell origin for idiopathic myelofibrosis in this patient.

DNA Fingerprinting. Comparative DNA fingerprint analysis of a patient’s tumor and constitutional DNA represents a new method for the detection of clonal markers (78). The feasibility of this approach depends on the properties of the minisatellite DNA probes which detect a large number of VNTRs scattered throughout the genome (79, 80). A study of gastrointestinal tumors showed differences between the tumor DNA and constitutional (peripheral blood and mucosa) DNA in about two-thirds of patients when screened with three fingerprint probes (81). Many of the differences resulted from the loss of bands in the tumor DNA; whether these correspond to major chromosomal deletions or to submicroscopic changes is not known. Mutant bands were also observed in tumor DNA samples; the precise mechanism responsible for their generation is not known, although some VNTR loci have been shown to have a high mutation rate (82). DNA fingerprinting is a method with potential for following serial genetic changes in tumors, particularly in those cases lacking an obvious cytogenetic abnormality.

Viral Integration Analysis

The molecular analysis of tumors containing viruses offers an interesting and novel approach to the study of clonality. An experimental system has been recently described in mice which illustrates the principles behind this approach (83). The clonality of various cell populations within the hematopoietic system was studied by reinfecting retrovirus-infected bone marrow cells into mice whose bone marrow had been depleted by irradiation. Repopulation of the bone marrow was derived from cells that had been clonally marked by unique retroviral integration sites. The appearance of new clones and loss of clones from some lineages were then traced by Southern blot hybridization of DNA from various hematopoietic cell populations with probes specific for the transducing retrovirus.

A similar method for assessing the clonality of Epstein-Barr virus-associated tumors has been developed over recent years (84). EBV DNA is a double-stranded linear molecule with homologous tandem repeats at each terminus. The linear DNA termini of the virus are joined intracellularly to form covalently closed episomal DNA following viral infection of susceptible host cells. The structure of the virus differs for each independently circularized genome on account of the presence of variable numbers of tandem repeated sequences present at each linear terminus; these differences can be readily detected by Southern blotting. The progeny of each infected cell contain multiple identical viral episomes with the same fused termini. If the original in vivo multiplicity of infection is assumed to be low (i.e., one virion/cell), then in a monoclonal population of cells (the progeny of one-EBV infected lymphoid cell) only one circular form of the EBV episome will be present, and this will be seen as a single band on Southern blotting. In contrast a polyclonal population of B-cells will contain viral episomes with different fused termini with a multiple band pattern on Southern blotting.

This method was used and a monoclonal EBV pattern has been found in carcinomas of the nasopharynx, the parotid gland (84), and in a few cases of Hodgkin’s disease (85), whereas polyclonal EBV-infected cell lines contained multiple forms. In a further study of Hodgkin’s disease EBV genome sequences were detected in Reed-Sternberg cells by in situ hybridization in about 20% of cases, and the fragment pattern was consistent with a monoclonal population of cells (86). A study of transplant-associated lymphoproliferative disorders has reported both the configuration of immunoglobulin gene rearrangements and fused EBV termini. The data were considered to be consistent with the notion that the lymphoproliferations initiate as polyclonal expansions of EBV-carrying B-cells, which progress to multicolonic lymphomas in most patients (87). In principle this approach could be envisioned using probes for the breakpoints of human papilloma virus in human cervical carcinomas or hepatitis B virus in liver tumors (88, 89).

Conclusion

The clonality of human tumors is a central issue in the understanding of tumorigenesis. This is exemplified by the leukemias in which clonal analysis can be used to study the stem cell nature and the differentiation potential of the neoplastic cells. Clonal analysis of particular tumors has been the starting point for the discovery of important genes such as the tumor-suppressor genes now being characterized in the retinoblastoma model. Further research into the clonal composition and evolution of tumors will undoubtedly provide new insights into tumor development and growth, as well as provide clonal markers relevant to the clinical diagnosis and follow-up of individual cases.
CLONALITY IN HUMAN TUMORS

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