1 An Overview of Molecular Cancer Pathogenesis, Prognosis, and Diagnosis

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Fundamentals of cancer biology

Cancer is a disease of the genome, arising from DNA alterations that dysregulate gene structure or function.[1,2] Damage to the cellular genome or altered expression of genes is a common feature for virtually all neoplasms.[3] Given that there is an inherent error rate in DNA replication, all multicellular organisms face the near certainty of developing a neoplasm if they survive long enough, as essential mutations for neoplastic transformation will eventually develop. Many mutations may be inconsequential, but cancer can develop when nonlethal mutations occur in a small subset of the coding and noncoding regions of the genome, perhaps affecting even just a few of the ~20,000 genes thought to comprise the mammalian genome.[4] There are many agents, in addition to deficiencies in DNA replication fidelity and error repair, that drive tumor formation. These agents include viruses, mutagenic chemicals, and radiation.

Unraveling the pathogenesis of cancer has not only helped us understand how a cell transforms into a tumor but has also promoted molecular tests that now help diagnose and provide prognoses for a variety of cancers in humans and animals.

Genetic injury

Genetic damage is a universal component of the pathogenesis of neoplasia, with somatic mutations in genes identified in 90% of cases, and germ line mutations identified in 20% of human neoplasia and both features found in a small percentage of neoplasms.[1,2,4] In some cases a single base pair mutation is sufficient to alter a critical amino acid, leading to altered protein function and an increased risk for neoplastic transformation.

Other types of mutations involve insertions, deletions, or duplications of gene segments. Structural chromosomal changes, such as translocations, which lead to chimeric transcripts or deregulation of gene expression through movement of promoters and enhancer regions adjacent to relevant genes can also drive malignant transformation. In addition, gene copy number increases or decreases (gene dosage) can also occur.

Epigenetics

DNA sequence mutations are not the only route to neoplasia.[5] Epigenetic mechanisms regulate gene expression without causing structural changes to the genome and also play a role in malignant transformation.[6] Epigenetic changes are reversible, heritable alterations of gene expression without mutation of the genome. Three main forms of epigenetic gene regulation include DNA methylation, histone acetylation, and microRNA expression.

Gene expression can be silenced, diminished, or increased by altering methylation patterns in the DNA. Aberrant methylation patterns, such as hypermethylation and hypomethylation, are common in a variety of neoplasms and are linked to abnormal gene expression levels. In particular, methylation of tumor suppressor genes leading to their suppression is recognized in a number of human cancers, including breast, colon, and renal carcinomas.

Histone proteins serve as spools that are wound with DNA strands to package cellular DNA into nucleosomes, which when

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compacted constitute the main components of chromatin. Gene expression can be altered by loosening or tightening the binding of the DNA strands to the histone proteins. Tightly wound DNA is either not transcribed or transcribed at lower levels than when it is more loosely associated with the histone proteins. Histone proteins that have been altered, often by acetylation, have a more relaxed binding pattern with their associated DNA and this facilitates gene expression.

MicroRNAs (miRNAs) are small, nontranscribed RNA molecules, approximately 22 nucleotides in length, that contribute to a complex termed the RNA-silencing complex, which binds to specific sequences in messenger RNA strands and directs gene silencing.[7] MiRNAs may regulate up to 30% of genes via posttranscriptional control. Amplifications and deletions of miRNAs are common in various human cancers and more work is needed to evaluate animal neoplasms.[8] Consequently, miRNAs can participate in tumor formation, as increased expression of miRNAs that target tumor suppressor genes leads to an increased tumor risk, as does decreased expression of miRNAs that target oncogenes.

Although none of these mechanisms alter the structure of the cellular genome they can significantly alter gene expression and have all been shown to be involved in neoplastic transformation.[8] A combination of mutation and epigenetic mechanisms is involved in the multistep process that leads to the emergence of a population of cells with a malignant phenotype known as malignant transformation.

Epigenetic perturbations may offer an explanation for some types of tumors associated with chronic inflammation or the presence of foreign material but no clear pattern of mutation, such as the fibrosarcomas and osteosarcomas that can develop in dogs infected with *Spirocirca lupi* or rare reports of sarcomas forming following metallic implants. Injection site sarcomas in cats seem to be associated with the inflammation induced by the vaccine adjuvant, although a subset of the affected cats have mutations in the tumor suppressor gene *p53*.[9–11]

Tumor heterogeneity, tumor progression, and clonal evolution

There are many pathways that can lead an individual cell to the malignant phenotype, but they all involve multiple genetic and epigenetic alterations. Tumor progression is a process by which cells that have developed a neoplastic phenotype acquire more characteristics that lead to malignancy and metastasis. Tumor growth starts clonally with a single cell that has undergone neoplastic transformation and the incipient tumor develops by clonal expansion of that cell. When a population of cells is identified as clonal it is a strong indicator that the population is neoplastic. However, this is not universally true, as clonal lymphocyte populations can be identified in some inflammatory conditions such as cases of feline infectious peritonitis and infections with *Ehrlichia* sp.

Initially, all cells in the neoplastic mass are identical, but due to the genetic instability the tumor cells acquire genetic and epigenetic changes that give rise to tumor heterogeneity (Figure 1.1).[12] In some instances this genetic change can be dramatic and sudden, as seen in chromothripsis, a phenomenon in which hundreds to thousands of genetic rearrangements can occur in one or a few chromosomes during a single event. Processes frequently affected include various types of DNA repair, telomere maintenance, DNA replication, and chromosome segregation. Some genetic changes are lethal to the affected cells, whereas others confer new functions and phenotypes, giving inherent growth advantages. Over time, the developing tumor mass becomes composed of a heterogeneous cell population and the tumor accumulates characteristics that make them more dangerous to the host.[12] As a neoplastic cell replicates, subclones emerge that are more locally aggressive, more likely to metastasize, and less responsive to therapy. Tumor progression has been attributed to a greater level of genomic instability in affected cells, which explains why early detection of the neoplasm is associated with improved prognosis. However, early detection is challenging in most clinical settings. By the time most malignant neoplasms are detected, using contemporary imaging methods, they most likely comprise a heterogeneous cell population, the neoplasms having completed the greater part of their growth. A single transformed cell must undergo at least 30 doublings to form a 1 g mass, an approximate cut-off for clinical detection, but only approximately 10 more doublings are needed to form a 1 kg mass (Figure 1.2). Since a 1 kg mass is regarded as a lethal tumor burden for a human, it is likely that fewer doublings are needed to form lethal cancers in small domestic animals.

Cancer is a multistep process and in some types of epithelial cancers there is a histologic phenotype that is characteristic of the different steps, including hyperplasia, dysplasia, and adenoma and carcinoma formation (Figure 1.3). Progressive accumulation of various mutations and epigenetic disturbances accompany these different histologic phenotypes. However, in some circumstances activation of an oncogene in an otherwise normal cell can lead to cell senescence and inhibit tumor formation.[13] Paradoxically, expression of an activated oncogene can lead to an exit from the cell cycle and termination of cell growth. Oncogene-induced senescence is consequently considered an authentic tumor suppressor mechanism *in vivo*. Ultimately, genetic and epigenetic alterations lead to a common pattern of features, or hallmarks, that distinguish neoplastic cell populations from normal cells. These hallmarks of cancer were initially proposed in 2000[14] and an updated review has been recently published.[15] Each of these hallmarks and their relevance regarding animal carcinogenesis, diagnosis, prognosis and therapy will be discussed in this chapter.

The hallmarks of malignancy

The six key elements of malignancies are shown in Figure 1.4.

Sustaining proliferative signaling: proto-oncogenes and oncogenes

Normal tissues are often capable of responding to injury or tissue loss by proliferation. Proliferation is driven by growth factors that bind specific cellular receptors, often tyrosine kinases, causing them to become activated and propagating a cascade of intracellular signals that culminate in mitosis. However, proliferation is controlled and limited, retaining normal structure and function. In cancer, proliferation is persistent and unregulated.

Cell proliferation and maturation are regulated by a subset of cellular genes. Proto-oncogenes are normal genes that encode proteins participating in one or more signal transduction pathways associated with important regulatory pathways.[16] Because of their central role in the life cycle of the cell, proto-oncogenes have been conserved throughout evolution and their DNA sequences vary little from yeast to humans. Disturbances in gene structure or expression can alter the cellular function of a proto-oncogene,

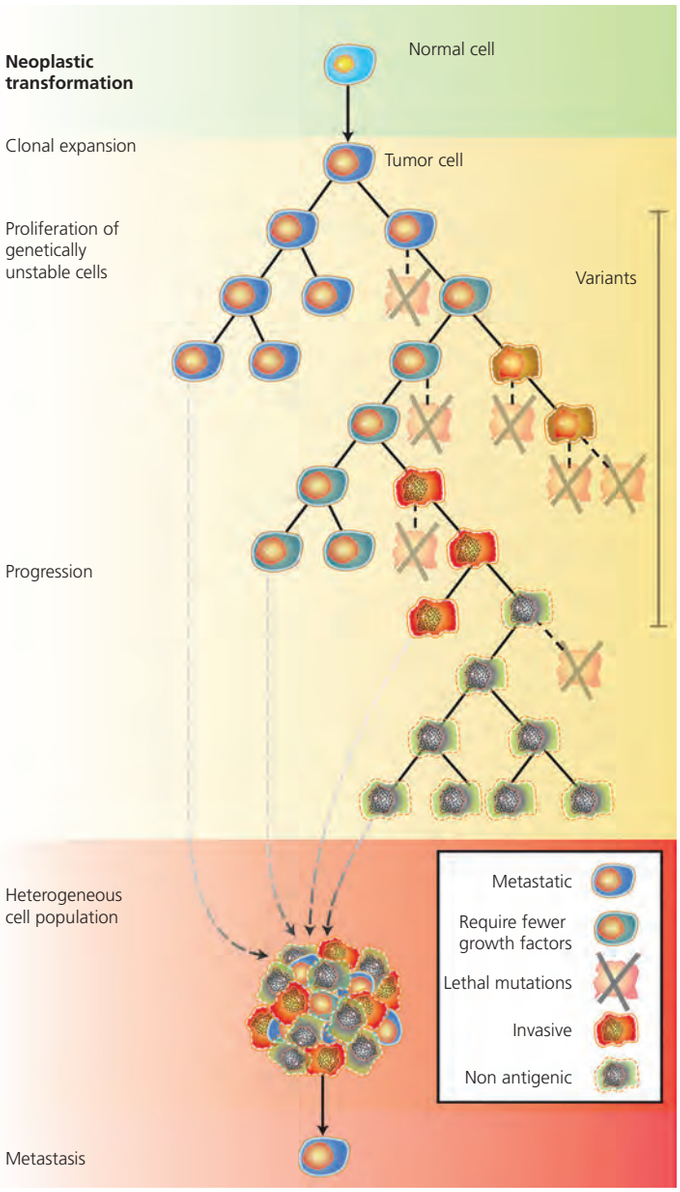

Figure 1.1 Tumor cell heterogeneity. Although tumors arise from a single cell, the inherent genetic instability in tumor cells gives rise to additional mutations and a heterogeneous population of cells with different genetic characteristics. Some mutations are lethal to developing cell lines and they die, but other mutations provide various features that facilitate the emergence of viable cell lines which may contain malignant characteristics including the ability to metastasize.

causing it to stimulate tumor formation. Once this alteration has occurred, the proto-oncogene is termed an oncogene. More than 100 oncogenes have been identified, and their numbers increase with continued genetic analyses of neoplasms. Typically the genes are referred to using a three-letter nomenclature. Many oncogenes were initially identified as part of the genome of retroviruses that caused cancer and they were named for the virus from which they were originally identified. For example, the proto-oncogene *myc* was originally isolated from the avian myelocytomatosis virus, and *ERB A* and *ERB B* were isolated from avian erythroblastosis virus. It should be remembered that it is not the oncogene, but the encoded protein that leads to cell transformation. The proteins encoded by oncogenes are referred to as oncoproteins.

Classification of oncogenes

Oncogenes can be grouped into five categories based on the types of oncoproteins they encode. These categories include growth factors, growth factor receptors, intracellular signal transducers, nuclear regulatory proteins (transcription factors), and cyclins. The proto-oncogene *sis* encodes the beta chain of platelet-derived growth factor (PDGF). When fibroblasts are infected with simian sarcoma virus, a retrovirus that contains the oncogene *sis*, there is an excess of sis oncoprotein produced. This protein leads to overstimulation of PDGF receptors on the cell surface in an autocrine fashion and can drive fibroblasts towards malignant transformation. In this circumstance the oncoprotein has a normal amino acid sequence but is produced in an abnormal, deregulated amount. Mutant forms of

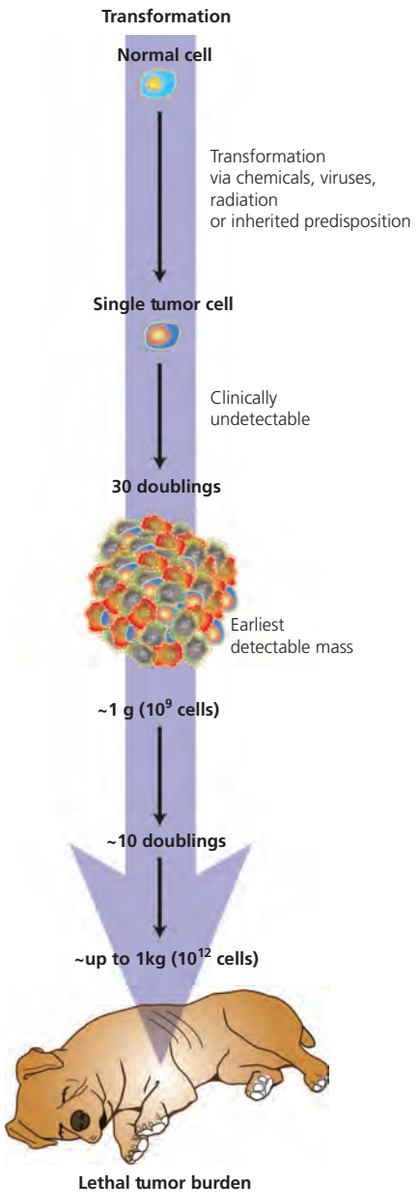

Figure 1.2 Tumor doubling. Tumor growth starts with a single cell that expands clonally. It takes approximately 30 doublings to form a 1 g mass, at which time most lesions can be detected clinically. Only 10 more doublings are needed to form a 1 kg mass, considered to be a lethal burden in humans. Likely, a smaller mass would be lethal in dogs or cats.

growth factors also occur, and they may inappropriately stimulate receptors by binding to them in an abnormal fashion.

Oncogenes may encode growth factor receptors. A typical growth factor receptor has three components: an extracellular growth factor binding domain, a transmembranous segment, and a cytoplasmic domain with kinase activity. Oncogene-encoded growth factor receptors, such as ERB B, are often truncated into a form that no longer has the extracellular receptor portion of the normal protein. These abnormal receptors do not require growth factor binding to be stimulated and are constitutively activated.

The intracellular signal transducers are located in the cytosol (e.g., ABL, RAF) or are membrane associated (e.g., RAS, SRC). Typically, these molecules are enzymes in the tyrosine kinase family. Point mutations or more gross structural alterations can constitutively activate these proteins, producing a level of activity that in turn leads to uncontrolled cell proliferation. Tyrosine kinase receptor activity is abnormal in several animal cancers, including mammary carcinomas and mast cell tumors, and several inhibitors of tyrosine kinase activity are being investigated, or are currently in use, as therapy.[17]

Transcription factors are nuclear proteins that regulate gene expression. They bind to selected sites on DNA in a complex with other proteins to facilitate gene expression. The oncoproteins encoded by *MYC*, *JUN*, and *FOS* are transcription factors that stimulate expression of genes necessary for cell division. Abnormal levels of expression, or mutations that alter the function of these proteins, can compromise growth control.

Cyclins are a series of proteins that precisely regulate movement through the cell cycle.[18] Individual cyclins are expressed for brief intervals at appropriate points in the cell cycle. The cyclins interact with and activate enzymes termed cyclin-dependent kinases (CDKs). The CDKs, in turn, activate proteins that are essential for progression through the cell cycle. Disruption in the function of cyclins leads to dysregulated control of cell replication. Several types of tumors in humans have been described with mutations in the genes that encode cyclins or CDKs.[19] Abnormal cyclin expression has been documented in canine and feline neoplasms, including cyclin A in mammary carcinomas and cyclin D1 in mammary tumors, squamous cell carcinomas, and, to a lesser extent, basal cell tumors.[20,21]

Alterations of gene structure (function)

Proto-oncogenes can be transformed into oncogenes following damage to their structure. Structural alterations can occur by mutation of individual nucleotides or alterations that may occur during changes to karyotype organization, such as chromosomal translocation events. Damage to individual nucleotides (i.e., point mutation) is the most common mutation sustained by proto-oncogenes. Chemical carcinogens and some forms of radiation exert their influence this way. Mutation of a single nucleotide can lead to the incorporation of a novel amino acid into a protein, and, if appropriately localized, the activity of the protein can be profoundly altered. One of the better characterized signal transduction pathways affected by mutation involves the *RAS* (derived from rat sarcoma virus) signaling pathway (Figure 1.5). All mammalian cells express three related RAS proteins, designated K-RAS, N-RAS, and H-RAS. Each of these proteins has a similar function, acting as an enzyme (GTPase) that phosphorylates GTP and acts as a switch regulating cell proliferation and survival. Any RAS family member can drive tumor development when they are mutated in specific codons.

Signaling via RAS begins when growth factors bind to specific cell surface receptors. This induces the receptors to dimerize, autophosphorylate, and undergo a conformational change. As a result of the conformational change, the receptors can interact with an associated bridging protein complex, which in turn transfers activation to the RAS protein located on the cytoplasmic surface of the cell membrane. Normally, the RAS protein is inactive and is bound to guanine diphosphate (GDP). When the RAS protein is stimulated it exchanges GDP for guanine triphosphate (GTP) and becomes activated. RAS protein is negatively regulated by GTPase-activating protein (GAP), a protein that enhances the hydrolysis of RAS-bound GTP to GDP. Activated RAS attracts a serine/threonine kinase, termed RAF (derived from rapidly accelerated fibrosarcoma), to the

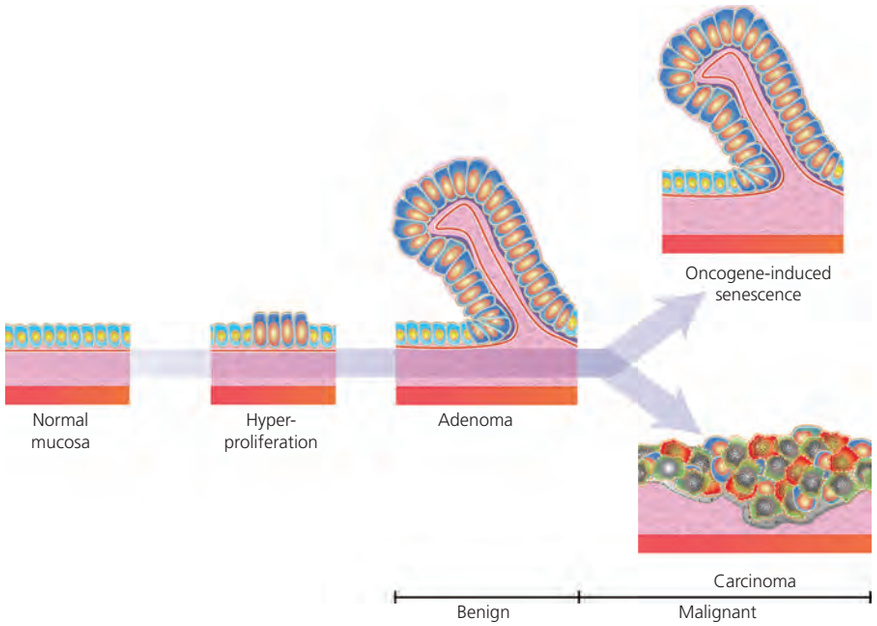

Figure 1.3 Histologic evolution of a carcinoma. The cellular development of cancer is a multistep process in most cases. There are several phenotypic steps in the evolution of colonic carcinoma including areas of hyperproliferation/hyperplasia, then dysplasia, followed by adenoma and, in a subset of these, carcinomas. The distribution of different phenotypes is not uniform throughout individual lesions and regions with different phenotypes may be seen when a lesion is sampled. Spontaneous growth arrest or resolution of tumors may occur, as overexpression of oncogenes can drive cellular senescence in some circumstances.

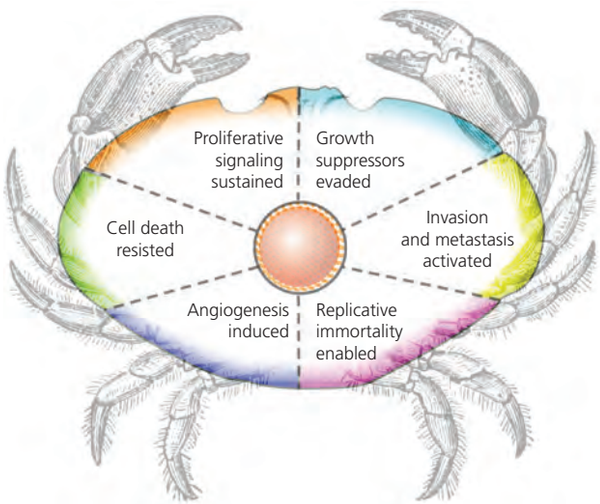

Figure 1.4 Hallmarks of cancer. These features are key elements of malignancies.

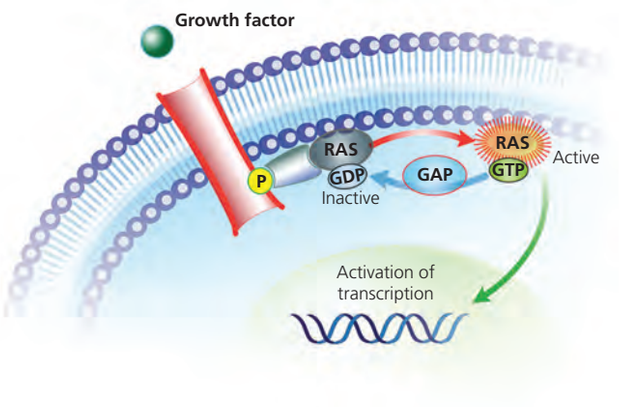

Figure 1.5 RAS oncogene. An example of proto-oncogene activation is shown in this overview diagram of the typical RAS signaling cascade. When a growth factor binds to its transmembrane receptor the receptor becomes activated. Receptor binding triggers activation of RAS via a bridging protein. Inactive RAS, which is bound to guanosine diphosphate (GDP), becomes activated via an exchange (red arrow) for guanosine triphosphate (GTP). Activated RAS acts through intermediary proteins to activate mitogen-activated protein kinases (MAP kinases) that lead to altered nuclear signal transduction and cell mitosis. In normal cells GTPase-activating protein (GAP) stimulates dephosphorylation of activated RAS to an inactive form that curtails signaling (blue arrow). Mutant RAS does not interact with GAP normally and consequently stimulates cell proliferation in an unchecked fashion.

inner aspect of the cell membrane, where RAF is phosphorylated by membrane-associated kinases. Activated RAF in turn phosphorylates mitogen-activated protein (MAP) kinases, and these kinases migrate to the nucleus, where they stimulate the synthesis of nuclear transcription factors, such as MYC. These transcription factors stimulate the expression of genes that cause resting cells either to enter the cell cycle and divide or to alter their differentiation or synthesis patterns. Control of cell signaling is fine-tuned by the balance of a matrix of stimulatory and inhibitory influences. Consequently, cell proliferation can be driven not only by stimulatory events, but also by the disruption of inhibitory pathways. The *RAS* gene offers a good example of this abnormality as well, as mutations in *RAS* typically impair the ability of the GAP protein to dephosphorylate RAS, causing it to remain in the active state, leading to a constitutive activation of RAS driving cell proliferation.

Mutations in *RAS* are common in human tumors. In some surveys 16–30% of all tumors are reported to bear *RAS* mutations.[22] Specific sites of human tumors bearing *RAS* mutations in 20% or more cases include the biliary tree, large intestine, small intestine, pancreas, and the skin. Evaluation of specific RAS isoforms reveals that they selectively appear in different tumor types.[23] Specific isoforms can be quite common in individual tumor types. Mutations in K-*RAS* are found in 22% of all human tumors with an incidence of 8% for N-*RAS* and 3% for H-*RAS*. N-*RAS* is mutated in human melanoma, acute myelogenous leukemia, thyroid neoplasia, and multiple myeloma. K-*RAS*, involved in pancreatic, colorectal, thyroid, and lung carcinomas and acute myelogenous leukemia among others, is the most frequently mutated *RAS* gene in human neoplasms. Approximately 61–90% of human pancreatic carcinomas contain a mutation in K-*RAS*. Mutations in *RAS* are not nearly as well documented in animals as humans, as most veterinary studies are limited by small sample sizes, but *RAS* mutations are reported to be less frequent in a variety of sarcomas of dogs and cats than they are in humans.[24–32] *RAS* mutations have been noted in several tumor types, including K-*RAS* mutations in approximately 15–25% canine lung tumors of different types[33–35] and N-*RAS* mutations in canine leukemia, where up to 25% of cases of acute myeloid leukemia or lymphoid leukemia have mutations.[36,37]

Altered RAS signaling is one pathway among several alterations in signal transduction that lead to cellular hyperproliferation. The ability of malignant cells to proliferate in a sustained fashion can also derive from the impact of an excess of growth factors. Mitogenic signals can be generated in an autocrine fashion in circumstances where malignant cells release growth factors that bind their own receptors and initiate signaling that leads to proliferation. In a more complicated paracrine fashion, malignant cells can signal nearby stromal cells, causing them to release mitogenic factors that in turn stimulate the tumor cells. In some cases, mutations lead to multiple copies of growth factor receptors on an individual cell, leading to excessive intracellular signaling in the face of a normal amount of growth factors. Alternatively, malignant cells may become independent of growth factors when they have mutated growth factor receptors or signal transducers such as RAF that are constitutively activated stimulating the downstream signaling cascade.

Another more clinically relevant example of abnormal proliferation caused by a mutation is found in canine and feline mast cell tumors. The proto-oncogene c-*KIT* (also referred to as CD117 and stem cell factor receptor) encodes the transmembrane tyrosine kinase receptor KIT. Following binding of its ligand, stem cell factor, the resulting signal transduction is involved in survival, maturation, migration, and proliferation of mast cells and other hematopoietic cells. Mutations in the c-*KIT* gene, in particular small internal tandem repeats in exons 11 and 12, lead to an abnormal receptor that does need ligand binding as a prerequisite for activation, and consequently it constitutively stimulates signal transduction.[38] There are several other mutations that also can activate c-KIT.[39] Abnormal localization of the gene product in the cytoplasm in neoplastic mast cells is also associated with increased proliferation of the affected cells and likely a poorer prognosis for dogs bearing these genetic alterations than those without such changes.[40] The presence of internal tandem repeats (ITRs) can be detected by a PCR-based assay, which identifies neoplastic mast cells in approximately 30% of malignant canine cutaneous mast cell tumors.[41]

Identification of the c-*KIT* ITR mutations can be used for diagnostic purposes, to assess prognosis and to monitor response to therapy. Not all malignant mast cell tumors bear this mutation so its role is not entirely clear. However, targeting the tyrosine kinase activity of c-KIT is recognized as a useful approach in a variety of human and veterinary applications. There are new chemotherapeutic agents that inhibit the tyrosine kinase activity of c-KIT now available in veterinary medicine, Palladia® (Zoetis) and Kinavet® (AB Science) that are used to treat cutaneous mast cell tumors in dogs as well as other malignancies with aberrant c-KIT activity.

Chromosomal translocation

Chromosome translocation results in the movement of one chromosome to another chromosome, or exchange of segments between different chromosomes in reciprocal translocation events. This process can deregulate transcription by bringing in close juxtaposition active cellular promoters and proto-oncogenes. One example occurs in both humans and mice: the proto-oncogene *MYC* is overexpressed in lymphomas of B-cell lineage due to translocation of an active cellular promoter from the immunoglobulin gene to another chromosome that contains MYC.

In some circumstances the functions of proto-oncogenes are altered by chromosome translocation. A well-characterized example of this process occurs in the distinctive translocation that produces the Philadelphia chromosome found in up to 95% of cases of human chronic myelogenous leukemia (CML), but it is not specific, as it is found in approximately 25% of cases of human acute lymphoblastic leukemia and rarely in acute myelogenous leukemia.[42] This rearrangement involves an exchange of chromosomal segments between the distal ends of human chromosomes 9q and 22q, resulting in a derivative chromosome 22 in which a fragment of the proto-oncogene (*abl*) on human chromosome 9 becomes juxtaposed to the breakpoint cluster region (*bcr*) on human chromosome 22. This fusion yields an abnormal hybrid gene that encodes a chimeric messenger RNA containing information from both genes. When the message is translated, a hybrid protein, termed a fusion protein, results. In this circumstance the fusion protein is an active oncoprotein that results in elevated tyrosine kinase activity, which is crucial to its oncogenic potential. To halt progression of the leukemia the BCR-ABL kinase antagonist imatinib mesylate (Gleevec®) is used in therapy. As a competitive inhibitor of the tyrosine kinase activity, the drug serves to block the proliferative signal given by the BCR-ABL protein, preventing the formation of new abnormal cells. While this effect does not apply to all patients with CML it is sufficiently effective that Gleevec therapy is now considered standard of care for patients with the Philadelphia chromosome.

Although CML is rare in domestic dogs, numerous cases have been shown to present with evolutionarily conserved cytogenetic change (structural chromosomal changes) resembling the Philadelphia translocation in human cases. In the canine aberration, termed the "Raleigh" chromosome, the canine genes *BCR* (dog chromosome 9) and *ABL* (dog chromosome 26) are juxtaposed and produce a fusion protein[43,44] (Figure 1.6). These data suggest that treatment with Gleevec or other tyrosine kinase inhibitors could be an option for therapy of BCR-ABL-positive canine cases. The Raleigh chromosome has since been identified in additional canine leukemias, including chronic monocytic and acute myeloblastic cases.[43,45] More study will be needed to determine the frequency of this translocation in canine CML and other leukemias. Cytogenetic analysis has been used to monitor response to therapy and revealed a marked reduction of circulating neoplastic cells with the translocation following therapy (vincristine and prednisolone) in one case of canine chronic monomyelocytic leukemia.[46]

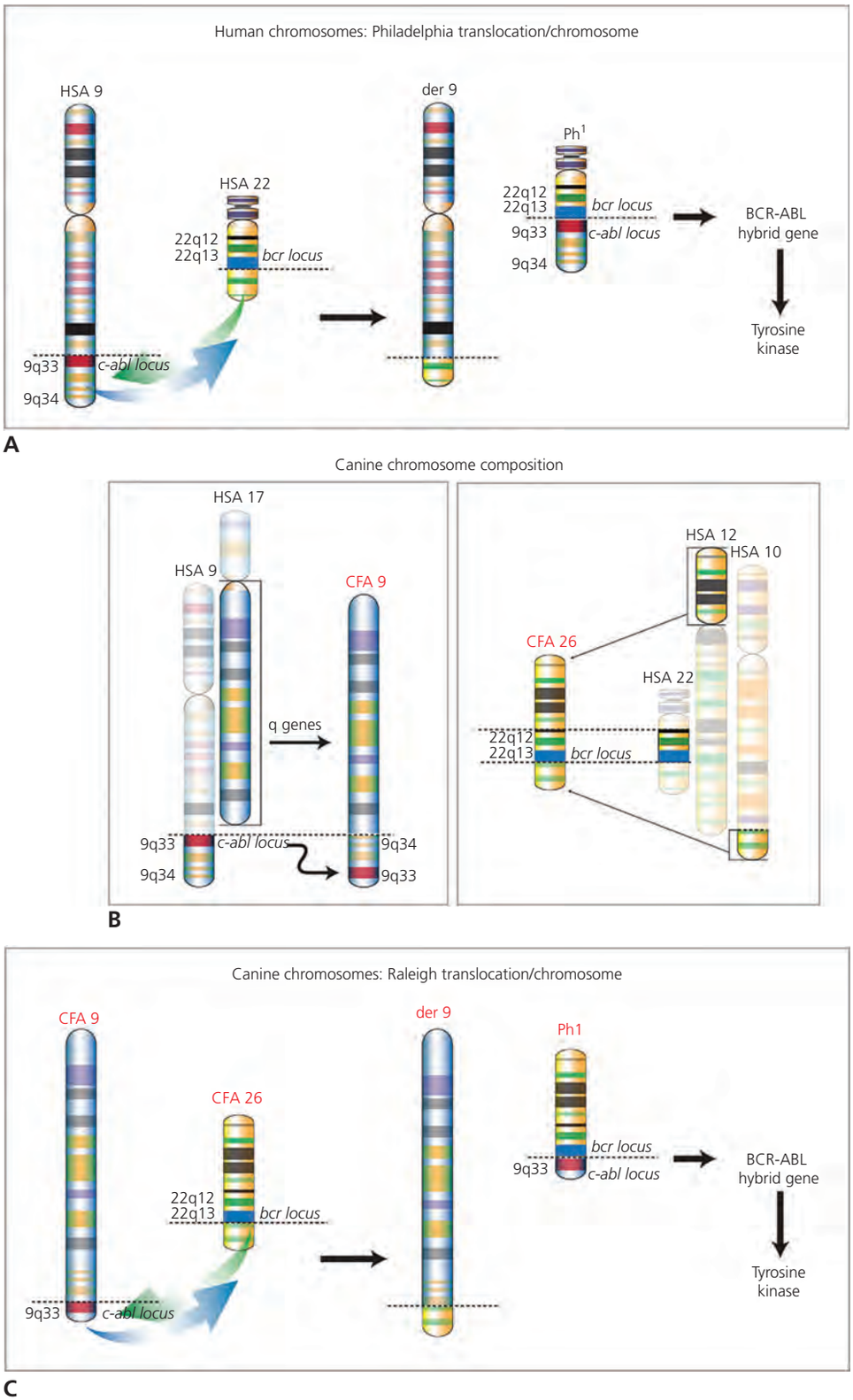

Figure 1.6 Raleigh chromosome. In human leukemias, a characteristic chromosome is the Philadelphia chromosome (Ph). This derivative chromosome, also referred to as the Philadelphia translocation, is the result of reciprocal translocation between human chromosomes 9 and 22, bringing the genes *BCR* and *ABL* (panel A) together to create activation of the tyrosine kinase of c-ABL. The evolutionarily conserved translocation (panel B) has been detected in canine leukemias, the result of a reciprocal translocation between regions of dog chromosomes 9 and 26 (shown in panel C). The canine event is referred to as the Raleigh chromosome and has been detected in chronic myelogenous leukemia and chronic myelomonocytic leukemia. Within these patients, the frequency of cells with the Raleigh chromosome has been shown to decrease in response to tyrosine kinase inhibitor treatment, indicating that its presence may be used to monitor cytogenetic remission.

Alterations of gene expression

Gene expression can be altered via gene amplification or deletion, promoter insertion, gene translocation, and regulatory miRNA. Each of these genetic mechanisms can lead to the deregulated synthesis of normal (i.e., wild type) proto-oncogene proteins. Given that many proteins encoded by proto-oncogenes function to stimulate cell proliferation, it is obvious that their overexpression would have the potential to lead to cancer formation.

For reasons that are not well understood, tumor cells often sustain excessive rounds of localized DNA replication that can result in the formation of multiple copies (hence the term gene amplification) of the same gene or genes. The duplicated genes (or amplicon) may be found in small chromosome-like structures termed double minutes, or may form concatenated (i.e., like beads on a string) structures within a chromosome that can be identified as homogeneously staining regions (HSRs). HSRs are portions of chromosomes that lack the characteristic banding pattern found in normal chromosomes. In general, gene amplification leads to the overproduction of the products encoded by the genes within the amplicon, increasing the potential for neoplastic transformation.

MicroRNAs

An additional and relatively new mechanism for influencing gene expression involves miRNA.[47] There are more than 1000 types of miRNA expressed in essentially all cells. Primarily they bind to message RNA and promote degradation of messenger RNA, thereby preventing translation and influencing gene expression. Specific miRNAs are associated with some human neoplasms, particularly colorectal cancer and chronic lymphocytic leukemia, but more work is needed to evaluate the role of miRNAs in neoplasia of veterinary interest.

Oncogenic viruses

In veterinary medicine there are several important oncogenic retroviruses. These include feline leukemia virus, bovine leukemia virus, and avian leukosis virus. When certain oncogenic retroviruses, known as nonacute retroviruses, insert their genome into cellular DNA, the regulatory elements normally controlling viral gene expression also affect the expression of nearby cellular host genes. Viruses and cells have two major types of these regulatory elements, enhancers and promoters. Both elements stimulate gene expression, but differ in their functional attributes. Promoters stimulate adjacent genes but must be properly oriented (upstream of the gene) to facilitate expression. Enhancers stimulate promoter activity, but unlike promoters, their capacity to stimulate transcription is orientation independent. Since, in general viral promoters and enhancers are more potent than their cellular counterparts, they can significantly increase and thus dysregulate cellular gene expression. When a retrovirus integrates within a region of genomic DNA flanking a proto-oncogene, transcription of the proto-oncogene can be deregulated, leading to cell transformation. In most circumstances, viral insertion events affect the regulation of gene expression, not the function of the gene or genes affected.

There is also a second type of oncogenic retrovirus, called acute transforming retroviruses, that are typically replication defective, but carry an oncogene derived from a host's proto-oncogene in their genome and rapidly transform infected cells. Feline sarcoma virus is an example of this type of virus.

Oncogenic DNA viruses generally differ from oncogenic retroviruses in that they contain authentic viral genes that encode oncoproteins capable of transforming infected cells. These viral proteins often act by interfering with the proteins encoded by tumor suppressor genes. Bovine papillomavirus and several primate herpes viruses are examples of oncogenic DNA viruses of veterinary importance.

Tumor proliferation

The phenotypic manifestation of the first hallmark of cancer (Sustaining Proliferative Signaling) is typically recognized as an increase in mitotic rate. Many tumor types have an increased rate of mitosis, enumerated by counting the number of mitotic figures observed in a specific number of high-power (40 × objective) microscopic fields (the mitotic count in 2.37 mm^2; see pp. 944–945) although there are several other immunohistochemical methods to assess cell proliferation, including staining for proliferating cell nuclear antigen (PCNA) or Ki67 that may be more readily interpreted than counting mitoses. The presence of an increased number of mitoses is considered in the diagnosis of benign or malignant forms of various tumors. For example, the mitotic count of soft tissue sarcoma in dogs is currently used as one of the principal criteria in the assessment of malignancy and to predict the likelihood of recurrence and or metastasis. However, increased mitoses alone are not necessarily an indication of malignancy since the tissue of origin requires consideration. For example, canine histocytomas are characterized by a high mitotic count yet these neoplasms typically regress spontaneously. A similar observation can be made for transmissible venereal tumors of dogs. There also are examples of tumors with a low proliferation index that behave aggressively, such as maxillary fibrosarcoma in dogs. In some cases, a high mitotic count may not correlate with the cellular proliferation of a mass. This occurs when there is arrest of mitosis, leaving elevated numbers of mitotic figures at a given point in time, but in the absence of completed cell division, no increase or a limited increase in cell population.

Evading growth suppressors

A second hallmark of cancer involves the ability to bypass potent growth inhibitory signaling.[48] The major agents of growth inhibition are a group of 25 or more tumor suppressor genes. Tumor suppressor genes play a critical role in the control of normal cell growth. They serve as the "brakes" to cell replication. When tumor suppressor genes are inactivated, cells lose regulatory control of cell proliferation. A single, intact, functional copy of a tumor suppressor gene is sufficient to maintain control of cell proliferation. When both alleles are lost or damaged the affected cell has a high risk of neoplastic transformation.

The discovery of tumor suppressor genes arose from the study of certain human families that presented with a significantly increased incidence of specific tumor types. Genetic analysis of these "cancer families" revealed that some family members were born with one mutated allele of a critical gene, and when a second mutation in the functional allele occurred spontaneously the affected individual was at a very high risk to develop neoplasia.[9] The first tumor suppressor gene to be discovered this way was the retinoblastoma or *RB* gene. Loss of both alleles led to the development of retinoblastomas in affected children. Loss of function of both alleles of another tumor suppressor gene, *TP53*, was identified in other kindreds and termed Li–Fraumeni syndrome. These individuals are at elevated risk for a variety of mesenchymal neoplasms, but mutated *TP53* is frequently identified in many human malignancies.[49,50]

At least one heritable cancer syndrome (renal carcinoma and nodular dermatofibrosis, or RCND, of German shepherd dogs) has been described in dogs with an autosomal dominant inheritance.[51,52]

The heritable factor for this syndrome maps to dog chromosome 5 (CFA 5), and specifically to the tumor suppressor gene folliculin (*FLCN*, previously *BDH* gene). This region in the dog chromosome overlaps a corresponding region in the human chromosome that was recently described as the heritable factor for the corresponding human disease (Birt–Hogg–Dubé syndrome). Inactivation of this tumor suppressor gene is critical to the development of this syndrome. It is probable that other comparable syndromes to those that are described in humans will eventually be identified in companion and laboratory animals, but it is unlikely these will account for more than 5–10% of all cancers in animal cases.

There are a few examples in dogs in which a specific cancer (melanoma, histiocytic sarcoma) is associated with the absence or decrease of tumor suppressor gene(s). It is likely there are inherited susceptibilities to cancer in many breeds. Examples are lymphoma in the golden retriever[53] and parathyroid neoplasia in keeshonds.[54] Ongoing cytogenetic studies are being conducted to assess the genetic basis of breed-related alterations in tumor risk.

To understand the relevance of tumor suppressor gene inactivation in tumorigenesis, a brief review of the normal cell cycle and how it differs from that in neoplastic cells is warranted. The cell cycle consists of a series of biochemically distinct temporal periods that prepare the cell for division.[55] Following mitosis, a cell may withdraw from the cell cycle and enter a quiescent stage (G_0 phase) or continue to proliferate. In most instances, cells in G_0 can be recruited into the cell cycle when necessary by interactions with one or more growth factors.

The first growth phase of the cell cycle is termed G_1, for the gap in time between mitosis and the next round of DNA synthesis. The duration of this phase of the cell cycle is more variable than the duration of other phases, ranging from 6 to 12 hours. During G_1, RNA and proteins are synthesized but no DNA is formed. Synthesis of DNA occurs in the S phase, during which the DNA content of the cell increases from diploid to tetraploid. The duration of the S phase is similar in all cells and takes from 3 to 8 hours. The S phase is followed by the G_2 phase, a pause of about 3–4 hours that precedes mitosis. During the G_2 phase the cell has two complete sets of diploid chromosomes. Mitosis, or the M phase, takes no more than an hour to complete in normal cells.

The ability of cells to restrict or slow their movement through the cell cycle is regulated. This can be observed when normal cells in tissue culture are damaged, for example in irradiation-induced genetic damage.[56] Irradiated cells in the early stages of the cell cycle respond by halting their progress prior to the S phase; this pause in the cell cycle has been termed the G_1/S checkpoint. During the pause, DNA that has been damaged by irradiation can be repaired before mutations are passed on to the genomes of daughter cells. In cells in which tumor suppressor genes are absent or not functioning properly, genetic damage is left unrepaired, which often leads to genetic instability in the daughter cells and additional oncogenic events. A similar checkpoint is present at the transition between the G_2 and M phases of the cell cycle.

The best characterized of the tumor suppressor genes are *TP53* gene, activated only in cases of genetic damage or hypoxia, and the retinoblastoma (*RB*) gene, which is constitutively involved in the cell cycle.[57,58] Both of these genes encode nuclear phosphoproteins that regulate cell cycle progression. When the RB protein (pRB) is in its hypophosphorylated form it inhibits entry of the cell into the S phase of the cell cycle by binding a transcription factor transcription factor E2 promoter-binding-protein (E2F) that stimulates mitosis-promoting genes (Figure 1.7). When a cell is stimulated to divide, pRB is hyperphosphorylated by cyclins, causing it to release E2F, which enables cells to enter the S phase. Following the S phase, pRB is dephosphorylated and is, once again, able to bind E2F and inhibit entry of the cell into the S phase. In tumor cells, the ability of pRB to bind E2F is disrupted and the checkpoint is eliminated. For example, oncogenic DNA viruses (discussed later) can disrupt cell cycle control by synthesizing viral proteins that block the uptake of transcription factors by pRb protein.

The *TP53* gene encodes a nuclear phosphoprotein that can regulate movement of the cell through the cell cycle. Although this phosphoprotein (p53) is not involved in regulation of the normal cell cycle, it plays an important role in cells that have sustained genetic damage or in conditions of hypoxia. In the absence of functional p53 these genetically damaged cells may undergo neoplastic transformation. Through mechanisms that are not well understood, p53 can detect when a cell sustains genetic damage by UV light, irradiation, or carcinogenic chemicals and then arrests the entry of the cell into the S phase from the G_1 phase of the cell cycle to allow time for the repair of cellular DNA damage via growth arrest and DNA damage-inducible protein (GADD45), which allows for DNA repair and cyclin-dependent kinase inhibitor

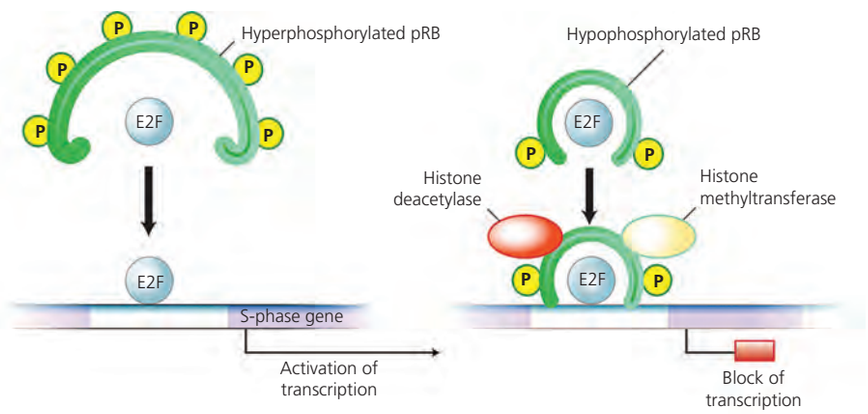

Figure 1.7 Tumor suppressor protein pRB. When pRB is hyperphosphorylated by cyclin-dependent kinases it releases members of the transcription factor E2F family that then bind to DNA and stimulate progress from G_1 into the S phase of the cell cycle. When pRb is hypophosphorylated it binds E2F and interacts with histone-modifying proteins, histone deacetylase and histone methyltransferase, inhibiting progress through the cell cycle. When the ability of pRB to bind E2F is disrupted by mutations or viruses, the checkpoint is eliminated and cells may then proliferate in an uncontrolled fashion.

1 (CDKN1 or p21) that inhibits phosphorylation of cell cycle-related kinases. If the extent of DNA damage is too excessive, p53 can promote cellular apoptosis. Although normally a short-lived protein, after genetic damage, p53 is modified in a way that causes it to have a significantly longer half-life. P53 will then accumulate in the nucleus, leading to cell cycle arrest by activating transcription of genes that inhibit specific cyclin-dependent kinases and prevent the phosphorylation of the RB protein.

Other effects include expression of genes involved in DNA repair or apoptosis. Cells carrying mutated *TP53* genes or cells infected with oncogenic DNA viruses that alter the function of p53 do not arrest before entering the S phase of the cell cycle and are less likely to undergo apoptosis (Figure 1.8). Affected cells can continue to replicate with damaged DNA, and those that do not develop lethal genetic changes are at risk for acquiring additional genetic damage, leading to neoplastic transformation.

The canine DNA sequence for *TP53* is 87% identical to the human sequence and has a similar intracellular role.[59,60] Because mutations in *TP53* occur in a high proportion (approximately 50%) of some types of human neoplasms, the frequency of *TP53* mutations in animals has been examined. Mutations in *TP53* of dogs have been detected most often in osteosarcomas, where they can be detected in approximately 40% of cases.[61] In canine melanomas mutations have been identified in several tumor suppressor genes,

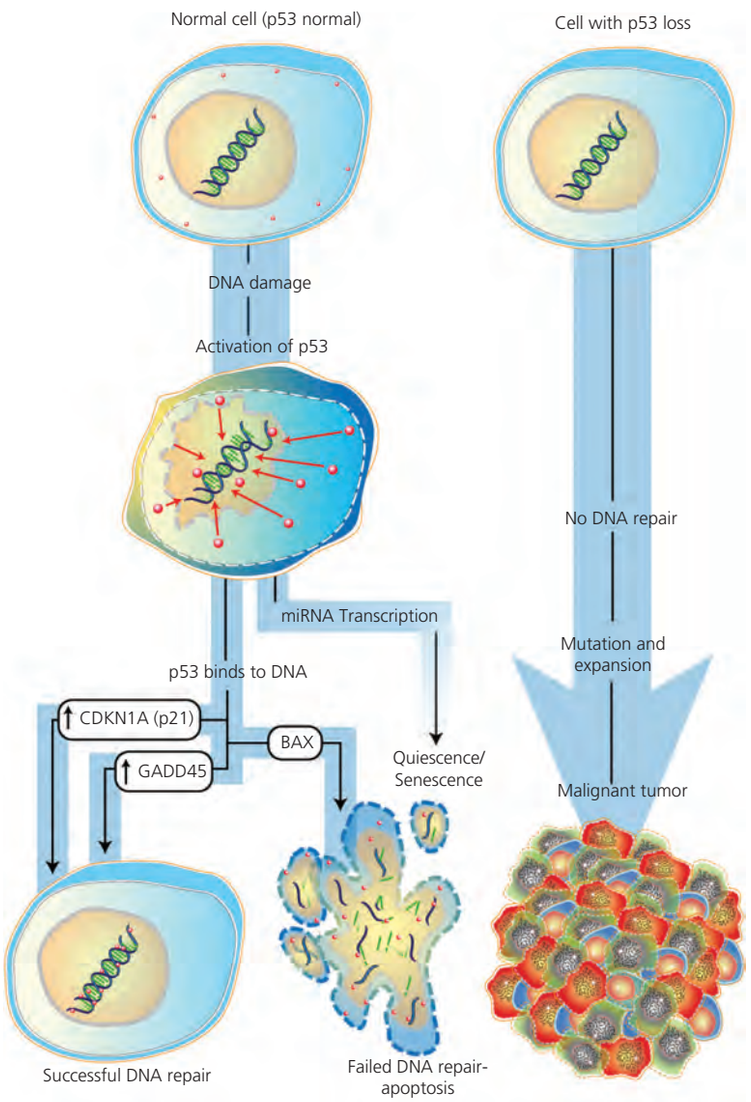

Figure 1.8 Tumor suppressor protein, p53. The tumor suppressor gene (*TP53*) encodes a protein, p53, which is crucial for repair or apoptosis of genetically damaged cells. Signaling is mediated through growth arrest and DNA damage-inducible protein (GADD45) that allows for DNA repair and cyclin-dependent kinase inhibitor 1 (CDKN1 or p21) that inhibits phosphorylation of cell cycle-related kinases and arrests progression through the cell cycle. When genetic damage is too severe to be repaired p53 can initiate apoptosis via activation of the apoptosis-stimulating gene *BAX*. Alternatively, activation of p53 in severely damaged cells can also trigger transcription of microRNAs (miRNA) that drive cell senescence. When *TP53* is damaged by chemicals, radiation, viruses, or inherited defects, p53 production may be abrogated or a mutant p53 protein produced. Mutant p53 does not function normally and affected cells with damaged DNA do not arrest the cell cycle to enable DNA repair. Mutated cells are able to progress though the cell cycle giving rise to daughter cells with mutations and eventual tumor formation. Thus, when the gene *TP53* is damaged or absent, tumor suppression is compromised.

including *TP53*.[62] *TP53* is also found to have altered expression or to be mutated infrequently in several types of canine and feline neoplasms, including canine mammary tumor,[63] canine and feline squamous cell carcinoma,[21,64] and canine mastocytoma.[65] Cytogenetic analysis has demonstrated loss of several tumor suppressor genes in canine histiocytic sarcoma.[66,67]

Resisting cell death

Genes that control programmed cell death play a significant role in tumor development when they fail to function normally.[68] B-cell lymphomas serve as examples of the importance of the genes that control apoptosis. These tumors are characterized by an increased expression of the gene *BCL2* (derived from B-cell lymphoma 2), which blocks apoptosis. *BCL2* is only one of a family of genes that participate in the regulation of apoptosis. The ability of oncoproteins such as BCL2 to block cell death pathways may enable cells that have sustained genetic damage to escape mechanisms that would stimulate normal cells to undergo programmed cell death. Alternatively, neoplastic lymphocytes that overexpress *BCL2* can persist and slowly form lymphoid masses, unlike normal lymphoid cells that have a finite lifespan. Consequently, cells eluding apoptosis could multiply and are at risk to accumulate additional genetic damage that can heighten malignancy. Overexpression of *BCL2* has been demonstrated in feline lymphoma, but was not associated with prognosis.[69]

Enabling reproductive immortality

Essentially unlimited replicative potential is a key feature in the formation of malignancies.[48,70] While normal cells are capable of no more than 60–70 doublings before they become senescent and die, malignant cells must be free of these growth constraints in order to continually grow and expand. The principal mechanism by which cells replicate without entering senescence involves the enzyme telomerase. Telomerase is typically inactive in somatic cells but is active in stem cells, germ cells, and cancer cells. Activation of telomerase is recognized in about 85–95% of human cancers.[70,71] Telomerase is a ribonucleoprotein complex that includes a reverse transcriptase, an RNA template, and additional proteins (Figure 1.9). This complex is responsible for adding back short sections of DNA of 50–200 nucleotides that are lost from the chromosomal telomeres (specialized nucleoprotein structures at the ends of chromosomes) during normal DNA replication cycles. Continued loss of the ends of chromosomes in normal cells will eventually trigger senescence and apoptosis by activating tumor suppressor genes encoding *TP53* and *pRb*. Cells that lack normal tumor suppressor gene activity do not arrest at appropriate cell cycle checkpoints, leading to acquisition of various mutations. Telomerase activity has not been extensively studied in veterinary oncology. There is evidence from one canine study that nearly all lymph nodes (97%) with a histologically confirmed diagnosis of lymphoma (various subtypes) had detectable telomerase activity and activity was greater than that seen in normal lymph nodes.[72] More work in this area is needed.

Inducing angiogenesis

Solid neoplasms depend on the blood vessels and supporting stroma that they recruit from adjacent tissue for their survival and growth.[48,73] Without vascularization, growing tumor masses are limited to about a 1–2 mm diameter. In normal tissue and in neoplastic masses angiogenesis is regulated by competing pro- and anti-angiogenic signaling. The transition to a pro-angiogenic status

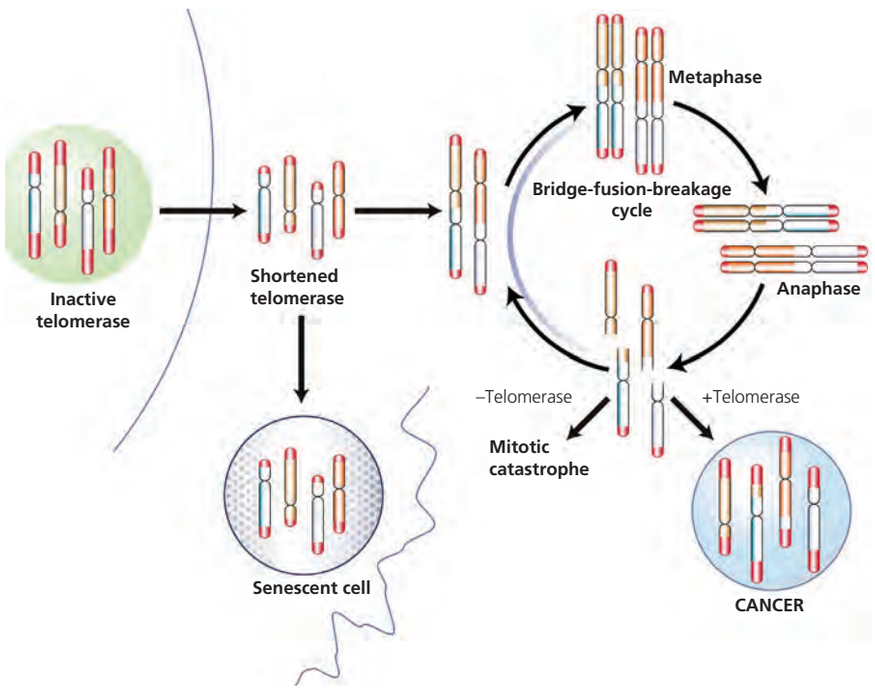

Figure 1.9 Telomerase. Telomerase is an enzyme that enables cells to replicate in an unlimited fashion. Cells with repressed telomerase activity such as somatic cells eventually reach senescence after a finite number of mitoses and undergo cellular senescence, a permanent growth arrest state, or apoptosis. Telomerase adds back short sections of DNA that were lost from the chromosomal telomeres (repetitive nucleoprotein sequences at the ends of chromosomes) during normal DNA replication cycles. Cells with telomerase activity such as stem cells, germ cells and cancer cells can potentially proliferate indefinitely and are potentially immortal.

occurs when anti-angiogenic signaling is overwhelmed. Tumor cells secrete growth factors such as vascular endothelial growth factor A (VEGF-A) and various types of fibroblastic growth factors (FGF) or stimulate other cells to release angiogenic factors that stimulate the vessels and supporting stroma in tumors (Figure 1.10). There are many cell types that participate in angiogenesis. In addition to the tumor cells and adjacent stromal elements, bone marrow–derived cells, primarily cells of the innate immune system, macrophages, neutrophils, and mast cells, and also myeloid precursors infiltrating at the margins of neoplastic lesions release angiogenic factors contributing to the ingrowth of new vessels.[73] Angiogenesis was once thought to be significant primarily when robust tumor growth was occurring, yet is now known to begin early in the process of tumorigenesis and is evident in preneoplastic and benign lesions.[74] The processes of angiogenesis and stroma formation are similar in tumors and wound healing, leading to the conceptual description of tumors as nonhealing wounds. There are some distinct differences in the structure and function of the vessels that are formed during each process.[75] In tumors, the blood vessels are poorly differentiated and are not distributed uniformly through the tumor. Tumor blood vessels tend to be more tortuous and dilated than normal vessels, with gaps in the endothelium rendering them persistently permeable, unlike vessels in healing wounds that have a transient phase of permeability.[76] Since most tumor cell entry into the bloodstream occurs between gaps in the endothelial cells it is likely that metastasis is facilitated by these abnormal vessels. Increased interstitial pressure due to the permeable vessels and the lack of lymphatics to carry away the leaked fluid lead to edema formation. This edema and the resultant interstitial fluid pressure tend to collapse the vessels within the tumor, thus obstructing local blood flow. The density of vascular supply to tumors is frequently minimally adequate and is deficient in arteriolar supply, in particular. As a result, irregular blood flow and perfusion cause localized areas of hypoxia and anoxia, leading to apoptosis or necrosis.

Without angiogenesis, tumors have to rely on cellular diffusion to provide needed nutrients and eliminate waste products. Angiogenesis plays an essential role in sustained tumor growth, as well as metastasis. Recruited endothelial cells do more than provide perfusion, as endothelial cells also secrete growth factors that can stimulate tumor cell growth. Angiogenesis, measured as the density of the microvasculature within a tumor, has been shown to be a significant prognostic indicator for some human neoplasms such as those of the lung and breast.[77,78] Because of this powerful effect on tumor growth, angiogenesis is an area of particular interest in tumor biology. Angiogenesis by itself, however, is not an indication of malignancy as even benign neoplasms have the ability to stimulate vascular growth.

Tumor stroma is composed of non-neoplastic connective tissue, blood vessels, and inflammatory cells.[48] While the vasculature is an essential component of stroma formation because of its nutrient support of the neoplasm, the greatest proportion of the tumor stroma is nonvascular. The noncellular components of the stroma include collagen types I, III, and V, glycosaminoglycans, proteoglycans, fibronectin, fibrin, and plasma proteins. Fibroblasts, endothelial cells, and inflammatory cells are the principal cellular constituents. Initially, the tumor stroma resembles granulation tissue with a high density of blood vessels and smaller numbers of fibroblasts. The persistent permeability of tumor vessels allows a continued leakage of macromolecules, engendering a perivascular deposition of fibrin that serves as scaffolding for migration of host stromal cells and tumor stroma formation. As this tissue matures, collagenous stroma predominates and vascularity diminishes, creating a desmoplastic or scirrhous response. For reasons that are unclear, the amount of

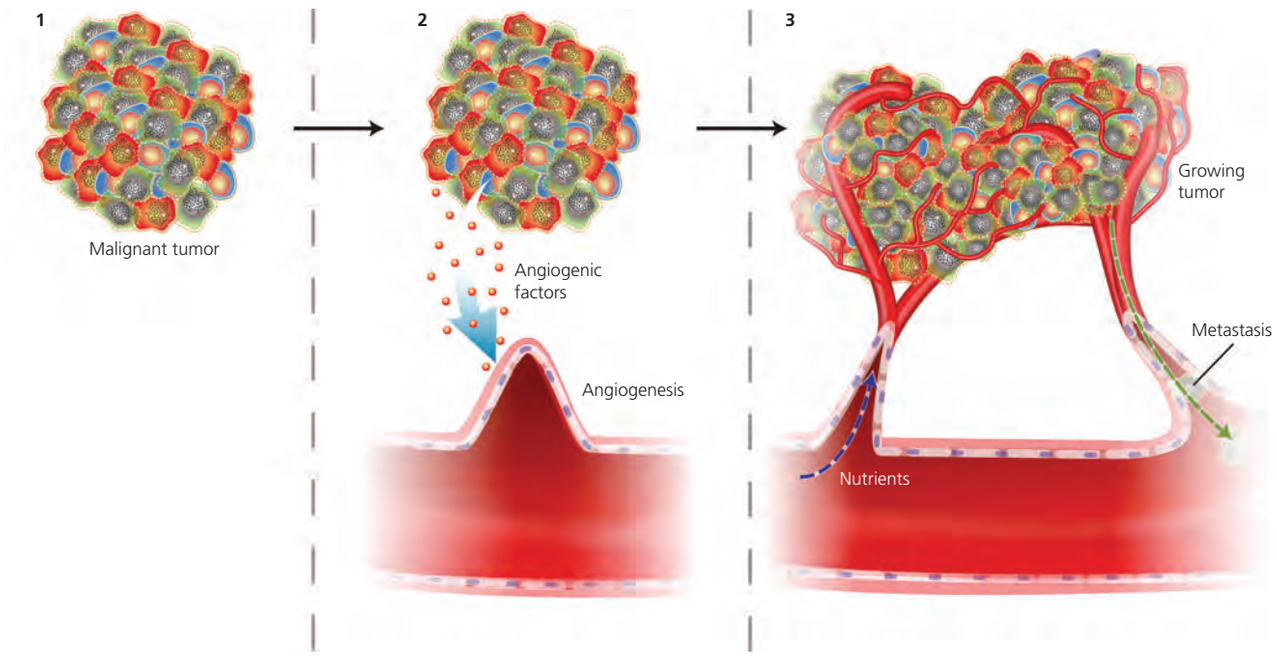

Figure 1.10 Angiogenesis. Tumor angiogenesis is a critical step for the growth of the primary mass as well as metastatic masses. Tumor cells release angiogenic factors that stimulate budding of new vessels that deliver oxygen and nutrients to the growing tumor cells and provide venous drainage to remove waste products. New vessels also provide an avenue for vascular metastasis. Tumor size is limited to approximately 1 mm in diameter without supporting blood vessels.

stroma produced by different neoplasms varies considerably. Certain carcinomas such as gastric, urothelial, and mammary carcinomas are more prone to develop desmoplasia (scirrhous response) than other neoplasms. The resultant masses are very firm to the touch, and the stroma can comprise a larger proportion of the mass than the tumor cells do.

A newly emerging understanding of epithelial–mesenchymal interactions is clarifying the role of fibroblasts and other stromal elements in tumor growth and desmoplasia. Fibroblasts or myofibroblasts adjacent to carcinomas, termed cancer-associated fibroblasts, have a fetal-like phenotype that differs from fibroblasts in other parts of the body.[79] In some cases tumor cells secrete extracellular vesicles containing genetic information that can re-program mesenchymal stem cells to produce extracellular matrix.[80] Tumor-associated fibroblasts stimulate tumor cell proliferation via release of growth factors and proteases in response to cytokines signaling by neoplastic epithelial cells and can also facilitate angiogenesis, invasion, and metastasis. Overall, there are multiple interactions between cancer cells and adjacent stromal elements and inflammatory cells that can facilitate tumor growth and metastasis in complex patterns (Figure 1.11).

Activating invasion and metastasis

Metastasis is an inefficient multistep process, and only a very small proportion of cells are able to complete the process.[81–84] Once a malignancy develops, a metastatic subclone may arise within the tumor through the process of tumor progression. In epithelial tumors a common initial step supporting invasion is the loss of intercellular adhesion due to impaired activity of cell adhesion factors such as E-cadherins. Conversely, cell surface adhesion molecules such as N-cadherin, associated with cell migration during development, may be re-expressed. A series of steps occur during the transition from noninvasive (*in situ*) carcinoma to a metastatic carcinoma (Figure 1.12). Initially, metastatic cells penetrate the basement membrane in a two-step process. First, metastatic cells attach to the basement membrane via laminin and fibronectin receptors among others; subsequently, they secrete hydrolytic enzymes (proteases) that degrade the basement membrane. The next step involves locomotion. Tumor cells migrate into the extracellular matrix facilitated by the release of products secreted by the tumor cells and host inflammatory cells, particularly macrophages. Connective tissues are unequally susceptible to invasive processes. Hyaline cartilage, for example, contains inhibitors of matrix degrading enzymes and is highly resistant to invasion. Eventually metastatic cells encounter a blood or lymph vessel. Entry of tumor cells into the bloodstream or lymphatics, termed intravasation, is only possible after attachment of tumor cells to the basement membrane of the vessel and degradation of this barrier. Extravasion is facilitated by the increased permeability of the new, but abnormal, blood vessels formed within tumors compared to vessels in normal tissue. Tumor cells can then pass through the junctions between adjacent endothelial cells or pass directly through the intact endothelium. Lymphatic vessels pose less of a barrier to entry than blood vessels because lymphatic vessels lack a basement membrane.

The mere presence of tumor cells in vessels does not ensure that those cells will eventually give rise to metastatic populations. Once tumor cells enter the vasculature, they encounter the array of host cells involved in immune-mediated killing of tumor cells. To survive, the tumor cells must evade intense scrutiny by the host immune response. One way tumor cells evade host defenses is by interacting with blood components, such as platelets and fibrin, to form thrombi. When the tumor cells are enclosed by fibrin, they may be protected from recognition by the immune system and have a better chance to survive in the hostile environment of the blood. Extravasation of surviving tumor cells may occur in a directed, nonrandom fashion.

Epithelial–mesenchymal transition

Recent research has revealed a process termed the epithelial–mesenchymal transition (EMT)[85] that regulates the acquisition of capabilities needed to facilitate invasion and metastasis. In keeping with the view that cancer cells do not possess unique behaviors or

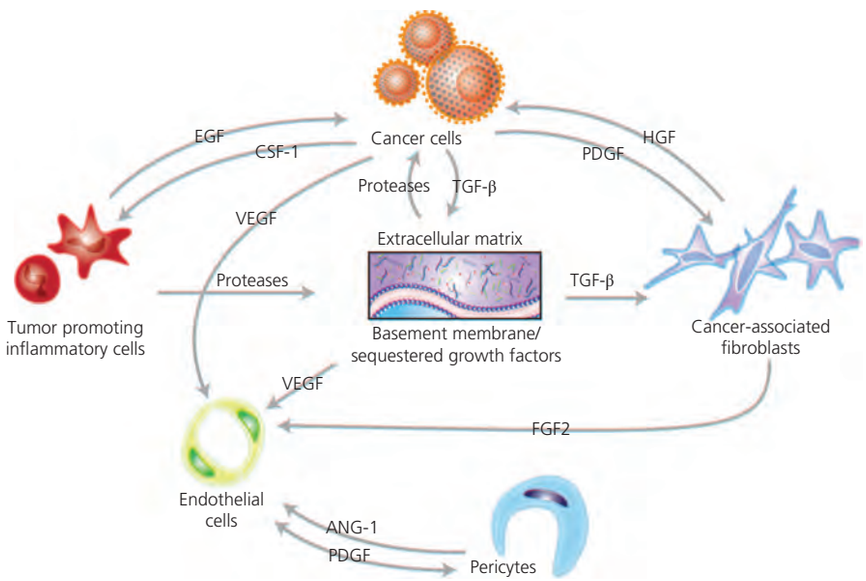

Figure 1.11 Tumor cell and stromal interactions. Interactions between tumor cells and the adjacent stroma play a key role in many facets of tumor evolution. Multiple interactions between the tumor cells and stromal and inflammatory cells mediate tumor growth, differentiation, and metastasis, as well as host tissue responses.

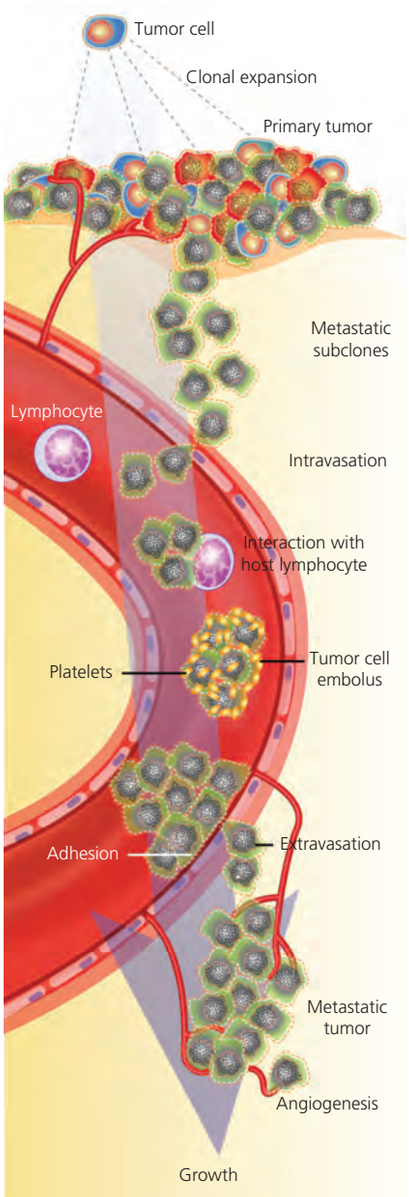

Figure 1.12 Metastasis. Invasion and metastasis are hallmarks of malignant tumors. Each step in the process of metastasis can involve progressive histological changes and/or molecular alterations, some of which are illustrated here.

capabilities, but rather co-opt normal cellular processes for the detriment of the host, EMT involves the emergence of capabilities normally only expressed in embryogenesis or wound healing. Malignant epithelial cells may stably or transiently acquire the ability to invade, resist apoptosis, and invade locally through this process. In addition to the altered behaviors, a phenotypic alteration can also be observed. Typical polygonal epithelial cells can be changed to spindle-shaped fibroblast-like cells with the ability to locomote, resist apoptosis, and secrete enzymes that digest the local stroma. The mechanisms that facilitate the gene expression driving these changes are not well characterized, but studies suggest that interaction with other cells in the local environment enable or facilitate the transition. Local environmental factors are likely to play an important role. Histologic and immunohistochemical examination of the invasive margins of some carcinomas reveals that EMT occurs only at the leading edge of the neoplasm and not in the center of the mass.

Malignancies have been likened to villages, rather than monotypic masses of proliferating cells, because of the important interactions between stromal and inflammatory cells and the tumor cells.[48] Cell–cell interactions include secretion of factors by mesenchymal stem cells in response to signals released by the tumor cells that enhance invasion of the tumor cells. Inflammatory cells, particularly those of the innate immune system, can also facilitate tumor development. Macrophages have been shown to facilitate breakdown of the extracellular matrix to enhance invasion. In an experimental model of mammary carcinoma, tumor-associated macrophages secrete epithelial growth factor to support mammary carcinoma growth and the tumor cells secrete CSF-1 to stimulate the macrophages. This interaction also facilitates intravascular invasion and metastasis.[86]

Once malignant cells have disseminated into the bloodstream, lymph flow, or other spaces they still have to undergo a series of steps to establish a viable mass at a new site. Recent studies have elucidated the predilection for certain tumors to metastasize to particular organs. Paget's 1889 theory of "seed and soil," which explains why certain tumors tend to metastasize to a particular set of organs, holds true today, although the mechanisms are now becoming clear. Some tumor cells are guided to particular organs because they bind to tissue-specific endothelial cell surface markers. In other tumor types, the cells bear receptors to specific chemokines and home towards organs that release these chemokines; they are less likely to be found in organs that do not release these chemokines.[87]

The newly extravasated tumor clone must next acquire a blood supply. A new vascular network is needed not only to provide nutrients to the growing tumor, but also to carry away waste products. Once a metastatic tumor has established a proper vascular supply, its growth may be limited by inhibitory growth factors, by a restrictive growth environment, or by a cytotoxic response by the host.

There are three principal pathways of metastasis: lymphatic, hematogenous, and direct extension.

Lymphatic metastasis

Lymphatic invasion occurs primarily at the periphery of the tumor. Lymphatic vessels offer little resistance to penetration by tumor cells because they lack a basement membrane. Clumps or single-cell tumor emboli may be trapped in the first lymph node encountered, or they may traverse or bypass lymph nodes to form a more distant metastasis, a condition termed skip metastasis. Tumor cells are usually first detected histologically in the subcapsular region of the lymph nodes. Based on extensive studies in humans and limited data from animals, carcinomas have a predilection for metastasis by the lymphatic route compared to sarcomas, although the mechanism is unclear. In dogs with mammary cancer, regional lymph nodes appeared to function as good filters since bypassing the node was found to be uncommon. An enlarged local lymph node does not necessarily mean metastasis has occurred; the node may be enlarged due to hyperplasia and/or metastasis. In most cases, an enlarged lymph node draining a region with malignancy is probably no longer immunologically effective, but there is no consensus regarding the value of the removal of such an enlarged node. Fine-needle aspiration by an experienced cytologist or biopsy for histologic examination is necessary to distinguish lymphoid hyperplasia from metastasis and to allow appropriate clinical staging and treatment planning.

Hematogenous metastasis

Tumor cells can enter the blood directly by invasion of blood vessels or indirectly via the lymphatic system that connects with venous tributaries at sites such as the thoracic duct and subsequently enter into the vena cava. Distribution of hematogenous metastases can be initially explained by the hemodynamic theory based on circulatory anatomy. Briefly, metastatic emboli from primary tumors spread via the vena cava drainage (mammary, skin, soft tissue, bone, thyroid tumors) unless they arise in the abdominal organs (gastrointestinal, splenic, and pancreatic tumors) drained by the portal vein. The majority of tumor cells are arrested in the first capillary bed they encounter. The first capillary filter of vena caval drainage is the lung, and the liver is the first microvascular field draining the portal vein system. From those sites, tumors can spread to secondary microvascular filters, such as bone marrow. However, in the human, and to a lesser extent also in domestic animals, preferential metastatic sites can also be explained by organ tropism or the "seed and soil" hypothesis described earlier.

Direct extension metastasis

The coelomic surfaces, covered with a film of fluid, are an ideal site for metastatic seeding. Neoplastic cells shed from a primary tumor can survive when implanted onto the serosal surfaces of body cavities or organs. Implantation of tumor cells in serous cavities is often accompanied by an accumulation of fluid. Peritoneal or pleural carcinomatosis is associated either with a primary tumor within a coelomic cavity (ovarian or pulmonary carcinoma) or with metastases from carcinoma elsewhere in the body (e.g., mammary carcinoma). Pleuritic carcinomatosis in dogs and cats with mammary carcinoma was found to be invariably associated with the presence of pulmonary metastasis.[88,89] The spread of mesotheliomas is often restricted to the same coelomic cavity as the site of origin. Mechanical transfer can also occur via contaminated surgical tools or fine-needle aspirates.

There are two naturally occurring clonally transmittable malignancies that can be spread by contact. These include transmissible venereal tumor of dogs, in which tumor cells are transferred by coitus but can then spread to other sites in some animals, most of whom are likely immunocompromised.[90] A more recently described example is the disease of Tasmanian devils called devil facial tumor disease, in which bite wounds appear capable of transmitting malignant mesenchymal cells that can eventually metastasize.[91]

Successful metastasis

A tumor cell that has arrived at a new site following dissemination still has a number of challenges before it can expand from a micrometastasis into a metastatic mass. First, the interactions with local stromal cells and inflammatory cells may no longer be present and, in some cases, the new cell cannot expand in the absence of these supporting elements. There may be a considerable time required for sufficient new mutations to develop that enable the micrometastasis to proliferate in the new environment. Inadequate ability to support angiogenesis is a common limitation. Primary tumors may secrete inhibitory factors that suppress growth of the tumor at the new sites. In such circumstances surgical or chemotherapeutic removal of the primary mass can stimulate growth of previously undetected microscopic metastases. These circumstances would explain the sudden appearance of metastases sometimes years after the primary lesion has been removed or treated.

Metastasis site selection

Since extravasation requires adhesion to endothelial cells or underlying basement membrane, tumor cell attachment may be directed to specific sites by receptor and ligand interactions. The release of chemokines can also direct some types of tumor cells to specific organs.[87] Clearly, the lung and the liver are common sites of metastasis for many types of neoplasms. Organ tropism seems to play a role in metastasis of melanomas in dog and human with frequent spread to the brain. Prostatic carcinomas in dog and human frequently spread to bones. Occult micrometastases are frequently present in these unique sites and in lymph nodes and lung at the time of the primary tumor diagnosis of these tumors.

Most osseous metastases have intertrabecular growth. Only at advanced stages do osteolysis or endosteal and periosteal bone formation occur.[92] The frequency of osseous metastasis may be underestimated when the bones are not carefully checked radiographically or during the postmortem examination. Bone metastasis in dogs is frequently underestimated, likely from failure to carefully examine the cut surface of long bones. In a detailed postmortem study, examination of transected bones revealed that 17% of dogs with visceral metastasis from a variety of neoplasms also had skeletal metastasis.[92] Dogs with epithelial malignancies with visceral metastasis also had bone metastasis in 24% of cases, often affecting more than one bone. Common sites are flat bones, including the ribs, the vertebrae, and the metaphyseal region of the long bones. Frequently, multiple sites in the bones are affected, and metastatic involvement of bone in this study was always accompanied by concurrent soft tissue metastasis.

Most primary tumors responsible for bone metastases in the dog are carcinomas, including those of mammary gland,[93,94] lungs,[92,94,95] and prostate.[92,94] Metastasis to bone from mammary[96] and pulmonary carcinomas,[97] along with various individual case reports have been reported in cats.[94]

A particularly impressive example of tissue tropism for metastasis is found in the pulmonary carcinomas of cats. These neoplasms can metastasize widely, but have a predilection for spread to the distal toes. The underlying mechanisms are not known.[98]

Paraneoplastic syndromes

Paraneoplastic syndromes are defined as systemic complications of neoplasia that are remote from the primary tumor.[99] Frequently, the effects of the paraneoplastic syndrome can be more injurious than the associated malignancy and may be the reason the animal was brought to the veterinarian. The common paraneoplastic syndromes in veterinary medicine are listed in Table 1.1.

Paraneoplastic syndromes may serve as diagnostic aids or as specific tumor markers for treatment response and failure. These effects are generally unrelated to the size of the tumor, the presence of metastasis, or the physiologic activity of the tissue of primary origin. Most of the examples in veterinary medicine are associated with the production of native (true) hormone from cells that normally produce that hormone or from the "ectopic" production of a hormone-like peptide by tumor cells that are not in an endocrine organ. Excessive insulin production by neoplastic islet cells and production of a parathormone-like peptide by neoplastic lymphocytes or apocrine cells of the canine anal sac are examples of each category, respectively. In order to definitively establish that a paraneoplastic condition is a result of a specific neoplasm, one or more criteria have to be met. These criteria include the following: (1) concentration of the product (e.g., calcium) decreases after removal or treatment of the neoplasm (e.g., an anal sac carcinoma that was

Table 1.1 Selected veterinary paraneoplastic syndromes and associated neoplasms

Paraneoplastic syndromes	Associated neoplasms
Endocrine	
Hypercalcemia of malignancy	Lymphoma
	Apocrine gland carcinoma of anal sac
	Mammary carcinoma
	Thymoma
	Others
Hypoglycemia	Hepatocellular carcinoma
	Salivary gland carcinoma
	Leiomyoma/leiomyosarcoma
	Plasma cell tumor
	Lymphoma
	Others
Ectopic ACTH	Pulmonary carcinoma
Cutaneous	
Paraneoplastic pemphigus	Lymphoma
Alopecia	Pancreatic carcinoma (cat)
	Others
Necrolytic migratory erythema	Glucaconoma
Hematologic	
Hypergammaglobulinemia	Multiple myeloma
	Lymphoma
Anemia	Many neoplasms
Erythrocytosis	Renal carcinoma
Neurologic	
Myasthenia gravis	Thymoma
	Others
Peripheral neuropathy	Insulinoma
	Others
Renal	
Glomerulonephritis	Multiple myeloma
	Polycythemia vera
	Others
Gastrointestinal	Gastroduodenal ulceration
	Mast cell tumors
	Gastrinoma
Miscellaneous	
Hypertrophic osteopathy	Pulmonary carcinoma
	Other thoracic masses
	Urinary bladder rhabdomyosarcoma

secreting the trophic hormone PTH-rp is removed and serum calcium decreases); (2) product concentrations are maintained after removal of the normal gland that controls the concentration of that product (e.g., calcium concentration remains high following removal of a parathyroid gland); (3) a positive arteriovenous concentration gradient of the hormone exists across the tumor; and (4) synthesis and secretion of the product by the tumor *in vitro* occurs. In veterinary medicine, the first criterion – decreased concentration of product after tumor ablation – is most commonly used to diagnose a paraneoplastic syndrome.

The pathogenesis of paraneoplastic syndromes is thought to result from several processes. De-repression of a gene may result in production of a substance with biologic activity. In fact there may be many products from a given tumor, but only the active substances are detectable. One example would be the production of hormone precursors that do not exhibit activity unless metabolized (i.e., prohormone production). Ectopic receptor production by a tumor has also been reported and accounts for displaced activity of a humoral substance (e.g., thymoma and acetylcholine receptor production). The third theory is termed "forbidden contact" and implies that there is exposure to substances that are normally sequestered from the body (i.e., antigens of normal or neoplastic origin) and therefore are recognized by the immune system as foreign. Immune complex formation from antigenic exposure to these normally sequestered antigens may result in a physiologic or pathologic event leading to clinical signs. Examples include anaphylaxis, coagulopathies, vasculitis, glomerulonephritis, and hemolytic anemia.

Endocrine syndromes are a frequent manifestation of paraneoplastic disease. Protein hormones, hormone precursors, or cytokines may be produced or metabolized by tumors. Some types of hormones, such as steroid hormones, thyroid hormone derivatives and catecholamines, are produced exclusively by tumors originating from glands that normally produced these substances. The frequency of biologically active peptide-producing neoplasms can be explained by the fact that most cells secrete peptide hormones that function in paracrine signaling. These peptide hormones may be expressed in excess when cells become malignant and their numbers increase by clonal expansion.

Cancer cachexia is one of the more common paraneoplastic syndromes encountered in veterinary and human medicine. Affected animals are anemic, weak, easily fatigued, lose weight, and have diminished immune function. There are characteristic metabolic changes associated with this syndrome that affect carbohydrates, proteins, and lipids.[100] Growth of the tumor occurs at the expense of the host. Increased serum lactate levels and insulin levels characterize abnormal carbohydrate metabolism. There is a loss of muscle mass and hypoalbuminemia in affected animals because protein catabolism exceeds protein synthesis. Typically these animals will have profound muscle wasting and prominent boney protuberances but surprising amounts of abdominal or subcutaneous fat. Starvation and parasitism drain adipose reserves first, resulting in serous atrophy of fat whereas protein and muscle loss are recognized at later stages of these conditions. Wound healing and immunity are also affected by altered protein metabolism. The loss of protein in cancer patients develops because amino acids are redirected from protein synthesis into gluconeogenesis. Although tumor cells are less capable of using lipids for energy than normal cells, cancer cachexia also promotes fat utilization. Cancer cachexia has been attributed to the effects of tumor necrosis factor, interleukins 1 and 6, and interferons gamma and alpha.[100] It affects 50–80% of human patients with malignancies.

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Veterinary cancer incidence and molecular approaches to diagnosis and prognosis

Genetics and cancer

Inherited tumor risk in dogs

Although it is not possible to accurately determine the number of animal cancers each year, several previous studies have attempted to determine overall incidence rates in various countries, especially for purebred dogs.[1–5] Overall, these broad population-based studies have highlighted those dog breeds that present with higher incidences of certain cancers. Such studies also reinforced the possible bias that may be introduced into regional surveys of pets by factors such as breed popularity, the impact of small population sizes, and diseases that impact breed longevity. There are indeed numerous purebred dogs that have an elevated incidence of certain cancers, compared to the overall purebred dog population, such as histiocytic tumors in Bernese mountain dogs, hemangiosarcoma in golden retrievers, keratoacanthomas in Kerry blue terriers, cutaneous mast cell tumors in pugs, T-cell lymphoma in boxers, urothelial carcinoma in Scottish terriers, and osteosarcoma in Irish wolfhound.

The development of many breeds of modern dog over a relatively short period of time (200–300 years) was based primarily on generations of inbreeding and line breeding, intended to maximize conformity to a breed standard. Selective breeding for phenotypes resulted in a very broad range of morphological types (breeds) within which genetic variation has been reduced. Sampling of genetic variation across all breeds would provide a level comparable to that present across human populations.[6] In individual breeds, however, the level of genetic diversity is variably restricted.[7] The process of breed formation over just the past two centuries is estimated to have caused a seven-fold greater reduction in genetic diversity than did the early domestication process, which lasted thousands of years.[8] This is compounded further by the use of popular sires and gene pool decline during the twentieth century. As a result, numerous traits in purebred dogs are a consequence of variants in just a few genes. Since many of these phenotypes are characteristics of the particular breed, their presence in the breed had been positively selected, resulting in high frequency of the genes that cause them. With such intense selection, it is perhaps not surprising that there are now over 350 inherited diseases recognized in dogs and this number will expand annually. While some diseases have simple inheritance patterns, cancers are likely to be more complex. In some breeds the genetic background of the breed may predispose to a higher risk for specific cancers, or cancers in general. It seems likely certain breeds may have suppressor genes missing and they would have multiple different types of cancer, while others may have a promoter gene that enhances the development of a specific cancer. The absence of a suppressor gene would parallel what is seen in Li–Fraumeni syndrome in human families and may explain why certain breeds (e.g., golden retrievers) are at higher risk of developing several cancers compared to the general dog population. For other breeds the risk may be associated with one or more specific genes, where the "insult" is fixed in the genome and inherited, requiring just one or a few additional "insults" to promote cancer initiation. This may explain why we see certain breeds with an extraordinary high incidence of one cancer or closely related cancers (e.g., Bernese mountain dog and flat-coated retrievers are less common breeds but they have a high incidence of histiocytic tumors). Rottweilers have an increased incidence of several types of neoplasia and there is a germ line mutation in the proto-oncogene *MET*, which encodes a tyrosine kinase in 70% of dogs of this breed but fewer than 5% of dogs of other breeds.[9]

Susceptibility to tumors has been traced to the family level in lifelong studies of laboratory beagles in a pattern that is similar to those in some human families.[10] There are inherited tendencies to develop melanomas in Sinclair and Hormel miniature pigs and Duroc-Jersey swine.[11] Although the specific genetic damage associated with the increased risk for tumors has been identified for some human families, inherited genetic abnormalities responsible for increased tumor susceptibilities in domestic animals are still emerging.

From concepts to clinics: molecular genetics usher in an era of opportunity

Improvements in our fundamental understanding of cancer biology present opportunities to lower the morbidity and mortality associated with this spectrum of diseases. For half a century, conventional cytogenetic approaches used to investigate human cancers provided

clues to the genetic basis of cancer. More recently, the introduction of molecular cytogenetics and other molecular genetic tools has revolutionized the way we are able to interrogate cancer cells to identify specific changes in chromosome and gene structure and/or function associated with cancer. Numerical chromosome changes (e.g., whole-chromosome aneuploidy, insertions, deletions) represent a deviation from the normal gene copy number, potentially leading to increased or decreased expression of genes associated with regulation of growth or survival. Structural changes (e.g., inversions, translocations) result in genome reorganization, which may cause genes that are separated in the normal/healthy genome to be brought into close proximity in the tumor genome with consequent effects on gene dysregulation. Abnormal patterns of gene expression can also result from mutations that affect single genes. In fact, every tumor will have a multiplicity of mutations that will create unique patterns of gene expression and contribute to its pathogenesis. The potential impact of faster, less costly techniques for DNA and RNA sequencing in veterinary oncology should not be underestimated. Currently the costs of whole-genome DNA sequencing and whole-transcriptome RNA sequencing are still prohibitive in a clinical setting for routine evaluation of individual tumor specimens. However, the surge in research activity in these areas will undoubtedly identify genes of particular interest in the genomes of domestic animals for targeted analysis, leading to the emergence of potential new targets for therapy.

Cytogenetics in cancer

The DNA of all animals is packed into nature's biological filing cabinets, chromosomes, the analysis of which is covered by the field of cytogenetics. In recent years the introduction of new molecular techniques and resources has led to the field of molecular cytogenetics, or cytogenomics, an area particularly suited to providing insights into the level of gross genome reorganization that occurs frequently in cancer cell populations. Using such techniques clonal chromosome aberrations have now been identified in over 65,000 cases of human cancer, representing over 70 different types of cancer (see http://cgap.nci.nih.gov/Chromosomes/Mitelman). Many of these recurrent chromosome aberrations were initially associated with histopathological or immunological subgroups, leading to their use as diagnostic signatures. In addition, the cytogenetic status of tumor cells is of established clinical value for prognosis, guiding therapy and assessing remission for a range of cancers, including ovarian cancer,[12] colorectal carcinoma,[13,14] gliomas,[15] melanoma,[16,17] and breast carcinoma.[18]

For example, in human leukemia patients, significant differences in the duration of treatment-free interval are reported for different cytogenetic events or deletions of chromosome 17p and 11q.[19] Detection of a *RUNX1–RUNX1T1* translocation [t(8;21)(q22;q22)] in individuals with acute myeloid leukemia is a cytogenetic marker associated with a more favorable prognosis.[20] However, acute myeloid leukemia patients with 8;21 translocation who also have a mutation of *KIT* or *FLT3* have decreased survival times.[21]

In human lymphoma patients, the *BCL6* transcriptional repressor (located at 3q27) is regarded as the most frequently involved oncogene in diffuse large B-cell lymphoma (DLBCL). Chromosome translocations that involve *BCL6* have been identified in up to 40% of people with DLBCL, but fewer than 10% of people with follicular lymphomas. The presence of a *BCL6* translocation has been reported to have no prognostic significance for DLBCL,[22] but in follicular lymphoma patients such events have been associated with an increased level of progression to DLBCL.[23] When considering treatment, early studies have suggested that a small molecule inhibitor that binds to the corepressor binding groove of the *BCL6* BTB domain may be effective against BCL-positive DLBCL.[24]

The ability to identify recurrent cytogenetic aberrations in cancers has been useful in helping to localize cancer-associated genes. This approach has led to the selection of the most appropriate therapeutic approaches for patients and subsequent monitoring for recurrent disease. Cytogenetics has thus proven to be a key approach to improvements in the clinical management of patients, sparing patients with good prognosis from unnecessary treatment and, conversely, allowing patients whose cytogenetic abnormalities indicate poor prognosis to receive more aggressive treatments to improve the probability of positive outcomes. The World Health Organization recognizes that genetic abnormalities are one of the most reliable criteria for the classification of tumors and has stressed the importance of further research into this area.

The increasing role of cytogenetics in the development of companion diagnostics and theranostics, which is the development of molecular diagnostic tests and targeted therapeutics in an interdependent, collaborative manner with the goals of individualizing treatment by targeting therapy to an individual's specific disease subtype and genetic profile, is evident from recent studies. For example, it was discovered that in non-small cell lung carcinoma (NSCLC), approximately 1 in 25 patients present with a chromosomal rearrangement that results in the fusion of the intracellular region of ALK (anaplastic lymphoma kinase) to the N-terminal portion of EML4 (echinoderm microtubule-associated protein-like 4).[25] This discovery led to the investigational use of ALK inhibitors to suppress the constitutive kinase activity in such patients. When treated with the selective MET/ALK inhibitor crizotinib, NSCLC patients with the ALK fusion gene had a 57% overall response rate and a >70% probability of having 6-month progression-free survival. The US Food and Drug Administration (FDA) have approved use of crizotinib for use in patients with late-stage NSCLS where the presence of this fusion gene has been determined. The FDA have also approved the use of a fluorescence *in situ* hybridization (FISH) assay to detect different ALK-associated fusions as a companion diagnostic for crizotinib therapy.

Recent progress in molecular cytogenomics of domestic animals (especially the dog) has allowed us to develop species-specific "toolboxes" that will accelerate progress in our understanding of cancer genetics in these species. In addition, the use of comparative genomics is allowing the transfer of key genetic information across multiple species, leading to a greater impact. Although the application of cytogenomics technologies to animal cancers has begun to make an impact for the benefit of veterinary medicine, progress has been limited by a lack of appropriate patient samples associated with standardized therapy and detailed clinical follow-up. For veterinary medicine to benefit from the full potential of clinical genomics, a greater level of collaboration between clinical and basic sciences is an essential prerequisite. The level of consistency of clinical management of cancer patients through tightly controlled clinical trials would provide the best opportunities to maximize progress towards the development of companion diagnostics/prognostics for veterinary health. Such clinical trials would ideally be conducted across multiple sites to enhance accrual rates. In the absence of sufficient resources to fully fund such trials, the next best alternative is the evaluation of biological specimens from patients with the same diagnosis, made using standardized diagnostic tests, and which are treated with a standard-of-care therapy. Sharing of clinical information, with appropriate informed consent of clients, would

then help to expedite the path to improved outcome indicators. More veterinary clinicians need to recognize that opportunities to drive their profession towards the most appropriate treatment/care plans for their cancer patients tomorrow requires that they be open to providing clinical information and appropriate biological specimens from their patients today. This requirement is already evident in the increasing number of multi-institutional clinical trials, especially through the Comparative Oncology Trials Consortium (COTC), and in the standardized collection of biological specimens from cancer-bearing dogs by the Canine Comparative Oncology and Genomics Consortium (www.CCOGC.org).

At the gross chromosome level, numerous studies have identified cytogenetic aberrations detected in a range of canine neoplasms,[26–37] including hematopoietic malignancies,[26–28] intracranial malignancies,[29] osteosarcoma,[31,32] hemangiosarcoma,[33] histiocytic malignancies,[34] urothelial carcinoma,[35] melanoma,[36] leukemia,[37] and mast cell tumors (Mochizuki *et al.*, unpublished).

In addition, cytogenetic characterization of canine cancer cell lines has been used to compare their status to the primary disease they are reported to represent.[36,38–41,79] (Poorman *et al.*, unpublished). Early work has also reported on cytogenetic changes evident in feline sarcomas[42] and intestinal lymphoma (Thomas *et al.*, unpublished).

As the studies above have shown, the increasing use of molecular tools, including arrays comprising thousands of genomic features, either DNA or RNA sequences, are now being used to analyze genome-wide patterns of changes to DNA content and transcript abundance. In combination, these approaches maximize the efficiency with which we can identify genetic alterations associated with a specific diagnosis and also provide insight into tumor pathogenesis. Assessment of genome-wide DNA copy number aberrations has been performed in numerous cancer types, in both domestic dogs and cats, as discussed above. In parallel, characterization of the level of transcriptional activity of genes, either via a genome-wide or a targeted approach, has begun to provide key signatures relating to diagnosis and prognosis in canine cancers.[20,43,44]

Furthermore, even though about 90% of the genome does not encode proteins, it nevertheless has important functions in maintaining homeostasis. At the turn of the century, noncoding DNA was discarded as mere "junk DNA," thought to be an anachronism inherited from our evolutionary forebears. We now know that this DNA in fact encodes molecules, such as microRNAs, that have important functions in gene regulation.[45,46] Indeed, gain or loss of function of microRNAs may turn out to be just as important in cancer causation as gain or loss of function of traditional protein-coding genes.[46–54]

Molecular diagnostics and prognostics

Molecular diagnostic testing entered the medical arena with the availability of assays to detect the presence of infectious agents. Although this remains the largest segment of the molecular diagnostics market, molecular testing in oncology is a rapidly growing area. Development of molecular-based assays to aid in cancer diagnosis and prognosis indicates that they will soon be considered as conventional as morphologic approaches to cancer diagnosis. Access to an increasing portfolio of molecular assays for cancers will have a profound impact on patient care and lead to the need for a more interdisciplinary approach to decision-making. Although a variety of biological techniques are considered as molecular diagnostics, all are based on the detection and analysis of either specific sequences of nucleic acid (DNA or RNA) or proteins. For diagnostic purposes the regions analyzed are associated specifically with the presence of a disease or subtype. To provide prognostic value, the regions assessed need to have characteristics that are associated with differing clinical progression and outcomes. In the area of oncology, molecular diagnostics based on DNA include identification of large numerical and/or structural changes to genome organization; ranging from whole-chromosome copy number changes (aneuploidy), partial-chromosome copy number change (segmental aneuploidy; deletions, duplications, amplifications), or chromosome rearrangements (translocations), down to changes that affect perhaps just one base of DNA. When considering RNA, a diagnostic parameter may be a specific level of transcription of a gene (or multiple genes) in a cancer subtype or more likely a multi-gene "signature." If such a signature is associated with clinical progression and outcome, it may also be considered to be of prognostic value.

Molecular approaches to cancer patient management will become a key tool, with assays for cancer prediction, diagnosis, and prognosis becoming intertwined temporally to: (1) identify patients at high risk, (2) provide early detection of a cancer, (3) select the most efficacious therapy options, and (4) spot early signs of relapse.

Complementary assays, using cytogenetics, immunohistochemistry, and gene expression platforms, are now in place to determine the status of an individual tumor. Since 2007, the College of American Pathologists and the American Society of Clinical Oncology have provided benchmark guidelines to ensure consistency of reporting across testing laboratories.[55] The association of molecular signatures with the patient's response to therapy is leading to the emergence of companion diagnostics, assays that can inform the clinician of the chances that a specific therapy will be effective for an individual patient (theranostics), based on known efficacy of treating patients/cancers with the same signature. For example, once it has been determined that a human breast carcinoma is overexpressing Her2, evidence-based efficacy data suggest that such cancers will respond favorably to trastuzumab (Herceptin). In general, the development of a companion diagnostic requires close cooperation between the providers of the molecular assay and the therapy. There are numerous stakeholders in the field of cancer patient care (clinician, pathologist, molecular assay developers, molecular assay technologist, pharmaceutical company), each with their own challenges. Communication between these is key to ensure that the ultimate stakeholder, the cancer patient, receives the most appropriate care to optimize the quality and duration of their life.

Evolutionarily conserved genomic changes in cancers

Perhaps the most widely investigated chromosome aberration associated with cancers in people is the Philadelphia chromosome, first described almost half a century ago in patients with CML.[56,57] This aberrant human chromosome (HSA) is the result of a translocation event that brings together the c-*abl* oncogene (located at HSA 9q34 (*ABL* locus)) and the breakpoint cluster region (*BCR*) (located at HSA 22q11) to form a derivative human chromosome 22, technically described as t(9;22)(q34;q11) and referred to as the Philadelphia (Ph) chromosome.[57] The juxtaposition of *BCR* and *ABL* is considered a hallmark feature of CML, reported in over 95% of CML patients.[58] The biological consequence of the generation of this fusion is elevation of tyrosine kinase activity, which results in the uncontrolled proliferation of white (predominantly myeloid)

blood cells. The identification of STI571 (imatinib mesylate) as a compound that acts as an antagonist to this fusion protein (bcr-abl tyrosine kinase) and prevents blast crisis,[59,60] led to clinical trials and the development of Gleevec®,[58] which (with some exceptions) is now generally considered standard of care for patients with the Philadelphia chromosome. Almost 90% of patients treated with Gleevec are free of disease worsening, with an estimated overall survival rate of 91%. A cytogenetic response, defined as a reduction of cells with the characteristic molecular abnormality, is seen in up to 60% of patients[61,62] and remains an important surrogate marker of survival in human CML patients.[63,64] Cytogenetic testing is used to initially diagnose the CML and then subsequently to monitor remission and identify any elevation in the number of cells harboring BCR-ABL during relapse/recurrence.

Although very rare in veterinary species, CML has been reported in dogs and conveys a poor prognosis.[27,65–67] A study of canine CML showed that dogs diagnosed with CML also presented with a functionally active BCR-ABL translocation.[27] These data suggest that, cost aside, treatment with Gleevec, or a similar TKI compound could be an option for therapy of canine CML assuming any toxicity issues are overcome. This study resulted in the first molecular cytogenetic test for the presence of a clinically significant genomic alteration in a veterinary cancer and has since been used to identify the Raleigh chromosome in numerous additional cases presenting with suspected CML. In addition, cases presenting with the Raleigh chromosome (Figure 1.6) have been followed during treatment with various compounds and the cell population containing the BCR-ABL event was almost cleared from detection during remission, and then returned at relapse.[27,33,68] These data demonstrate that appropriate therapies do have the desired impact on the cancer cell population, opening the door for broader studies of treatment efficacy in animal cancers, determined by molecular as well as conventional clinical response.

The presence of RB1 deletions in canine patients presenting with chronic lymphocytic leukemias and MYC-IgH translocations in canine patients diagnosed with Burkitt lymphoma have also been reported, supported by functional data.[27] These findings reinforce the concept that as mammals, humans and dogs may be considered temporally separated, differential organizations of the same collection of ancestrally related genes. Since we have shown that genetic "lesions" associated with human cancers may be similarly associated in cancers of veterinary species, therapies developed for malignancies with specific cytogenetic signatures in human cancers may become applicable to provide improved treatments for cancers in our pet dogs and cats. Cytogenomic screening of cancers in our pets could become common practice in veterinary oncology, used to aid diagnosis, selection of the most appropriate therapy, monitoring of residual disease, and for prognostication.

Molecular assays in veterinary oncology

The availability of high-quality genome sequences for the domestic dog and cat laid the foundations for the development of a series of new resources for cancer research for both species. Recent studies using genomics have led to the identification of inherited genetic risk factors associated with canine cancers, cytogenomic changes associated with specific diagnosis and/or prognosis of cancer in dogs and cats, and a series of new molecular tests that will provide new aids to diagnosis and clinical management of pets diagnosed with cancer. Examples of such assays are described below.

PARR (PCR for antigen receptor rearrangement)

PARR is a polymerase chain reaction (PCR)-based assay developed to detect clonal expansion of lymphocytes in dogs and cats suspected of having lymphoma. The concept of PARR is based on the assumption that the lymphoid neoplasm is the result of clonal expansion of B or T lymphocytes. Ideally, the DNA is isolated from specimens representing the lesion, including aspirates (bone marrow or lymph node), incisional and excisional biopsies, provided that they yield a sufficient amount of cellular DNA. In certain circumstances DNA may be isolated from peripheral blood of the lymphoma patient, but this approach is to be treated with caution. Material to be tested is usually transferred to the laboratory fresh or frozen, but may also be formalin-fixed, paraffin-embedded (FFPE) tumor specimens, or cytologic preparations on glass slides. Each sample is processed to obtain cellular DNA, which serves as the template for PCR analysis. The assay uses PCR primers specific to immunoglobulin antigen receptors in B cells and T-cell receptors in T cells.[69] Rearrangement of these genes is a natural part of lymphocyte differentiation, resulting in polyclonal cell population. With a clonal expansion of cells the usual multitude of rearrangements present in these genes is eradicated, generating a single PCR amplicon. Detection of a monoclonal proliferation strongly favors a diagnosis of lymphoid neoplasia, while a polyclonal proliferation supports hyperplasia. As with all assays it is important to be aware of the limitations of detection. For dogs, PARR is reported to be at least 90% specific, but has only 75% sensitivity for lymphoid neoplasia.[70–72] In cats, the specificity is similar, but the sensitivity is reduced to 65%. These data mean that PARR will miss 25% and 35% of actual lymphoma cases in dogs and cats, respectively. Approximately 10% of cases with positive test results (clonality) do not have lymphoma (false positives). Monoclonal proliferation with inflammatory or hyperplastic diseases is uncommon but has been reported with feline infectious peritonitis and canine ehrlichisosis. As with all tests, PARR is not 100% specific or sensitive and results of PARR need to be correlated with clinical signs, cytology, histopathology, and other test results. However, with access to just a fine-needle aspirate of an enlarged node, PARR is an option for clinicians to determine clonality which would be indicative of lymphoma or to assess recurrence of lymphoma following treatment. Molecular clonality is not a primary or sole diagnostic test and it is not needed when the results of histology or cytology and/or immunophenotyping are definitive. However, determination of clonality is useful when morphology and immunophenotyping are not definitive.

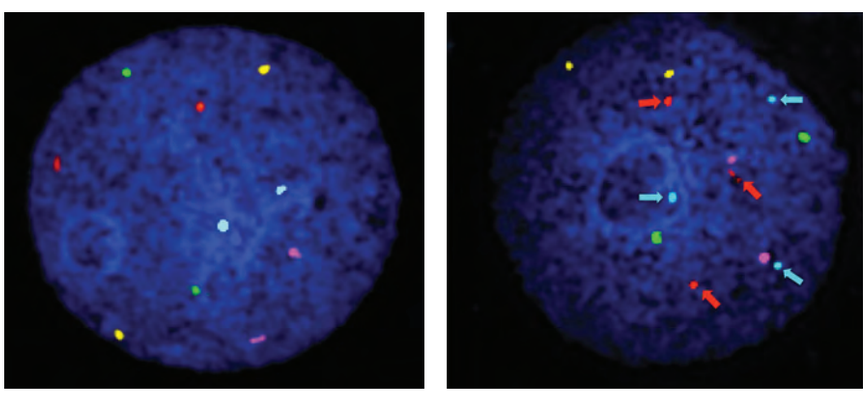
Cytogenetic assay for prognosis in canine lymphoma

Lymphoma is estimated to affect in excess of 250,000 pet dogs each year in the United States, and is one of the most common canine cancers. In a recent survey of over 150 veterinary oncologists in the United States (Breen, unpublished) the three most common reasons for dog owners hesitating to opt for treatment of the lymphoma are (1) concerns over whether their dog will be even more sick during chemotherapy, (2) the cost of treatment, and (3) uncertainty of outcome. Currently, it is widely accepted that up to 90% of all canine multicentric lymphomas will enter remission if treated with standard-of-care chemotherapy, and median survival is 9–12 months. In addition, most B-cell lymphoma cases are expected to have a longer survival time than most T-cell cases, but there are exceptions. A review of current indicators for survival has been provided by Valli *et al.*[73]

To assist oncologists and owners in making a more informed decision about treatment options and potential outcomes we developed a cytogenetic assay to help predict the duration of remission with two different treatments, either single-agent doxorubicin or multi-agent CHOP (cyclophosphamide, hydrodoxorubicin, oncovin/vincristine, prednisone) therapy. Genome-wide DNA copy number profiling of diagnostic biopsy specimens from dogs diagnosed with multicentric lymphoma revealed numerous recurrent aberrations, including whole-chromosome aneuploidy and segmental aneuploidy.[26,28] In a parallel study to evaluate the prognostic significance of these copy number changes we evaluated patients from two cohorts; the first cohort comprised 160 FFPE diagnostic biopsy specimens from dogs with confirmed lymphoma (any subtype). In all cases the biopsy was taken prior to any treatment and then the dog was subsequently treated with single-agent doxorubicin. In the second cohort, 100 FFPE diagnostic lymph node biopsy specimens were obtained from dogs diagnosed with multicentric lymphoma and prior to treatment. For this cohort, all dogs were subsequently treated with standard-of-care multi-agent CHOP therapy. Both cohorts comprised dogs with B- and T-cell lymphoma and of various subtypes. For both cohorts, the dogs were clinically evaluated at regular intervals during and after their chemotherapy, and the duration of their first remission recorded. Using cells obtained from the FFPE specimens interphase nuclei were screened using multicolor FISH analysis to determine the mean copy number of selected regions of the canine genome (Figure 1.13). In the doxorubicin-treated patients, the mean copy number of two regions of the genome (located on dog chromosomes 1 and 6) correlated significantly with the duration of first remission in a positive linear relationship; dogs with low mean copy number of both regions had shorter first remission times than those with higher mean copy number. In the CHOP cohort, the region on chromosome 1 was not associated with the duration of first remission, while the mean copy number of the region on chromosome 6 remained significantly associated. These data were used to develop a molecular cytogenetic assay in which both regions are evaluated simultaneously in cells derived from lymph node samples and the data used to provide a predicted duration of remission if a dog is subsequently treated with either single-agent doxorubicin or multi-agent CHOP therapy. This assay should be widely available in 2017.

Cytogenetic assay to separate histiocytic malignancies from lymphoma

Histiocytic neoplasms, benign and malignant, arise primarily from dendritic cells found in the skin and visceral organs. The incidence of all histiocytic malignancies is rare in the general dog population, but remarkably high in several purebred dogs, including the Bernese mountain dog, flat-coated retriever, rottweiler, and golden retriever. Malignant tumors of histiocytic origin generally have a very poor prognosis (typical survival is just a few weeks post diagnosis) and are considered generally unresponsive to current therapeutic options. In the Bernese mountain dog, 66% of deaths are reported to be due to cancer,[74] of which 47% are attributed to histiocytic malignancies, with a further 29% due to lymphoma.[74] These data indicate that, strikingly, 75% of all cancers and 50% of deaths in this one breed are due to just these two cancers. Correct diagnosis of a histiocytic neoplasm currently requires specialized immunohistochemistry (IHC). However, IHC is not always readily available, as specific antibodies are required and special tissue preparation such as frozen sections are required for some of the antibodies. IHC can be time consuming and requires a particular skill set. The ability to accurately distinguish between canine lymphoma and histiocytic malignancies is an important determinant of treatment and outcome; the prognosis for lymphoma is better than that of disseminated histiocytic sarcoma. Genome-wide evaluations of DNA copy number in canine lymphoma and histiocytic malignancies across a range of breeds have led to the identification of regions of the canine genome that are aberrant in one of these two cancers but not the other. Specifically, histiocytic neoplasms present with a high frequency of deletion of dog chromosomes 2, 16, and 31; none of these deletions are evident in canine lymphoma. In addition, dog chromosome 31 is frequently increased in copy number in lymph node cells of confirmed lymphoma patients. Using these features, a cytogenetic assay was developed to simultaneously assess the mean copy number status of regions of dog chromosomes 2, 16, and 31 (Figure 1.14). The assay has 97.2%



A B

Figure 1.13 Lymphoma cytogenetic prognostic assay. Multicolor FISH of canine interphase nuclei of cells aspirated from lymph nodes of (A) a healthy dog and (B) a dog with lymphoma. Enumeration of the five differentially labeled single locus probes indicates that in (A) all five have a normal copy number of 2, while in (B) the two probes labeled in red and aqua (arrows) both have an abnormal copy number of 3. Probe enumeration in 100 cells allows derivation of mean copy number value for each probe. With standard-of-care doxorubicin-based chemotherapy for lymphoma, 95% of dogs with low mean copy number (<1.6) of both probes labeled in red and aqua have shorter first remission times (<90 days), and 95% of dogs with higher mean copy number (>2.5) have remission times over 9 months. Assays like this will help oncologists and owners make informed decisions on how an individual patient with lymphoma may respond to specific therapy (theranostics).

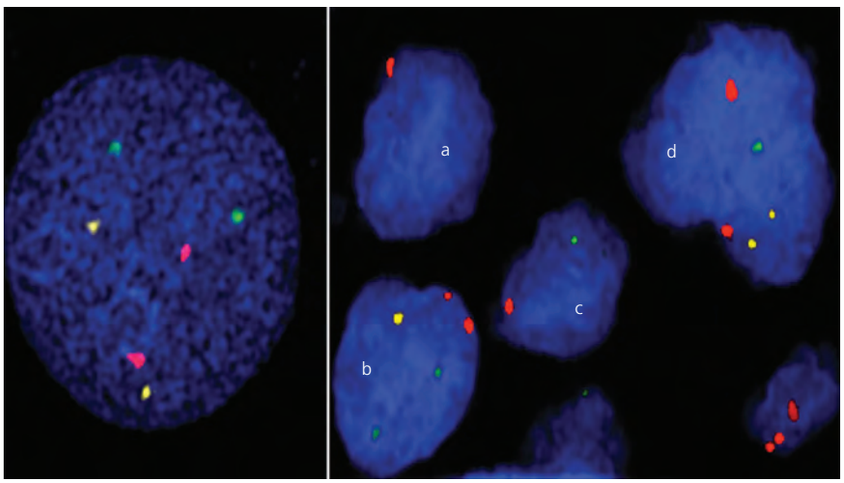


Figure 1.14 Histiocytic sarcoma cytogenetic assay. Three-color FISH of canine interphase nuclei designed to detect cells with DNA copy number aberrations characteristic of cells derived from a canine histiocytic malignancy. This assay was designed to help differentiate canine histiocytic sarcoma from lymphoma. (A) Nucleus of peripheral lymphocyte of a healthy dog. (B) Nuclei of cells derived from a fine-needle aspirate of canine lymph node from a dog with a confirmed histiocytic neoplasm. The three probes comprising the FISH assay represent regions of CFA 2 (red, R), CFA 16 (green, G), CFA 31 (yellow, Y). Although all three probes have a copy number of $n = 2$ in the healthy cell (i.e., R2/G2/Y2), it is clear from panel B that the cells labeled a–d each have one or more numerical abnormalities with copy numbers as follows: (a) R1/G0/Y0, (b) R2/G2/Y1, (c) R1/G1/Y0, and (d) R2/G2/Y1. Enumeration of >100 cells yields mean copy numbers for each probe of <2.0 in >90% of histiocytic neoplasms, while >90% of canine lymphomas a have a balanced or mean copy number >2.0 for each probe.

specificity and 97.3% sensitivity to distinguish between histiocytic neoplasia and lymphoma.

The significance of this assay for veterinarians lies in the ability to readily distinguish these two types of cancer, especially for those breeds that are at high risk of developing both cancers and/or where there is an uncertain diagnosis based on morphology alone. In combination with an assay to predict duration of remission to chemotherapy in canine lymphoma patients treated with standard of care, such an assay has the potential to offer considerable value to patient management, adding new approaches to refine diagnosis, and even prognosis. This assay, available since 2015, is being extended to confirm the presence of a histiocytic malignancy and exclude other round cell neoplasms.

Diagnostic assay for urothelial carcinoma

Urothelial carcinoma, also referred to as transitional cell carcinoma (TCC), is the most common bladder neoplasm in the dog, although compared to other cancers it is uncommon, accounting for <2% of all cancer diagnoses.[75,76] The tumor is invasive and is one of the most aggressive tumors in veterinary medicine. At the time of initial diagnosis approximately 20% will have clinically detectable metastases and 50% will have metastases detectable via autopsy. Although cases of urothelial carcinoma may initially respond well to chemotherapy, cyclooxygenase inhibitors, and/or surgery, most dogs die within the first year of treatment. In the absence of tumor biopsy, accurate diagnosis of urothelial carcinoma is challenging. Cytological examination of urine is often inconclusive and the bladder tumor antigen test has less than ideal specificity, frequently indicating false positives, especially when inflammation is present.[77] A robust diagnosis of urothelial carcinoma currently requires a sample of the tumor to be evaluated histologically or cytologically. Since urothelial carcinoma is a tumor that may "seed" to other locations, any form of mechanical disturbance of a potential mass is avoided where possible. A highly desirable means to diagnose the presence of urothelial carcinoma is one that may be performed on a free-catch urine sample, on the basis that malignant cells are shed into urine. Ideally this test would be affordable and detect cases early in the tumor's life, when the presenting signs may be mimicking cystitis (hematuria). A step forward has occurred with evidence that specific microRNAs from FFPE samples are increased in urothelial carcinoma, but not inflammatory bladder disease.[78] In human oncology, a cytogenetic test was developed to identify the presence of bladder cancer cells in urine and became available in the form of a multicolor assay, called Urovysion (http://www.abbottmolecular.com/us/products/oncology/fish/bladder-cancer-urovysion.html developed by Abbott Molecular). Human patients with confirmed bladder tumors are known to present with aneuploidy of chromosomes 3, 7, and 17 as well as 9p21. The Urovysion test is based on detection and quantification of these four regions of the genome using four differentially labeled FISH probes, chromosome enumeration probes for chromosomes 3, 7, and 17, and a locus-specific probe for 9p21. This assay is based on the use of cells pelleted from a free-catch urine sample, which are then fixed and prepared on glass microscope slides. Twenty-five morphologically abnormal cells are evaluated for probe copy number and criteria provided to determine the status of the patient sample. The clinical interpretation of the data is evaluated within the context of the medical history and additional diagnostic laboratory test results.

Studies of canine urothelial carcinoma biopsies have revealed aneuploidy of several dog chromosomes.[35] Among these aberrations, high-frequency aneuploidy of dog chromosomes CFA 13, 19, and 36 within the same cell was a cytogenetic signature not evident in numerous other canine cancers and so was used to develop a FISH assay to aid the diagnosis of urothelial carcinoma in a biopsy specimen or to detect the presence of urothelial carcinoma cells in urine. Enumeration of CFA 8 in urothelial carcinoma revealed cells to be either diploid ($n = 2$) or tetraploid ($n = 4$) and so was included in the

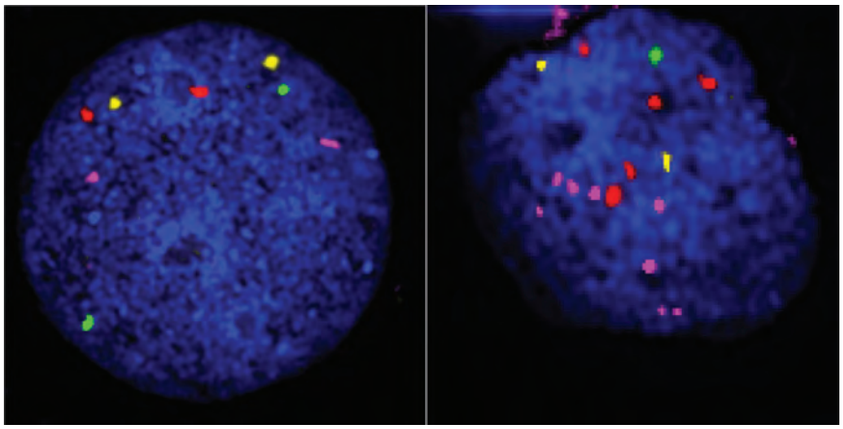


Figure 1.15 Cytogenetic signature of canine urothelial carcinoma. Four-color FISH of canine interphase nuclei designed to detect cells with DNA copy number aberrations characteristic of cells derived from canine urothelial carcinoma. (A) Nucleus of peripheral lymphocyte of a healthy dog. (B) Nucleus of a cell voided in the urine of a dog with a confirmed urothelial carcinoma. The four probes comprising the FISH assay represent CFA (*Canis familiaris*) 8 (yellow), CFA 13 (red), CFA 19 (green), and CFA 36 (pink). While all four probes have a copy number of $n = 2$ in the healthy cell, panel **A**, it is clear from panel B that there are several abnormalities. In this case the neoplastic cell has the expected two copies of CFA 8 (internal control), but has the urothelial carcinoma signature of copy number increases of 13 and 36 and loss of 19: in this example there are five distinct signals representing CFA 13, one signal representing CFA 19, and at least eight distinct signals representing CFA 36. This assay will help detect and or confirm urothelial carcinoma in a free-catch sample of urine.

FISH assay to identify the ploidy status of each cell. In combination, enumeration of the FISH probes representing regions of these four chromosomes (8, 13, 19, 36) provided an assay that had over 99% specificity and sensitivity when evaluating tumor biopsies. Evaluation of cells collected from free-catch urine samples (Figure 1.15) confirmed that this FISH assay for canine urothelial carcinoma retained a specificity of over 99% and a sensitivity of >99%.

Similar approaches are being used to develop additional cytogenetic assays designed to provide diagnostic and prognostic information for a range of canine cancers, including, for example, canine leukemia subtypes, mast cell tumors, osteosarcoma, oral melanoma, intracranial tumors, and hemangiosarcoma. In addition, studies looking at the cytogenomics of various feline cancers are leading to assays that will provide new tools to aid management of cats diagnosed with injection site sarcoma, gastrointestinal lymphoma/inflammatory bowel disease and mammary carcinoma.[42]

Conclusions

In recent years, remarkable progress has been made in our understanding of the complex pathogenesis of neoplasia. The molecular mechanisms involved in the neoplastic transformation and regulation of cells have been identified for numerous tumor types. This is beginning to be applied to risk assessment, tumor diagnostics, and anticancer therapy. It is hoped that this new understanding will permit more precise identification of the early stages of neoplasia when, it is presumed, therapy can be more effective.

Therapies that can be developed to specifically target abnormal properties of cancer cells may spare normal cells and may avoid the side effects of many contemporary treatments. Moreover, as the genetic lesions responsible for cancer development and progression are identified, conventional diagnostic techniques and grading algorithms will, it is hoped, be complemented by stronger predictors of outcome.

Currently, with few exceptions the gold standard for a diagnosis and determining malignancy remains histologic diagnosis. As molecular studies of animal cancers are pursued, and data become more widely accessible, it is to be expected that new signatures of malignancy will emerge to aid in the determination of a malignant versus benign phenotype. Provision of an accurate diagnosis remains the key benchmark for the pathologist. However, pathologists are seeing only a snapshot in the temporal course of any cancer. Tumors are dynamic yet we may only sample at one point in time and the diagnosis of a benign mass may progress to one of malignancy. Evaluations will need to consider the timeline of transformative events to leverage comprehensive information of patient samples. Detection and quantification of such events will accelerate our understanding of the biological significance of neoplasms, whether assessed through a microscope or molecular means. Whether considering the natural course of the disease, response to primary therapy, or additional response to rescue therapy, the veterinary profession needs to engage in a coordinated way to tackle animal cancers as a team.

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