

Interpretation/Diagnosis

1) What is your diagnosis based on the cytologic findings and location of the lesions?

The cytologic diagnosis was: “round cell neoplasm, most suggestive of plasma cell neoplasia”, based on the cytomorphologic similarity of the principal population to aspirates from mammalian plasmacytomas.

2) What are the main differential diagnoses?

Differential diagnoses included other round cell neoplasms such as lymphoproliferative disorders and histiocytic tumour, and malignant mesenchymal tumours such as soft tissue sarcoma and extraskkeletal osteosarcoma.

3) What further tests would you recommend to clarify the diagnosis?

ALP staining, histopathology and immunohistochemistry were performed to further characterise the lesions.

Additional information

A total body radiographic study was performed using a single dorso-ventral projection (Figure 7). This revealed a generalized decrease in skeletal opacity suggestive of a degree of osteopenia. No fractures or bony exostoses around the skull were identified. No other soft tissue mass lesions were observed and there was no evidence of mineralisation within the internal organs. Furthermore, no focal lytic “punched out” bony lesions were observed (Figure 7). Lateral projections, which could have been helpful to evaluate possible organomegaly (liver, spleen and lungs), were not obtained.

A blood smear was examined and appeared unremarkable apart from a suspected lymphopenia. The volume of collected blood was insufficient to permit quantitative haematologic analysis.

Cytologic examination of the three slides obtained from the sampled nodules revealed moderate numbers of well-preserved, oval to round cells arranged mostly individually and in non-cohesive clusters. Within these clusters was a small amount of grainy to fibrillar, magenta, intercellular material. Cell borders were variably distinct. The cells varied between 10 to 20 µm and had a moderate amount of smooth, mid basophilic cytoplasm which had a “hyalinised” appearance and sometimes appeared to overlie the nucleus. In some cells, the periphery of the cytoplasm had a grainy, magenta hue of similar quality to the intercellular material. The nucleus varied from 6 to 12 µm diameter, was eccentric and round or indented, or occasionally had an irregular nuclear outline. Chromatin was coarse and cells had 1 to 3 small indistinct nucleoli. The cells displayed moderate anisocytosis and anisokaryosis. Frequent binucleate and occasional trinucleate cells were found. Low numbers of erythrocytes and rare heterophils were also present. The background contained stippled eosinophilic proteinaceous material. No

microorganisms were found.

ALP staining performed on one slide was negative. The positive control slide was from a canine liver aspirate, as no suitable positive control from a tortoise was available. Although ALP staining on reptilian cells has not been specifically validated in the authors' laboratory, ALP expression in osteoblasts is well conserved across species, so the negative result was considered plausibly valid.¹ Furthermore, there was no evidence of bony involvement on imaging, and the superficial nature of these masses rendered an extraskeletal osteosarcoma unlikely.

An incisional biopsy of two of the dermal masses was obtained and submitted for histopathology. This revealed a non-encapsulated, poorly demarcated mass expanding within the dermis. The neoplastic cells were arranged in sheets within the pre-existing stroma, extending close to but not reaching the overlying basement membrane. The cells were round with distinct borders, moderate amounts of eosinophilic to glassy cytoplasm and round to ovoid, often eccentric nuclei with stippled chromatin. There was moderate anisocytosis and anisokaryosis. The mitotic rate was low. The neoplastic cells were observed extending to the surgical margins of the mass (Figure 8). Toluidine blue staining was performed and illustrated metachromatic granules in occasional cells. These were interpreted as pre-existing or reactive mast cells, rather than representing the neoplastic population. Overall, the histopathology findings were compatible with a round cell tumour. A plasma cell tumour was considered the most likely diagnosis, supporting the cytologic findings.

To further characterize the tumour, immunohistochemistry (IHC) for plasma cells (MUM 1 immunolabelling) was performed. The neoplastic population was negative for MUM1, arguing against a plasma cell origin. A few scattered individual cells exhibited MUM1 positivity and were interpreted as non-neoplastic plasma cells within the lesion.

Additional IHC markers were evaluated. The neoplastic cells were negative for CD18, Iba1, PAX5, vimentin, desmin, melan A and S100, but showed intense cytoplasmic positive immunoreactivity for CD3 (Figure 9). In the absence of available suitable positive tortoise control material, positive external controls for all markers were successfully performed on canine tissue. Positive internal controls within the test case tortoise tissue were evident for MUM1, Iba1, desmin, PAX5 and CD3, as follows. For MUM 1, PAX5 and CD3, focal areas of lymphoid-rich inflammation within the mass provided evidence of positive staining non-neoplastic plasma cells, B cells and T cells respectively. A consensus of three histopathologists designated these distinct populations from the neoplastic cells on the basis of small, mature morphology, location, and correlation with the haematoxylin and eosin-stained sections. Within this area, there were also occasional stromal dendritic cells providing the positive internal control for Iba1. Elsewhere within the mass, blood vessel mural cells provided the positive internal control for desmin. However, no convincing, or only ambiguous positive internal controls could be found for the remaining markers (S100, CD18, vimentin and melan A) (Figure 10). The immunohistochemical antibodies clones were as follows: MUM1p (RTU/DAKO) for MUM1; CA16.3C10 (University of California, Davis) for CD18; Anti Iba1 Rabbit (Fujifilm Wako Chemicals) for Iba1; 1EW (Leica Biosystems) for PAX5; LN10 (Leica Biosystems) for CD3; V9 (Leica) for vimentin, DE-R-11 (Leica Biosystems) for desmin; A103 (Leica Biosystems) for melan A; and EP32 (Leica Biosystems) for S100.

Based on these findings, the tumour was eventually classified as a “CD3+/MUM1-plasmacytoid round cell tumour”.



Figure 7: Radiographic studies, dorsoventral projections, of the total body (left) and head (right).

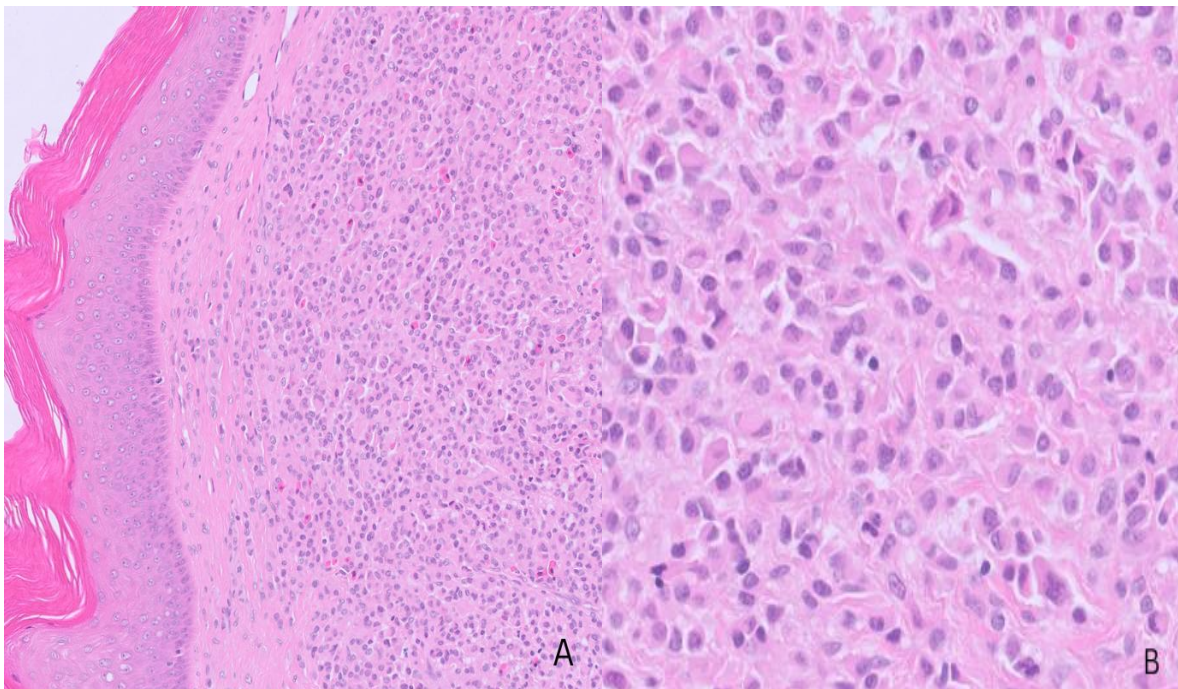


Figure 8: Skin mass (haematoxylin and eosin). (A) Expanding the dermis are sheets of neoplastic cells interspersed within the pre-existing stroma, extending close to but not reaching the overlying epidermal basement membrane (100x). (B) Inset: The cells are round with distinct borders, moderate amounts of eosinophilic to glassy cytoplasm and round to ovoid, often eccentric nuclei (400x).

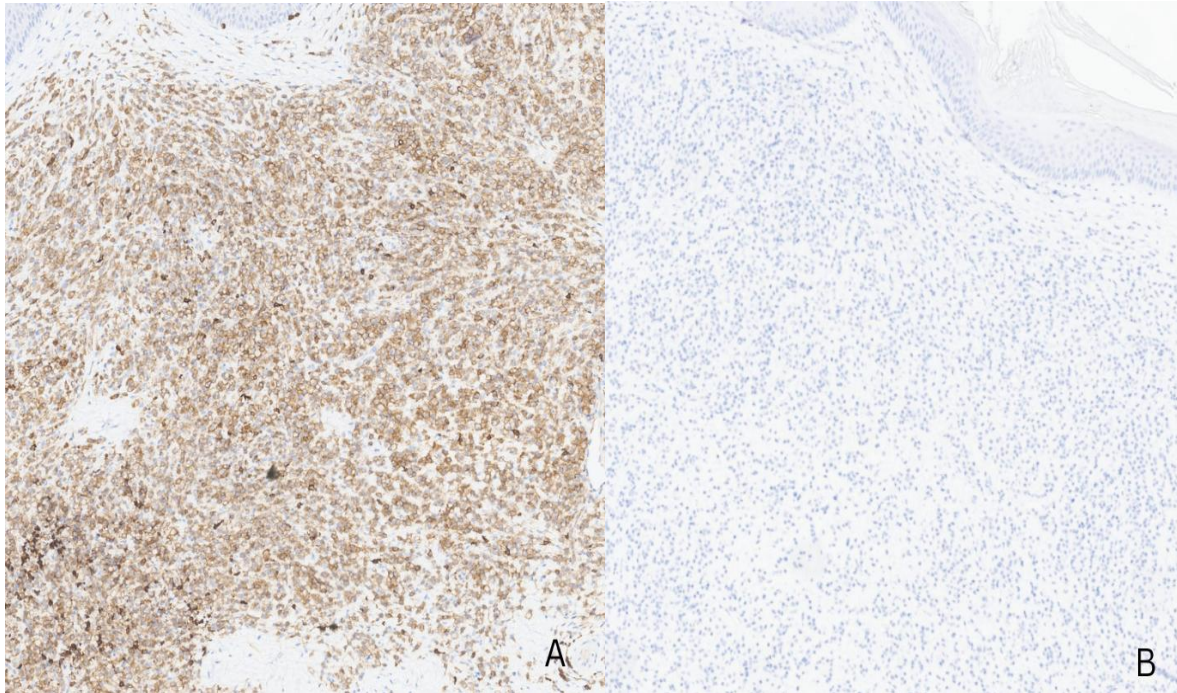


Figure 9: Skin mass (IHC) (A) Neoplastic cells showed intense cytoplasmic immunoreaction to CD3 (100x). (B) MUM1 staining was negative in neoplastic cells (100x).

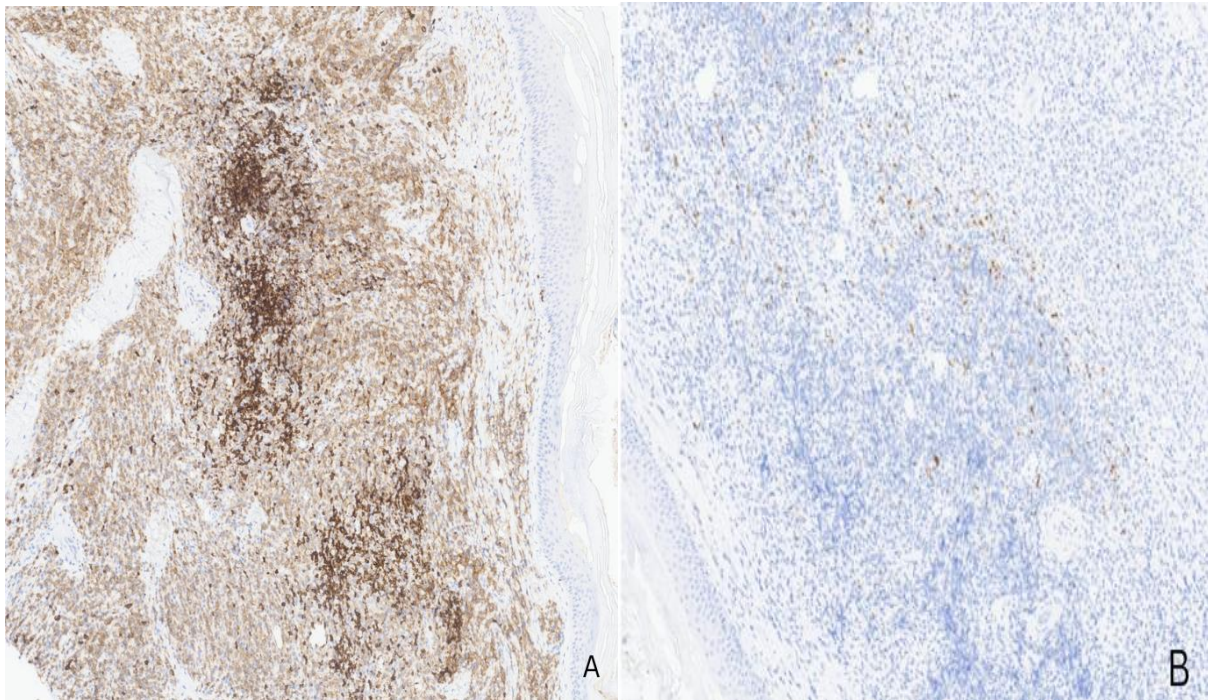


Figure 10: Skin mass (IHC, internal positive internal controls) (A) Focal aggregates of non-neoplastic smaller mature T-cell lymphocytes staining dark brown with CD3 (100x) amongst the larger, less intensely staining neoplastic cells. (B) Occasional non-neoplastic plasma cells showing moderate nuclear and cytoplasmic immunoreactivity to MUM1 (100x).

Follow up and clinical outcome

Five months following the investigations, no significant growth of the lesions has been observed, and the tortoise remains clinically well.

Discussion

Neoplasia in testudines is extremely rare, possibly due to innate resistance to oxidative stress and protein dysregulation. Indeed, they have been proposed as a model for exploring the evolution of longevity and natural cancer suppression mechanisms.² There is thus a paucity of information on tortoise neoplasia in the literature.³⁻⁶

Neoplasia in reptiles appears more common in snakes, followed by lizards, chelonians and crocodilians.⁵⁻⁸ One study showed a slightly higher incidence of neoplasia in turtles compared with tortoises.⁵ Both the overall incidence of neoplasia in chelonians and the rate of metastasis in reported cases of neoplasia in tortoises appear to be low.⁶⁻⁷

Cutaneous neoplasms in reptiles and in particular in lizards, appear relatively frequent when compared with tumours arising at other anatomic locations.^{5,7,9-10} However, this may reflect in part the easier detection of cutaneous tumours.

Of reptilian cutaneous neoplasms, papillomas, fibropapillomas, squamous cell carcinomas (SCCs) and chromatophoromas are most commonly reported.^{5,11} Less

common cutaneous neoplasms in reptiles include cutaneous fibromas and mastocytosis (one case report in a common snapping turtle [*Chelydra serpentina*] and in an African fat tailed gecko [*Hemitheconyx caudicinctus*], respectively), and sporadic cases of round cell tumours, melanomas and myxosarcomas.^{5, 11-12}

In testudines, the most frequently reported neoplasm is cutaneous squamous cell carcinoma, followed by mast cell tumours, fibromas and adenocarcinomas.¹¹ A mucinous subtype of melanophoroma has been described in a Hermann's tortoise, and also in bearded dragons (*Pogona vitticeps*) and red-bellied cooters (*Pseudemys rubriventris*).¹¹ Additionally, a single case of cutaneous mast cell tumour has been described in a Giant Galapagos tortoise (*Geochelone nigra vicina*), presenting as a solitary cutaneous mass at the base of the neck.¹³ In the same species, a case of subcutaneous cervical osteoma has been also described.¹¹ There is also a single report of a cutaneous adenocarcinoma in a desert tortoise (*Gopherus agassizii*). Of non-cutaneous tumours, chelonians have been reported to exhibit a higher prevalence of endocrine tumours compared with other reptiles, with thyroid adenocarcinoma the most common.¹¹

No plasma cell tumours have been previously described in tortoises, though have been reported in other reptiles. A plasma cell neoplasm of unknown location was described in a Nile monitor (*Varanans niloticus*).^{5,14} Another case of disseminated plasma cell neoplasia involving the lungs, liver and stomach has been reported in an East Indian water lizard (*Hydrosaurus aniboinensis*).¹⁵ The diagnosis of plasma cell tumour was not immunohistochemically confirmed in either case. A round cell tumour with plasmacytoid morphology has been described in a green tree python (*Morelia viridis*), presenting macroscopically as an off-white nodular coelomic mass. Histopathology examination revealed sheets of round cells with eccentric nuclei and amphophilic cytoplasm consistent with plasma cells.¹¹ The histologic appearance of these cells closely resembled those in the presented case.

Immunohistochemistry (IHC) was employed in the present case. This technique has been previously utilised in the investigation of a variety of reptilian neoplasms, albeit with variable degrees of success. In a reported case of rhabdomyosarcoma in a Texas gopher tortoise (*Gopherus berlandieri*), the neoplastic cells lacked immunoreactivity for both MyoD1 and myogenin markers. The authors suggested one possible explanation was the uncertain cross-reactivity of commercially available antibodies with reptilian antigens, as most antibodies are developed against human or rodent epitopes.¹⁶ In that case, only external positive controls were used and although these confirmed the ability of the antibodies to detect mammalian MyoD1 and myogenin, they did not validate antibody reactivity against reptilian antigens. The lack of internal or chelonian-specific positive controls further raises uncertainty as to whether the negative immunolabelling reflected a true absence of antigen expression or insufficient antibody cross-reactivity resulting from phylogenetic differences between mammalian and reptilian epitopes.¹⁶

Additional factors that may affect immunohistochemical results and contribute to incomplete antibody cross-reactivity include antigen preservation and fixation time. Prolonged formalin fixation can negatively affect immunohistochemistry through antigen cross-linking and epitope masking. However, one study demonstrated that extended formalin fixation times of up to seven weeks did not significantly impair antigen detection for the most common antibodies.¹⁷

Another reported case in which immunohistochemical characterization of a mass proved challenging, involved an intracoelomic neoplastic lesion in an Asia minor spur-thighed tortoise (*Testudo graeca iberica*). The neoplastic cells were negative for the majority of the immunohistochemical markers applied, including pan-cytokeratin, CD3, CD79, vimentin, chromogranin A and synaptophysin. Appropriate positive controls from mammalian tissue were employed for the markers, except for cytokeratin, for which peritumoral glands and ducts served as internal positive control tissue.¹⁸

In contrast, immunohistochemistry has proven useful in other reported cases of reptilian neoplasms. In one study, keratoacanthomas (KAs) in lizards showed a consistent immunohistochemical pattern with the expression of cyclooxygenase-2, E-cadherin, and pan-cytokeratin, and IHC proved valuable to distinguish KAs from squamous cell carcinomas (SCCs).¹⁹

The melanocytic markers PNL2, S100, melan A and HMB45 have been employed in bearded dragons and other reptiles with varying success. S100 and melan A have been demonstrated useful in the diagnosis of reptilian amelanotic and poorly pigmented melanophoromas, and a small number of cases also had immunoreactivity for PNL2 and HMB45. However, IHC has not proved able to distinguish the different subsets of chromatophoromas.²⁰⁻²²

Iba-1 and CD204 have been shown to be valuable in the diagnosis of reptilian neoplastic processes. In a central bearded dragon (*Pogona vitticeps*), the majority of the neoplastic cells of a retrobulbar histiocytic sarcoma showed immunoreactivity for Iba-1, whereas a small number of cells exhibited a weak positivity for CD204.²³

Of lymphoid markers, CD3 has been used successfully to identify T-cell lymphoma in reptiles, whereas CD20, CD79 and PAX5 have been variable effective at identifying B cell neoplasms.²⁴⁻²⁸

In the present case, histopathology supported the initial cytological suspicion of a plasma cell tumour. However, the neoplastic cells were negative for MUM1. A panel of additional markers targeting B-cell and T-cell, histiocytic, mesenchymal, melanocytic and muscle lineages, was also evaluated. The neoplastic cells were negative for all tested immunohistochemical markers, except for CD3. Despite the absence of validated antibodies for this species, the presence of positive internal controls for CD3 and MUM1 indicated successful binding of these antibodies to tortoise tissue. This suggests technical reliability within the histologic sections and increased our confidence in the interpretation of the positive immunohistochemical results. On the other hand, the absence of internal controls for some of the remaining markers suggests that the negative results should be interpreted with caution, as they may represent either true negative findings or false negative due to a technical failure.

The immunohistochemistry results, the dermal location with lack of epitheliotropism, and the presence of a strongly plasmacytoid morphology both on histopathology and cytology, supported the classification of this tumour as “CD3+/MUM1- plasmacytoid round cell tumour”. With the exception of CD79 and PAX5, which have demonstrated utility as B-cell markers in selected case reports, a consistent panel of reliable B-cell markers has not been established in reptiles^{11, 25-27} Consequently, despite the lack of immunoreactivity of this neoplasm for the B-cell markers MUM1 and PAX5, a plasmacytoid round cell tumour with aberrant CD3 expression was considered likely. In

the human literature, rare B-cell neoplasms with plasmacytoid morphology and aberrant CD3 expression in the immunohistochemistry, have been described, highlighting the potential limitations of immunophenotyping interpretation in atypical round cell tumours.³⁰ It would have been interesting to perform serum protein electrophoresis to investigate the possibility of any monoclonal antibody production associated with a potential plasma cell tumour, but serum was not available.

The principal differential diagnosis was a non-epitheliotropic T-cell lymphoma characterized by an unusual plasmacytoid morphology. This was due to the CD3 positivity and previous reports describing lymphomas with plasmacytoid morphology. If this was indeed a T-cell lymphoma, the findings would be consistent with the previously published reports of reptilian lymphoma: in the few cases where immunohistochemical characterisation was pursued, the majority were of T-cell lineage.^{5, 24, 25, 26, 28}

The dermal localization of lymphoma appears to be uncommon in reptiles, where lymphomas are typically reported as multicentric or leukemic, sometimes with oral involvement^{10, 25, 27, 29} In the present case, no oral lesions were identified and there were no overt findings to support a multicentric or leukemic form of lymphoma. Histopathological evaluation revealed that the neoplastic cells were confined to the dermis, with no evidence of epithelial invasion; thus, this was not consistent with an epitheliotropic T cell lymphoma.

Lymphomas in reptiles are often characterized by blast-like cell morphology. Plasmacytoid morphology of lymphoid neoplasm has also been described and may therefore be consistent with the morphology observed in this case.⁴ Furthermore, previously reported plasma cell tumours in reptiles were not immunohistochemically characterized, and were diagnosed solely on the basis of their plasmacytoid morphology on histopathology.^{5,14,15} As a result, the possibility that these neoplasms were of lymphoid origin cannot be excluded.

In conclusion, this case highlights the challenges associated with the use and interpretation of immunohistochemistry in reptiles, the utility of the CD3 marker in this species, and that caution must be employed when classifying reptilian round cell tumours with plasmacytoid morphology. It thus underscores the need for the development and validation of reliable immunohistochemical markers, in particular B cell markers, as well as the use of species-specific control tissues, for the accurate characterization of neoplasms in chelonians and other reptiles. Given the scarcity of available data on the application of immunohistochemistry in chelonians and the limited occurrence of neoplasia in tortoises, we believe that our findings contribute valuable information and may serve as a foundation for future applications of immunohistochemistry in this species.

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