Secondary erythrocytosis associated with high plasma erythropoietin concentrations in a dog with cecal leiomyosarcoma

Kota Sato, DVM, PhD; Yoshiaki Hikasa, DVM, PhD; Takehito Morita, DVM, PhD; Akinori Shimada, DVM, PhD; Kiyokazu Ozaki, DVM, PhD; Katsumoto Kagota, DVM, PhD

- ➤ Expression of erythropoietin by neoplastic cells is associated with secondary erythrocytosis in dogs.
- ➤ Immunohistochemistry and polymerase chain reaction assays can be used to detect erythropoietin polypeptides and mRNA in tumors from dogs with secondary erythrocytosis.
- ➤ Tumors involving intestinal tissues should be considered a possible cause of secondary erythrocytosis in dogs.

A 14-year-old male mixed-breed dog weighing 14 kg (30.9 lb) was referred to the Veterinary Teaching Hospital of Tottori University because of hematemesis of sudden onset, hematochezia, and anorexia. Physical examination revealed brick-red mucous membranes and a short capillary refill time. Hematologic abnormalities included high RBC count (8.98×10^6/µl; reference range,[1] 5.5 to 8.5×10^6/µl), high PCV (70%; reference range, 37 to 55%), and high hemoglobin concentration (19.6 g/dl; reference range, 12 to 18 g/dl); the total plasma protein concentration (7.1 g/dl; reference range, 6.0 to 8.0 g/dl) was normal.

The dog was treated with tranexamic acid[a] (10 mg/kg [4.5 mg/lb] of body weight, PO, q 12 h) and lactated Ringer's solution containing 5% glucose[b] (100 to 200 ml/d, IV). Continuous mild hematochezia resulted in a decrease in the PCV to 54% on day 13. However, RBC count increased slightly (10.4×10^6/µl), and the **mean corpuscular volume (MCV)** was low (58 fl; reference range, 60.0 to 77.0 fl). As the hematochezia resolved, both the RBC count (15.9×10^6/µl) and PCV (78%) again increased. Because total plasma protein concentration (7.1 to 7.8 g/dl) remained within reference limits throughout this time, we suspected that the dog had an absolute erythrocytosis. The WBC count (9.2 to 12.2×10^3/µl; reference range, 6.0 to 17.0×10^3/µl) was within reference limits throughout this period, and the platelet count (235 to 590×10^3/µl; reference range, 200 to 500×10^3/µl) was normal or only slightly high. The reticulocyte count was as high as 4% (reference range, 0 to 1.5%), indicating active erythropoiesis.

From the Departments of Veterinary Internal Medicine (Sato, Hikasa, Kagota) and Veterinary Pathology (Morita, Shimada), Faculty of Agriculture, Tottori University, 4-101 Koyama Minami, Tottori 680-8553, Japan; and the Research Institute of Drug Safety (Ozaki), Setsunan University, 45-1 Nagaotohge-cho, Hirakata, Osaka 573-0101, Japan.
Address correspondence to Dr. Hikasa.

A bone marrow aspirate was obtained from the iliac crest and examined. Erythroid cells frequently contained mitotic figures, implying that active erythrogenesis was taking place, whereas the granulocyte fraction consisted mainly of mature neutrophils. Although the **myeloid:erythroid (M:E)** ratio (1.09) in bone marrow smears was within reference limits (0.75 to 2.5), it was close to the lower reference limit. In addition, the M:E ratio in clinically normal dogs is generally between 1.0 and 2.0, and an M:E ratio < 1.0 indicates intensification of erythrogenesis.[1]

Plasma EPO concentration was determined with a commercial radioimmunoassay kit used for determination of EPO concentrations in humans.[c] Concentration was slightly high (28.5 U/L), compared with values for 5 clinically normal mixed-breed dogs (12.5 to 21.6 U/L), despite the erythrocytosis. Analysis of a blood sample from the femoral artery revealed normal oxygenation (Pa_{O_2}, 93 mm Hg; reference range, 85 to 100 mm Hg), suggesting that low oxygenation was not the cause of the high plasma EPO concentration. The arterial pH (7.37; reference range, 7.31 to 7.42) was normal, but the Pa_{CO_2} (20.1 mm Hg; reference range, 35 to 45 mm Hg) and bicarbonate concentration (12 mmol/l; reference range, 18 to 24 mmol/l) were low, suggesting that the dog had compensated metabolic acidosis. Although the cause of the metabolic acidosis was not clear, high blood hemoglobin concentration or the disturbance of blood flow associated with increased viscosity may have affected acid-base balance.

Pulmonary and cardiac disorders were not found using electrocardiography, radiography (including angiocardiography), or echocardiography. In addition, abnormalities of hemoglobin and RBC membrane proteins were not found with electrophoretic analyses. These findings were most consistent with a diagnosis of inappropriate secondary erythrocytosis. We suspected erythrocytosis was a result of abnormal secretion of EPO by the kidney secondary to local hypoxia or secretion of EPO by a neoplasm in the kidney and other tissues. Radiography (including angiography), ultrasonography, urinalysis, and serum biochemical testing did not reveal any abnormalities that could cause local hypoxia in the kidneys and did not reveal any evidence of a neoplasm in the kidneys or other tissues.

Periodic phlebotomy (every 1 to 2 weeks; 100 to 200 ml) and infusion of an equivalent volume of saline (0.9% NaCl) solution was used to maintain the PCV in the range of 60 to 65% for approximately 2 years. The clinical course was generally satisfactory, although anorexia and emesis were occasionally observed when the phlebotomy interval became long. Urinary tract

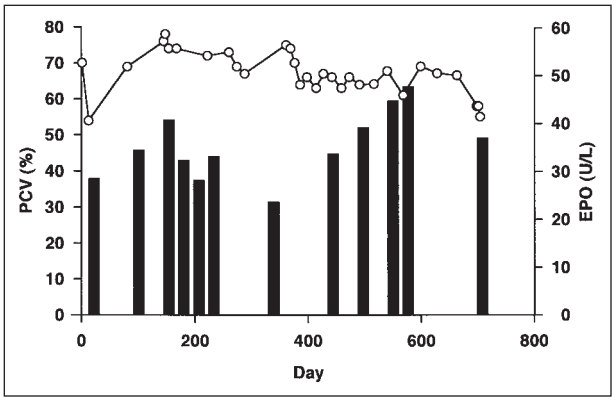

Figure 1—Changes in PCV (open circles) and plasma erythropoietin concentration (solid bars) in a dog with erythrocytosis secondary to inappropriate erythropoietin secretion by a cecal leiomyosarcoma.

infection was observed several times during this period. When urinary tract infection was observed, ampicillin (10 to 20 mg/kg [4.5 to 9 mg/lb], PO, q 12 h) was given until the condition resolved. The serum EPO concentration remained high throughout this period (23.6 to 47.7 U/L; **Fig 1**). In addition, a decrease in MCV (47 to 50 fl) was observed. The RBC appeared thin and hypochromic during examination of blood smears, possibly as a result of the loss of iron associated with repeated phlebotomy. Two years after initial examination (at 16 years of age), the dog developed uremia secondary to acute renal failure (urea nitrogen, 240 mg/dl [reference range, 10 to 28 mg/dl]; creatinine, 8.7 mg/dl [reference range, 0.5 to 1.5 mg/dl]) and died.

A complete necropsy was performed. A single reddish-white mass (3 cm in diameter) was found in the wall of cecum. No neoplastic nodules were seen in other organs. Samples of the mass, liver, spleen, kidney, heart, lung, tonsil, esophagus, stomach, small and large intestines, urinary bladder, adrenal glands, pancreas, lymph nodes, brain, and spinal cord were collected and fixed in neutral-buffered 10% formalin. They were then dehydrated, embedded in paraffin wax, sectioned at a thickness of 4 µm, and stained with H&E or **periodic-acid Schiff (PAS)** stain. Immunohistochemical analysis was carried out, using the labeled streptavidin procedure.[d] Specific antisera that were used were anti-human EPO monoclonal antibody[e] and anti-human smooth muscle actin monoclonal antibody.[f] For electron microscopy, tissues from the cecal mass that had been fixed in neutral-buffered 10% formalin were transferred to phosphate-buffered 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope.

Histologically, the cecal tumor was poorly circumscribed, moderately cellular, and located in the muscle layer. The neoplastic cells consisted of spindle or ovoid cells, some of which had cigar-shaped central nuclei. Some cells had abundant pale basophilic material in their cytoplasm; this material was intensely stained with PAS stain (**Fig 2**). Immunohistochemically, the cells were diffusely and strongly positive for α-smooth muscle actin, indicating that the tumor was of smooth muscle cell origin. Vacuoles within the cells that were identified as pale basophilic material with H&E stain were positive for EPO. Ultrastructurally, fine granules that were of low electron density were found within vacuolar structures in the cytoplasm. The vacuoles did not contain specific structures reminiscent of organelles such as endoplasmic reticulum, ribosomes, Golgi complexes, or mitochondria.

A **reverse transcriptase-polymerase chain reaction (RT-PCR)** assay was used to detect EPO mRNA in the tumor. Total RNA was extracted from the cecal tumor and purified with a commercial kit.[g] The RNA (DNase I treated; 2 µg) was reverse transcribed for synthesis of first-strand cDNA with reverse transcriptase.[h] The cDNA was used for the PCR assay with primers specific for canine EPO (5'-ACCTGGAAGAGGATGGATGTTG-3' [nucleotides 214 through 235] and 5'-TGCAGGCCTCCCCTGTGTACAGTGTC-3' [nucleotides 550 through 525), prepared on the basis of the published nucleotide sequence[2] (GenBank accession number L13027). Amplification was carried out, using a hot-start method,[i] for 40 cycles, with each cycle consisting of denaturation at 94 C for 30 seconds, annealing at 60 C for 30 seconds, and elongation at 72 C for 1 minute. Products were analyzed by means of agarose gel electrophoresis, followed by ethidium bromide staining. Amplification products (312 base pairs) corresponding to the expected partial nucleotide sequence of canine EPO were clearly observed when cDNA reverse transcribed from tumor RNA was used as the template (**Fig 3**). No amplification products were obtained when reverse transcriptase was omitted, demonstrating that amplification products were derived from EPO mRNA but not genomic DNA. The nucleotide sequences of the amplification products were determined with a commercial DNA sequencing kit[j] and automated DNA sequencer.[k] Sequences were identical to the published nucleotide sequence for canine EPO cDNA. Results of the RT-PCR assay, therefore, indicated that the tumor expressed EPO, which stimulated erythropoiesis and resulted in erythrocytosis in this dog.

Causes of erythrocytosis include dehydration, pulmonary and cardiac disorders, venoarterial shunts, and polycythemia vera.[3-5] In humans, polycythemia vera (primary erythrocytosis) is often accompanied by increases in WBC and platelet counts and a decrease in plasma EPO concentration.[6] In the dog described in the present report, obvious increases in WBC and platelet counts were not observed, and plasma EPO concentration was increased, indicating that erythrocytosis was not a result of polycythemia vera in this dog. Similarly, this dog did not have any clinical signs of pulmonary or cardiac disorders. High plasma EPO concentrations were detected continuously, even though results of blood gas analyses were normal, indicating that erythrocytosis was caused by ectopic EPO secretion, and we suspected secondary erythrocytosis attributable to ectopic production of EPO by a tumor. During a postmortem examination, a tumor was found in the cecum, and microscopic and immunohistochemical observations confirmed that it was a leiomyosarcoma. To

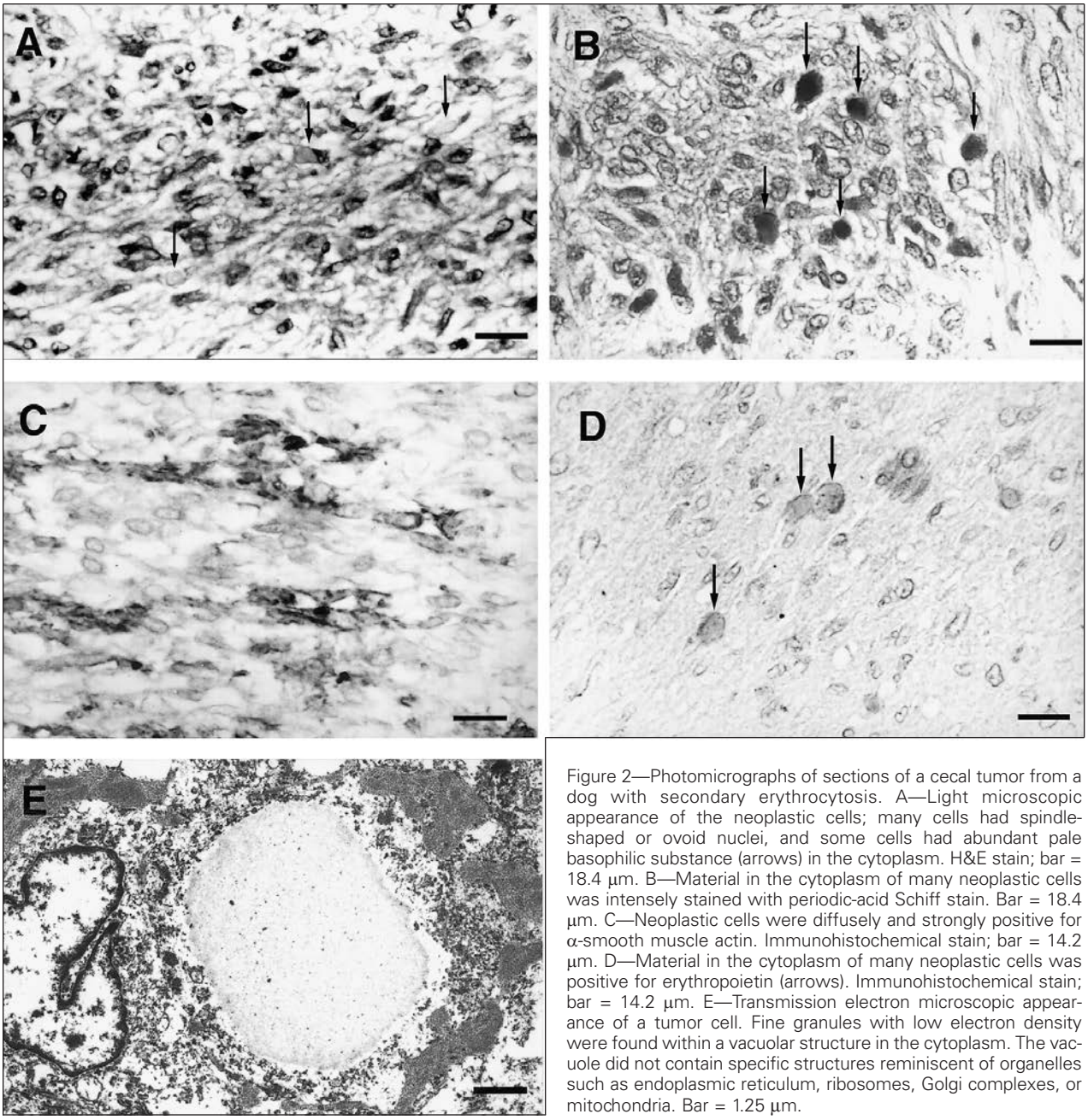

Figure 2—Photomicrographs of sections of a cecal tumor from a dog with secondary erythrocytosis. A—Light microscopic appearance of the neoplastic cells; many cells had spindle-shaped or ovoid nuclei, and some cells had abundant pale basophilic substance (arrows) in the cytoplasm. H&E stain; bar = 18.4 μm. B—Material in the cytoplasm of many neoplastic cells was intensely stained with periodic-acid Schiff stain. Bar = 18.4 μm. C—Neoplastic cells were diffusely and strongly positive for α-smooth muscle actin. Immunohistochemical stain; bar = 14.2 μm. D—Material in the cytoplasm of many neoplastic cells was positive for erythropoietin (arrows). Immunohistochemical stain; bar = 14.2 μm. E—Transmission electron microscopic appearance of a tumor cell. Fine granules with low electron density were found within a vacuolar structure in the cytoplasm. The vacuole did not contain specific structures reminiscent of organelles such as endoplasmic reticulum, ribosomes, Golgi complexes, or mitochondria. Bar = 1.25 μm.

our knowledge, this is the first time that erythrocytosis secondary to a neoplasm in the cecum of a dog has been reported. These findings suggest that leiomyosarcomas can be a source of ectopic EPO.

Secondary erythrocytosis attributable to ectopic secretion of EPO has been identified in association with tumors of various tissues, such as renal cell carcinomas,[7] Wilm's tumors,[8] cerebellar hemangiomas,[9] pheochromocytomas,[10] uterine leiomyomas,[11] and leiomyomas of cutaneous tissues.[12,13] In addition, EPO polypeptides and mRNA have been found in neoplastic cells from renal and hepatic carcinomas in humans.[14,15] Kidney and liver cells normally contain EPO-secreting cells, but neoplastic conversion of these cells apparently increased production of EPO in these tumors. Erythropoietin was detected in a cerebellar hemangiosarcoma at the protein and mRNA level,[16] and ectopic EPO protein was identified in a uterine leiomyoma by immunohistochemical means.[17] Several dogs with secondary erythrocytosis associated with renal neoplasms (eg, carcinoma,[18] lymphosarcoma,[19] fibrosarcoma,[20] and adenocarcinoma[21]) and nasal fibrosarcoma[22] have been described. A diagnosis of ectopic EPO production was made on the basis of resolution of the erythrocytosis following resection of the tumors[17,20-22] or measurement of EPO activity in the tumor.[22] In the dog described in the present report, EPO protein was detected in tumor cells by immunohistochemical means, and EPO mRNA expression in the tumor was confirmed by use of the RT-PCR assay.

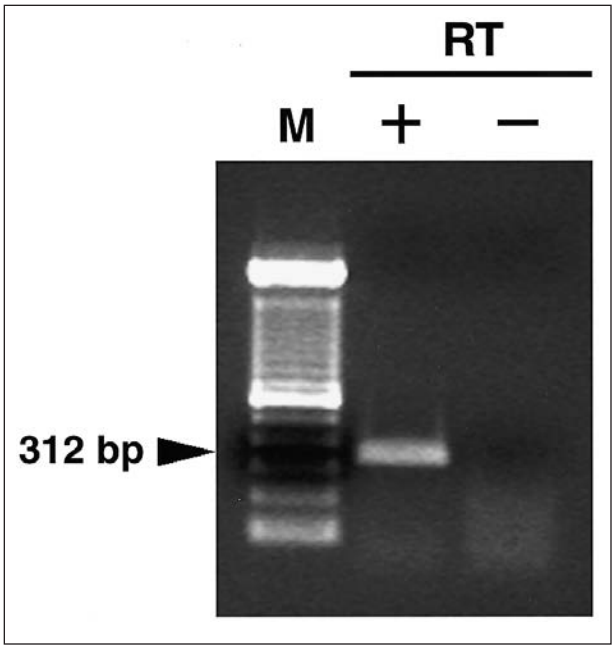

Figure 3—Electrophoretogram of amplification products obtained with a reverse transcriptase-polymerase chain reaction assay incorporating primers designed to amplify a 312-base pair (bp) portion of the canine erythropoietin cDNA. The starting material for the assay was RNA extracted from a cecal leiomyosarcoma in a dog with secondary erythrocytosis. The assay was performed with (+) or without (–) addition of reverse transcriptase (RT). M = 100-bp molecular ladder.

Ectopic EPO expression and the high plasma EPO concentrations strongly suggest that the cecal leiomyosarcoma was the primary cause of erythrocytosis in this dog. Moreover, the RT-PCR assay for detection of EPO mRNA may be useful for diagnosis of paraneoplastic erythrocytosis.

Morphologic features of the tumor cells from the dogs were interesting, because some cells contained large vacuoles filled with a PAS-positive substance. Erythropoietin is a highly glycosylated peptide, and glycosylation is important for its activity, because nonglycosylated EPO is degraded faster and cannot activate effective hematopoiesis.[23] Staining of this intravacuolar substance with PAS stain indicated that the accumulated substance was modified by some degree of glycosylation. Because high immunoreactivity to anti-EPO antibody was observed in the same vacuoles of the tumor cells, EPO produced in the tumor likely was highly glycosylated, suggesting that it was capable of inducing hematopoiesis.

In humans, normal[24] or mutant[25] EPO can be found in tumors associated with erythrocytosis, suggesting that EPO production itself is important for development of erythrocytosis. In the dog described in the present report, no mutation was found in the cDNA amplified by the RT-PCR assay. However, the presence of EPO mRNA in the tumor suggests that enhanced transcription of the EPO gene or hyperstability of EPO mRNA in the tumor was associated with the disorder. The molecular mechanism of ectopic EPO expression by tumor cells has not been fully elucidated.

[a]Vasolamine, Daiichi Pharmaceutical, Tokyo, Japan.
[b]Solulact D, Terumo, Tokyo, Japan.
[c]Recombigen EPO kit, Iatron, Tokyo, Japan.
[d]Dako LSAB kit/HRP, Dako, Glostrup, Denmark.
[e]Monoclonal antihuman erythropoietin, Genzyme Co, Boston, Mass.
[f]Monoclonal antihuman α-smooth muscle actin, Dako, Glostrup, Denmark.
[g]RNeasy Mini kit, Qiagen, Hilden, Germany.
[h]SuperScript Reverse Transcriptase II, Life Technologies Inc, Rockville, Md.
[i]KOD–plus-DNA polymerase, Toyobo, Osaka, Japan.
[j]Cy5 Thermo Sequenase Dye Terminator kit, Amersham Pharmacia Biotech, Piscataway, NJ.
[k]ALFexpress DNA sequencer, Amersham Pharmacia Biotech, Piscataway, NJ.

References

1. Jain NC. The dog: normal hematology with comments on response to disease. In: Jain NC, ed. *Schalm's veterinary hematology.* 4th ed. Philadelphia: Lea & Febiger, 1986;103–125.
2. Wen D, Boissel J-PR, Tracy TE, et al. Erythropoietin structure-function relationships: high degree of sequence homology among mammals. *Blood* 1993;82:1507–1516.
3. Jain NC. Clinical and laboratory evaluation of anemias and polycythemias. In: Jain NC, ed. *Schalm's veterinary hematology.* 4th ed. Philadelphia: Lea & Febiger, 1986;563–576.
4. Hasler AH, Giger U. Polycythemia. In: Ettinger SJ, Feldman CE, eds. *Textbook of veterinary internal medicine.* 5th ed. Philadelphia: WB Saunders Co, 2000;203–206.
5. Ogilvie GK. Paraneoplastic syndrome. In: Ettinger SJ, Feldman CE, eds. *Textbook of veterinary internal medicine.* 5th ed. Philadelphia: WB Saunders Co, 2000;498–506.
6. Hammond D, Winnick S. Paraneoplastic erythrocytosis and ectopic erythropoietins. *Ann N Y Acad Sci* 1974;230:219–227.
7. Nseyo UO, Williams PD, Murphy GP. Clinical significance of erythropoietin levels in renal cell carcinoma. *Urology* 1986;28:301–306.
8. Murphy GP, Mirand EA, Sinks LF, et al. Ectopic production of erythropoietin in Wilm's tumor patients in relation to clinical stage and disease activity. *J Urol* 1975;113:230–233.
9. Anagnostou A, Chawla MS, Pololi L, et al. Determination of plasma erythropoietin levels: an early marker of tumor activity. *Cancer* 1979;44:1014–1016.
10. Waldman TA, Rosse WF, Swarm RL. The erythropoiesis-stimulating factors produced by tumors. *Ann N Y Acad Sci* 1968;149:509–515.
11. LevGur M, Levie MD. The myomatous erythrocytosis syndrome: a review. *Obstet Gynecol* 1995;86:1026–1030.
12. Eldor A, Even-Paz Z, Polliack A. Erythrocytosis associated with multiple cutaneous leiomyomata: report of a case with demonstration of erythropoietic activity in the tumour. *Scand J Haematol* 1976;16:245–249.
13. Venencie PY, Puissant A, Boffa GA, et al. Multiple cutaneous leiomyomata and erythrocytosis with demonstration of erythropoietic activity in the cutaneous leiomyomata. *Br J Dermatol* 1982;107:483–486.
14. Da Silva JL, Lacombe C, Bruneval P, et al. Tumor cells are the site of erythropoietin synthesis in human renal cancers associated with polycythemia. *Blood* 1990;75:577–582.
15. Muta H, Funakoshi A, Baba T, et al. Gene expression of erythropoietin in hepatocellular carcinoma. *Intern Med* 1994;33:427–431.
16. Trimble M, Caro J, Talalla A, et al. Secondary erythrocytosis due to a cerebellar hemangioblastoma: demonstration of erythropoietin mRNA in the tumor. *Blood* 1991;78:599–601.
17. Yoshida M, Koshiyama M, Fujii H, et al. Erythrocytosis and a fibroid. *Lancet* 1999;354:214.
18. Peterson ME, Zanjani ED. Inappropriate erythropoietin production from a renal carcinoma in a dog with polycythemia. *J Am Vet Med Assoc* 1981;179:995–996.
19. Nelson RW, Hager D, Zanjani ED. Renal lymphosarcoma with inappropriate erythropoietin production in a dog. *J Am Vet Med Assoc* 1983;182:1396–1397.
20. Gorse MJ. Polycythemia associated with renal fibrosarcoma in a dog. *J Am Vet Med Assoc* 1988;192:793–794.

21. Crow SE, Allen DP, Murphy CJ, et al. Concurrent renal adenocarcinoma and polycythemia in a dog. *J Am Anim Hosp Assoc* 1995; 31:29–33.

22. Couto CG, Boudrieau RJ, Zanjani ED. Tumor-associated erythrocytosis in a dog with nasal fibrosarcoma. *J Vet Intern Med* 1989;3:183–185.

23. Dube S, Fisher JW, Powell JS. Glycosylation at specific sites of erythropoietin is essential for biosynthesis, secretion, and biological function. *J Biol Chem* 1988;263:17516–17521.

24. Noguchi Y, Goto T, Yufu Y, et al. Gene expression of erythropoietin in renal cell carcinoma. *Intern Med* 1999;38:991–994.

25. Funakoshi A, Muta H, Baba T, et al. Gene expression of mutant erythropoietin in hepatocellular carcinoma. *Biochem Biophys Res Commun* 1993;195:717–722.