Molecular Diagnostics of Hematologic Malignancies in Small Animals

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• Feline • c-kit

Over the past 20 years, human pathologists and oncologists have become increasingly reliant on genetic testing to diagnose, prognosticate and choose treatment for almost every kind of cancer. We are beginning to adopt such testing in veterinary medicine and, as a result, can often offer clients more cost-effective and less invasive diagnostics, and more reliable prognostic information.

Our ability to inexpensively identify various types of genetic changes associated with cancer not only adds to our diagnostic armament but also expands the kinds of questions we can ask in both the laboratory and clinically. For example, molecular testing allows us to distinguish a reactive population of lymphocytes from a neoplastic one and also allows us to determine if this patient’s lymphoma is the same one he was treated for 1 year ago or if it is a newly arising tumor. The number of tumor cells in the peripheral blood of lymphoma patients can be precisely quantified and followed throughout treatment. Detection of a mutation in the c-kit gene in canine mast cell tumors gives us prognostic information, and in addition helps clinicians choose the type of chemotherapy that will be most efficacious in that patient. Most polymerase chain reaction (PCR)-based tests are relatively inexpensive and may help save owners money in the long run.

Molecular based diagnostic tests in hematologic malignancy can be placed into 3 broad categories: (1) detection of individual mutations in oncogenes, (2) detection of clonality in lymphoma and leukemia by taking advantage of their unique antigen receptor genes (immunoglobulin and T-cell receptor genes, and (3) detection of chromosomal translocations, deletions, and duplications. Clonality testing and detection of the c-kit oncogene in mast cell tumors are both now in routine use and widely available to practitioners. The uses and limitations of both of these assays will

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be discussed here. Many of the general principles would apply to any type of genetic testing that eventually becomes available.

**DETECTION OF MUTATIONS IN INDIVIDUAL ONCOGENES**

In 1999, 2 reports described the presence of mutations in the gene c-kit in canine mast cell tumors.¹ ² c-kit is a receptor tyrosine kinase for stem cell factor (SCF), which stimulates mast cell growth. Both reports described small duplications of DNA within the c-kit gene called internal tandem duplications (ITDs). These mutations were found within exons 11 and 12 of the c-kit gene (Fig. 1, referred to hereafter as the exon 11

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**Fig. 1.** The c-kit gene and protein. (A) C-kit is a transmembrane protein which, when activated by SCF, is phosphorylated on the cytoplasmic tail. Internal tandem duplications (ITDs) are seen in the region of the protein encoded by exon 8 (extracellular) and exon 11 (transmembrane). (B) c-kit mutations are found in exons 8, 9, 11, and 17, but currently available assays only detect ITDs in exon 8 (always a 12-base insertion), and exon 11 (a variety of different insertions). The arrows illustrate the location of the PCR primers used to detect the two different mutations (not drawn to scale). (C) Amplification of exon 11 by PCR reveals a wild-type product (132 bases) in all cases and a larger product in dogs with an exon 11 ITD. Similar results would be seen with exon 8 primers (not shown).
mutation). When SCF binds to unmutated c-kit, the cytoplasmic portion of the receptor undergoes autophosphorylation. In the presence of the exon 11 ITD, the receptor is constitutively phosphorylated, regardless of whether SCF is present (see Fig. 1). c-kit phosphorylation activates signaling pathways that stimulate mast cell growth. Therefore, the presence of this mutation is directly responsible for the uncontrolled proliferation of mast cells. The finding that some mast cell tumors are caused by constitutive activation through a receptor tyrosine kinase lead investigators to hypothesize that tyrosine kinase inhibitors would be useful therapies against this disease.

A comprehensive analysis of mutations in 50 canine mast cell tumors demonstrated that while the exon 11 ITDs were the most common (half of the cases), other c-kit mutations could also cause constitutive activation of the c-kit gene. This included 8 examples of an identical 12–base pair ITD in exon 8 (16% of all mutations detected), as well as 8 examples of a single base change in exon 9 (16% of all mutations detected). Each of these was shown to cause constitutive phosphorylation of c-kit, suggesting that each might be rational targets of tyrosine kinase inhibitors. There were additional single base changes and rare deletions and insertions detected in exon 11 and 1 in exon 17 that did not undergo functional analysis. Together these 50 c-kit mutations represented 26% of the 191 mast cells tested. The patient population was dogs with a grade II or III mast cell tumor that had failed surgical resection or was unresectable. The prevalence of c-kit mutations in the population of mast cell tumors as a whole is probably lower.

**Technical Aspects of c-kit Mutation Analysis**

The discovery of dysregulated tyrosine kinase activity in the c-kit receptor led to the investigation of tyrosine kinase inhibitors as therapy and the eventual development of tyrosine kinase inhibitors such as Palladia (toceranib phosphate, Pfizer Animal Health) for use in this disease. Detection of c-kit mutations has therefore become a routine part of mast cell tumor diagnostics, because the presence of these mutations can guide therapy choices. Several laboratories now offer c-kit mutation analysis, most commonly for the exon 11 ITD. The assay is carried out by isolating DNA from cytology specimens or formalin-fixed, paraffin-embedded biopsy samples. The portion of the gene that most commonly carries the mutation is amplified by PCR using primers bracketing the region, and the products are separated by gel electrophoresis (see Fig. 1). All samples should show amplification of the wild-type, unmutated gene, since only 1 chromosome is likely to be affected by the mutation. In addition, there will invariably be nontumor tissue in the sample, which will have only the wild-type gene. This amplification can serve as the positive control, revealing adequate quantities of good-quality DNA. If the mutation is present, there will be a second, larger product visible as well (see Fig. 1). The size of the exon 11 ITD differs within each novel mast cell tumor.

Additional testing for the exon 8 ITD is offered by some laboratories. The principle is the same as detection of exon 11 mutations, but this mutation may be more difficult to detect using standard agarose gel separation methods, since the insertion is 12 bases. Single base resolution is possible using DNA sequencing instrumentation, and this method may be more appropriate for exon 8 analysis.

The methodology for these assays, however, will not detect single base pair changes, such as have been reported in exon 9. Therefore, the absence of a positive result in the commonly offered c-kit mutation assays for exon 8 and 11 does not mean that an activating mutation is not present. Assays for exon 9 have been developed (A. Avery and R. Burnett, unpublished observations, 2011), but
this mutation appears to have low prevalence in the population and exon 9 testing is not routinely carried out.

The detection methods currently offered require that approximately 10% of the cells in the sample be mast cells (A. Avery and R. Burnett, unpublished observations, 2011). This is because the mutated c-kit gene gives rise to a larger PCR product than does the wild-type gene. These products compete with one another during the amplification process, and amplification of the smaller, wild-type product is more efficient. Therefore, when the number of mast cells is low, a mutation may be missed. For this reason the standard assay is not likely to be useful for detecting metastasized mast cells in draining lymph nodes or for minimum residual disease detection. Tumor-specific primers, based on the specific mutation present, could be constructed for this purpose since such reagents are significantly more sensitive.

c-kit Mutations and Prognosis Using Conventional Therapy

The presence of a mutation in exon 11 of c-kit has been associated with poor prognosis in 2 studies conducted by the same group. Webster and coworkers demonstrated that in cases treated by surgical excision alone (no chemotherapy), the presence of a mutation in exon 11 was highly correlated with a poor outcome. Subsequently, the same group evaluated the response to vinblastine and prednisone in 28 dogs and found that dogs with an exon 11 mutation had a disease-free interval of 6.5 months compared to those without this mutation (11 months). Immunohistochemical staining for c-kit and histologic grade were also independently prognostic in this study. On the other hand, a large study found a trend toward a worse outcome when a mutation was present, but this trend did not reach significance. There was no control for treatment, however, so an effect of the mutation may have been missed.

c-kit Mutation Status and Response to Receptor Tyrosine Kinase Inhibitors

These reports compared the response of mast cell tumors to conventional chemotherapy (vinblastine and prednisone) with no therapy at all. Receptor tyrosine kinase inhibitors, such as Palladia, specifically target the c-kit receptor and therefore were predicted to be most effective in mast cell tumors with a constitutively activated c-kit gene. Consistent with this notion, Hahn and colleagues found that in 202 dogs with recurrent or nonresectable grade II or III mast cell tumors, first-line treatment with the tyrosine kinase inhibitor masitinib was more effective than placebo regardless of whether a c-kit mutation was present. Dogs that had received other therapy prior to masitinib, however, only responded if the c-kit mutation was present. This study included all c-kit mutations, not just those found in exon 11. In another study, dogs with recurrent mast cell disease responded better to the tyrosine kinase inhibitor Palladia if they carried the exon 11 c-kit mutation than if they did not. The effect of activating c-kit mutations on a patient’s response to tyrosine kinase inhibitors compared with conventional chemotherapy has not yet been reported.

Detection of c-kit mutations in mast cell tumors is now routinely used for prognosis and guiding treatment. Most commonly, mutation status is determined together with immunohistochemical staining to examine proliferation markers and the cellular location of c-kit. These factors help establish prognosis and the need for additional therapy. At least one clinical study demonstrated that dogs with a single base pair change in exon 9 of c-kit responded very favorably to imatinib, although testing for this mutation is not currently offered.
c-kit Mutations Can Be Used as a Tumor Fingerprint

Patients often suffer from recurrent mast cell tumors, but it is not clear if these are derived from a single clone. Zavodska and coworkers\textsuperscript{13} addressed this question in 2 patients by exploiting the fact that exon 11 mutations differ from tumor to tumor, thus providing a unique fingerprint to a tumor that carries it. They described 2 patients with mast cell tumors that recurred over a period of 3 years. One of these patients had 1 mast cell tumor occur each year for 3 years, with no therapy between the appearances of each tumor, and the tumor recurring in a different location each time. Since the tumor harbored a c-kit mutation, this allowed the investigators to demonstrate that the same mutation was present in each of the 3 tumors, indicating that the tumors were all derived from the same clone. Therefore, the neoplastic mast cells were never eradicated. A second case also involved a dog with multiple mast cells, which had a cytologically more aggressive appearance (grade III). Again, each of the individual tumors had the same tandem duplication, indicating they all arose from the same neoplastic clone. This approach could be extended by creating tumor-specific PCR primers based on the mutated sequence. Such primers would be far more sensitive than the standard assay and could detect distant metastases and the presence of neoplastic cells in the blood and can even be used to quantify tumor cells.

c-kit Mutations in Cats

Feline mast cell tumors also have c-kit mutations. The overall rate of mutation, based on 62 tumors, was 68%, higher than that seen in dogs.\textsuperscript{14} The majority of these mutations involved an ITD of exon 8, similar to that seen in dogs, although a number of other mutations were also identified. Seven of 8 cats with c-kit mutations had objective clinical responses to the tyrosine kinase inhibitor imatinib, but there have not yet been controlled trials to determine if the presence of a c-kit mutation results in better clinical responses with tyrosine kinase inhibitors. Although c-kit testing in cats is not currently offered commercially, an assay to detect the majority of exon 8 mutations would be straightforward.

c-kit in Gastrointestinal Stromal Cell Tumors

c-kit is expressed in many different types of tumors. In particular, activating mutations in exon 11 of c-kit have been found in approximately 70% of human gastrointestinal stromal cell tumors (GISTs), and these cases respond well to tyrosine kinase inhibitors compared with the response by mutation-negative tumors. To date, the relevant region of c-kit has been sequenced in 21 cases of canine GIST, and mutations found in 8 (38%) of these.\textsuperscript{15,16} Seven of the 8 involved a 3– or 6–base pair deletion and would therefore be detectable by some laboratories offering c-kit mutation analysis, depending on their level of resolution of the amplified gene product. It is important to note, however, that it has not yet been established that GISTs in dogs will respond to tyrosine kinase inhibitors such as Palladia, or if the mutation has any prognostic significance.

CLONALITY ASSAYS

Principals of Clonality Assays

A clonality assay demonstrates that a group of cells is derived from a single clone. The term is usually used to refer to detection of the unique genes found in each individual B or T cell—immunoglobulin genes in B cells and T-cell receptor genes in T cells. The portion of these genes that encodes the antigen binding region is the portion that varies between cells, in both size and sequence. Once a B or T cell is mature and divides in response to antigenic stimulation, the immunoglobulin and T-cell receptor genes are passed on to the daughter cells.\textsuperscript{17,18}
In the course of a normal immune response to a pathogen, B and T cells are activated, expand, and eventually die leaving behind a small number of residual memory cells. On the other hand, when a cell becomes neoplastic, it is no longer responsive to growth controls and can expand significantly more than the cells during an immune response. Therefore, if one can establish that the majority of cells in a particular collection of lymphocytes have the same immunoglobulin or T-cell receptor gene, it is most likely that these cells are neoplastic rather than reactive.19

When immunoglobulin and T-cell receptor genes rearrange during the course of B-cell and T-cell development, respectively, the length and sequence of the resultant gene differ from cell to cell. There are many reasons for this, including the fact that nucleotides are added between V, D, and J segments as they rearrange into a contiguous formation. The clonality assay takes advantage of this fact. In a sample consisting of many different lymphocytes, as in a reactive process (the lymph nodes of a dog with chronic pyoderma or poor dental hygiene, for example), there will be multiple different-sized T-cell receptor and immunoglobulin genes (Fig. 2). On the other hand, in a sample consisting of neoplastic lymphocytes, the immunoglobulin gene or the T-cell receptor gene (depending on whether it is a B-cell or a T-cell lymphoma) will be a single size.

**Technical Aspects of Clonality Assays**

Clonality assays are accomplished by isolating DNA from cells suspected to be neoplastic and then, using PCR primers directed at the conserved regions of T-cell receptor or immunoglobulin genes that flank the hypervariable regions of these genes, amplifying the variable regions. The PCR products are separated by size using a variety of possible methods. The presence of a single-sized PCR product is indicative of clonality, whereas the presence of multiple PCR products supports a reactive process (see Fig. 2). This assay has now been reported by a number of laboratories20–23 and used to answer a variety of clinical questions.24–26 We have termed this assay the PARR assay (PCR for Antigen Receptor Rearrangements) in order to distinguish it from other types of clonality assays.21 It should be noted, however, that this term is not used in the human literature.

The PARR assay differs from more commonly performed PCR assays—for example, those that are used to detect DNA from infectious agents where the result is read as “positive” or “negative”—in that the quality of the results relies very heavily on the source of the DNA (formalin-fixed paraffin-embedded tissue vs frozen or fresh samples), the primers used, the PCR cycling protocol, and particularly on the resolution of the PCR product separation technique. Because each of these factors will differ between laboratories, it is essential that a laboratory carrying out clonality analysis provide an assessment of the sensitivity and specificity of their assay. PCR products may look clonal if the products are not separated with sufficient resolution, if there was too little DNA, or if the DNA was poor quality. Thus the finding of a clonal product needs to be accompanied with information on how many non-neoplastic samples the laboratory has analyzed, and how many of these would be called clonal under that laboratory’s conditions. Ideally, separation techniques that give single base resolution, such as capillary electrophoresis, should be used, but at minimum resolution of three base pairs is necessary. Agarose gels, commonly used for most conventional end point PCR assays, do not afford this kind of resolution.
Limitations of the PARR Assay

Sensitivity
There are two types of sensitivity limitations in the PARR assay. First is the ability of the PCR primers used in these assays to detect all possible V and J regions that are used in the generation of immunoglobulin and T-cell receptor gene. Depending on the primers and conditions used and the nature of the tumor, the sensitivity of the assay for detecting clonal populations of B or T cells in confirmed cases of lymphoma or leukemia ranges from 63% to 100%.\textsuperscript{21,27,28} Failure to detect a clonal product in a case of unequivocally diagnosed lymphoma may be due to utilization of rare V or J genes, polymorphism within the species in V and J genes, or, in the case of immunoglobulin genes, somatic hypermutation of those genes in the site where the

**Fig. 2.** Rearrangement of immunoglobulin genes. (A) There are approximately 80 V region genes, 6 D region genes, and 5 J region genes (Bao and colleagues\textsuperscript{42} and A. Avery, unpublished observations, 2011) in dogs (this figure does not represent the actual numerical order of the genes). A single V, D, and J are brought together at random to create a single VDJ gene segment that encodes the antigen binding portion an antibody, and the intervening sequence is removed. (B) In the process of bringing together V, D, and J genes, a variable number of nucleotides (depicted in black) are added between V and D and between D and J. As a result, each individual B cell will have a VDJ gene segment with a unique length. When DNA from a heterogeneous population of B cells is isolated and amplified with primers bracketing the VDJ gene segment (small arrows), the PCR products will be different lengths. The bottom panel shows the PCR products separated by size using capillary gel electrophoresis and illustrates multiple different-sized PCR products. (C) When a population of B cells is composed of cells derived from a single clone, all the VDJ gene segments will be identically sized. PCR amplification of the VDJ gene segment will yield a single-sized product as shown in the bottom panel. All the principles illustrated here apply to T-cell receptor genes. For the clonality assay, the T-cell receptor gamma chain is amplified, although in theory T-cell receptor beta could also be used.

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primers bind. Since the original descriptions of the clonality assay, the complete canine genome has become available and is well annotated. This resource has allowed investigators to develop primers that will detect a higher percentage of T-cell receptor and immunoglobulin genes and, when widely used, should increase the sensitivity of the PARR assay.

The second type of sensitivity limitation is in how many individual tumor cells can be detected within a background of normal cells. As with all aspects of the assay, this limit will probably differ between laboratories, but in the original description of the assay it was estimated that approximately 1 tumor cell in 100 normal cells could be detected. The use of tumor specific primers can detect a significantly smaller proportion of neoplastic cells (1:10⁴), but this kind of assay is not in routine clinical use.

Determining phenotype
B-cell lymphomas have clonal immunoglobulin gene rearrangements but should not have clonal T-cell receptor rearrangements, and vice versa. Thus in theory, the clonality assay can be used to determine the phenotype of a lymphoma. The original descriptions of this assay reported high fidelity of the assay to lineage—for cases of confirmed lymphoma, the type of rearrangement was consistent with the immunophenotype in all cases (n = 42) except for 1 case in which both immunoglobulin and T-cell receptor rearrangements were identified. Subsequently, several reports have suggested that a higher percentage of cases will have clonal rearrangements of both immunoglobulin and T-cell receptor genes in both dogs and cats. Each of these studies had different case selection criteria (Valli and colleagues specifically looked at indolent lymphomas, whereas the original report included any immunophenotyped case of lymphoma), which may explain the differing results. Nonetheless, although flow cytometry, immunocytochemistry, and immunohistochemistry are the tests of choice for determining the phenotype of a lymphoma, in most cases of lymphoma and lymphocytic leukemia, the lineage can be deduced from the PARR assay. It is important to note, however, the simply identifying a lymphoma as B or T cell in origin can be prognostically misleading. While there is a great deal of data supporting the idea that B-cell lymphomas overall have a poorer prognosis than T-cell lymphomas, there are many exceptions. Ponce and coworkers demonstrated that Burkitt lymphoma (a B-cell origin tumor) had the gravest prognosis of all the histologic subtypes, B or T cell, and small clear-cell T lymphoma had the best. Furthermore, Rao and colleagues described a subset of B-cell lymphomas characterized by flow cytometry with a median survival of 136 days, closer to that typically reported for T-cell lymphomas. Therefore, if the goal of phenotyping is to establish prognosis, histology or flow cytometry is preferable to clonality testing.

The PARR assay should not be used to establish whether a tumor is lymphoid or myeloid in origin in cytologically ambiguous cases. Burnett and coworkers reported an acute myeloid leukemia with a clonally rearranged immunoglobulin gene, and similar findings have been reported in human myelogenous leukemias. Thus, while the presence or absence of a clonally rearranged immunoglobulin or T-cell receptor gene can contributed to lineage determination, it should not be the sole determinant.

Uses of the PARR Assay
The PARR assay is most commonly used to aid in distinguishing reactive (polyclonal) from neoplastic (monoclonal) lymphocytes when these distinctions are difficult to make with other means. For example, while cytologic examination of lymph node
aspirates can often be diagnostic for lymphoma, in cases where the majority of cells are small and mature, it is difficult to make the diagnosis of lymphoma definitively. Additional diagnostics in such cases can include histology, flow cytometry, or clonality assays. Unfortunately, to date there has been no systematic effort to compare the diagnostic utility of each of these methods, and the choice of which will be guided by the question being asked, owner finances, and the availability of sample. PARR is useful because the amount of sample required is small and the assay can be carried out on almost any tissue. On the other hand, as noted earlier, flow cytometry and histopathology can give more prognostic information than can PARR.

One example of the utility of PARR assays is in the diagnosis of indolent lymphoma. Indolent lymphomas that have been described in dogs include marginal zone lymphoma, mantle cell lymphoma and follicular lymphoma (B-cell origin) and T zone lymphomas (T-cell origin). These tumors can be difficult to diagnose by histology because they can retain follicular architecture and, especially in the early stages, can be mistaken for hyperplastic lymph nodes. Valli and colleagues used the presence of a clonal lymphocyte population assessment to reclassify several cases of indolent lymphoma that had originally been diagnosed as hyperplastic but contained clonally rearranged antigen receptor genes. In this study, detection of immunoglobulin rearrangements in cases of histologically diagnosed B-cell indolent lymphomas ranged from 80% to 100% (depending on the histologic subtype) and 63% of indolent T-cell lymphomas. It is important to note that neither diagnostic procedure (histology or clonality) is 100% specific or sensitive, so the final clinical diagnosis in such ambiguous cases would require systematic clinical follow up. Nonetheless, this study suggests that indolent lymphoma is likely underdiagnosed, and clonality assays may help to correct this. It would be useful to see a wider survey of cases diagnosed with lymphoid hyperplasia, coupled with clinical follow-up information, to determine to what degree indolent lymphoma may be underdiagnosed.

Clonality has also been used to demonstrate that the entity known as cutaneous lymphocytosis in dogs is likely an indolent cutaneous lymphoma. This study described 8 dogs presenting with cutaneous lesions that were composed of well-differentiated T cells and a histologically benign appearance. Nonetheless, in 7 of the 8 dogs, the lymphocyte population was shown to be clonal using PCR for T-cell receptor genes. Clinical follow-up of these cases showed that the disease did not regress in any of the patients, and progressed in several leading to euthanasia. Thus, the clinical progression in these cases supported the clonality results, and illustrates the utility of this kind of analysis.

**PARR can be used to determine the relatedness of lymphocyte populations**

Other than being used to distinguish reactive from neoplastic lymphocyte expansions, the PARR assay has been used in human medicine to detect lymphocytes that will eventually become lymphoma in pre-neoplastic tissues. The best example of such a study was the demonstration that the lymphoid hyperplasia seen in early *Helicobacter pylori* infection contains the B cells that eventually become neoplastic in MALT-lymphoma associated with this infection. This association was established by sequencing the hypervariable regions of B cells present in the pre-neoplastic lesions and demonstrating that one of these sequences was identical to the hypervariable region seen in the B-cell lymphoma that eventually developed. Our laboratory used this same principle to demonstrate the progression of a multicentric B-cell lymphoma to multiple myeloma. In this case, the dog originally presented with cytologically confirmed B-cell lymphoma and normal immunoglobulin levels. The dog was treated with a CHOP chemotherapy protocol and achieved
clinical remission. Several months later the dog re-presented with a monoclonal gammopathy, lytic rib lesions, and plasma cells in multiple sites. The progression from multicentric B-cell lymphoma to multiple myeloma had not been reported in the literature, so we wanted to determine if these were 2 separate tumors. Sequencing of the hypervariable region of the immunoglobulin gene demonstrated that the B-cell lymphoma and the multiple myeloma were derived from the same clone. Although sequencing is not a routine part of PARR analysis, it is possible to tentatively conclude that 2 tumors in the same patient are related if the clonal PCR product is the same size. Therefore, the relatedness of 2 lymphoproliferative disorders appearing sequentially in the same patient is relatively simple to investigate.

**PARR can be used to quantify tumor and monitor response to treatment**

The PARR assay, in its current form, is not useful for clinical staging, in that cases of stage III lymphoma with PARR positive blood had similar outcomes to cases with PARR negative blood. A modification of the PARR assay, however, has offered some useful insights into the response of dogs to treatment with chemotherapy. The investigators in this study sequenced the hypervariable regions of the immunoglobulin gene involved in each case of B-cell lymphoma they followed. They then designed primers that would anneal specifically to the hypervariable region of each tumor; this is in contrast to the primers used for the standard PARR, which bind to the conserved regions in order to detect as many tumors as possible with as few primer combinations as possible. The advantage of making tumor specific primers is that the sensitivity for detecting tumor cells increases substantially, and the number of tumor cells can be quantified.

Only 1 dog was tested before treatment, so it is not possible to evaluate the magnitude of the initial response to chemotherapy. Blood from all dogs were tested during treatment, and at all times tumor was detectable in the peripheral blood, even if the patients achieved full clinical remission. In most cases, the number of tumor cells ranged between 1 and 10 cells/µL during remission, but those numbers increased drastically when coming out of clinical remission—achieving concentrations of 1000 cells/µL. An increase in the number of cells in the blood correlated very well with increasing lymph node size and loss of clinical remission.

While the production of tumor-specific primers may not be practical or affordable for a large number of owners, detecting minimal residual disease using this method can be useful in a research setting. For example, if it can be shown that the degree of tumor burden reduction in the earlier stages of treatment is correlated with eventual outcome, then quantifying tumor burden can be a useful way of testing the efficacy of novel chemotherapies without needing to follow a patient through the entire course of treatment. Quantifying the response to treatment may also be a first step toward understand why some tumor types have an overall poorer outcome.

**PARR in the Diagnosis of Feline Intestinal Lymphoma**

Feline intestinal lymphoma presents a pervasive diagnostic dilemma. Severe inflammatory bowel disease can have many of the histologic features of intestinal T-cell lymphoma, including significant increases in the T-cell population of the lamina propria and an increase in intraepithelial lymphocytes. Even with full-thickness biopsy samples, some cases can be difficult to diagnose. Establishing that the intestinal lymphocytic infiltrate is clonal may help distinguish between the 2 entities.

Several studies have now been reported in which clonality has been assessed in feline patients with histologically diagnosed lymphoma and histologically diagnosed inflammatory bowel disease. Moore and coworkers examined 28 cases of intestinal
T-cell lymphoma and 9 cases of inflammatory bowel disease and found that 78% of the lymphoma cases had a clonal T-cell population (22 of 28), and none of the inflammatory bowel disease patients contained clonal T-cell populations. In a larger, more recent study, this same group looked at 120 cases that were chosen based on a histologic diagnosis of lymphoma and found the same sensitivity for detecting clonal T-cell populations (78%). Sensitivity for detecting clonal B-cell populations in the less common B-cell lymphomas was 50%. Although clonality assessment proved helpful in making diagnostic decisions, this study also highlighted the importance of histopathology in predicting outcome in cases of feline lymphoma—patients whose lymphoma was classified as large cell had a median survival of 1.5 months, whereas the small cell lymphomas had a median survival of 28 months. Cases with mucosal involvement only also had a significantly better survival time than did cases with transmural involvement. Nonetheless, clonality assessment on mesenteric lymph node or intestinal aspirates may be a useful way to establish malignancy if owners are unwilling or unable to pursue more invasive diagnostics.

Refractory celiac sprue in human patients provides a potential parallel to feline inflammatory bowel disease. This is a subset of celiac disease that does not respond to gluten withdrawal. Refractory sprue does not have the histologic features of lymphoma. Nonetheless, in one early study, a majority of patients with this disease who have a clonally expanded T-cell population in their small intestine experienced a malignant course, eventually dying of malnutrition. Thus, although they do not receive a histologic diagnosis of lymphoma, all the other features of the disease indicate malignancy. Subsequently, the presence of clonally expanded T-cell populations in patients with refractory sprue was shown to be 78% sensitive for the eventual development of histologically defined lymphoma.

While the etiology of feline IBD and intestinal lymphoma is probably not the same as human refractory sprue, experience in the latter suggests that using a histologic diagnosis of lymphoma as the gold standard may warrant reconsideration. As yet there are no studies in which clonality has been assessed as an independent prognostic factor in a series of cases of severe inflammatory bowel disease, but such studies would be clinically very useful. Ultimately, a combination of histology, immunohistochemistry, and clonality determination may prove to be necessary to obtain accurate diagnostic and prognostic information in cases of feline intestinal disease.

Additional Comments About Clonality Testing

There are several applications that are not appropriate for any of the tests described here. These are not intended as a first-line diagnostic procedure and often are not necessary. For example, although PARR testing can provide lineage information (B vs T cell) when it is positive, if the diagnosis of lymphoma or leukemia is unambiguous, flow cytometry, immunocytochemistry, or immunohistochemistry is a better test to determine the phenotype because more information can be obtained. Therefore, PARR testing is less useful for confirmed cases of lymphoma. These assays are also not intended as screening tests for healthy animals, as they have not been evaluated in this capacity. These assays can, however, provide a wealth of clinically useful information (in resolving ambiguous cases) and be useful research tools for better understanding the biology of hematologic malignancy.

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