

Preliminary Data on the Association of Microvessel Density and Vascular Endothelial Growth Factor A in 12 Canine Appendicular Osteosarcoma

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Abstract

Angiogenesis as a prognostic indicator in tumours has been extensively studied, demonstrating a positive correlation for various malignant tumours. Yet, in osteosarcomas (OSAs), its role remains a topic of debate. VEGFA is considered the most important pro-angiogenic factor involved in the development of the vasculature. In order to investigate the possibility that intra-tumoral microvessel density (MVD) expression may be related to VEGFA and may provide useful prognostic information in canine OSA, 12 histological specimens of primary canine appendicular OSA were immunostained using an endothelial marker CD31 and VEGFA antibody. No significant differences in mean MVD were found when comparing with various clinicopathological features, development of pulmonary metastasis and patient prognosis. Notably, the number of vessels counted in VEGFA-High expression specimens was significantly greater than those in the VEGFA-Low expression ($p < 0.05$). In this study, we were able to demonstrate that canine appendicular OSA is a relatively vascular tumour and that the local MVD in primary canine appendicular OSA is significantly correlated with VEGFA immunostaining expression in the tumour tissue. These observations suggest that VEGFA secreted by canine appendicular OSA cells elicits angiogenesis. However, the degree of MVD does not provide prognostic information. It is likely that angiogenesis plays a key role in the tumorigenesis of canine appendicular OSA and may be a potential target for novel anti-angiogenic therapies.

Keywords: Microvessel Density (MVD); Vascular Endothelial Growth Factor A (VEGFA); Angiogenesis; Canine; Osteosarcoma (OSA)

Introduction

Osteosarcoma (OSA) is the most common bone tumour in the dog, encompassing 85% of all skeletal malignancies [1]. It is characterized by very aggressive local tissue destruction and distant metastasis. Appendicular tumours are reported to be the most common, with 75% of all canine OSA [1-3]. It has long been known that solid tumours need tumour's surroundings blood supply for their growth and spread, and that the expression of angiogenic molecules to induce new vessel formation is a characteristic of highly aggressive and metastatic tumours [2,3]. Vascular Endothelial Growth Factor A (VEGFA) is the best characterized pro-angiogenic factor and is considered the most important factor involved in the development of the vasculature. It does so by stimulating endothelial cell proliferation, migration and capillary tube formation [4,5]. Expression of VEGFA has been suggested as a means of evaluating the prognostic importance of angiogenesis in OSA [6,7]. Several studies of numerous types of human carcinomas, including breast, gastric, colorectal and prostate cancers [5-20] have reported correlations between intra-tumoral microvessel density (MVD) and angiogenic growth factor expression, tumour growth and the occurrence of distant metastasis. This indicates that MVD reflects important information on the degree and function of tumour angiogenesis [8-10,15,19]. However, the role of angiogenesis in sarcomas, especially OSAs is still unclear. Results and conclusions vary and are conflicting. This is shown in a few existing reports [21-23]. In canine OSA, the only study that has been performed suggested that MVD in primary tumours maybe an indication of tumour progression and metastasis [2], but the relationship of this parameter with VEGFA expression and overall survival has not been previously described.

In this article, we directed our attention to the association of the MVD in 12 primary tumours with VEGFA expression, clinicopathological features, and the prognosis for dogs with appendicular OSA.

Materials and Methods

Patients and specimens

A total of 12 specimens were harvested from dogs with spontaneous appendicular OSA who underwent surgery at the Veterinarian Teaching Hospital in the Department of Veterinary Sciences (University of Turin) between 2010 and 2017. Following anamnesis and physical examination, all patients were initially staged with a complete blood count, serum biochemistry profile, urinalysis, abdominal ultrasound, and two-view limb radiographs. It was ensured that all studied dogs were free of visible metastasis prior to surgery using three-view thoracic radiographs; Computed Tomography (CT) was additionally available in case of non-conclusive radiographs during the latter 4 years of the study. Swollen regional lymph nodes were aspirated and examined cytologically to exclude metastasis. Closed biopsies of all cases were performed by fine-needle aspiration or trephine for diagnosis. None of the patients had received any preoperative treatment.

Treatment consisted of amputation or limb sparing followed by adjuvant doxorubicin (30 mg/m², 4-5 administrations, 21 days apart) or cisplatin (70 mg/m², 4-5 administrations, 21 days apart) as single agents or in combination (4 cycles, 21 days apart, each cycle consisting of cisplatin 50 mg/m² at day 1 and doxorubicin 15 mg/m² at day 2), as

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long as metastasis was not detected. Patients were re-staged clinically and radiographically every 3 months up to 1 year after the conclusion of chemotherapy and then every 6 months for a minimum of 2 years. None of the cases were lost to follow-up. Specimens harvested after surgery were fixed in formalin 10% for at least 24 h, embedded in paraffin, sectioned (4 µm) and stained with haematoxylin and eosin (H&E). The histological diagnosis was defined according to the WHO guidelines [24], while the grade was established using the scale of Loukopoulos and Robinson [25].

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was carried out on 4 µm paraffin sections of canine appendicular OSA samples prepared on 3-aminopropyltriethoxysilane-coated (APES) slides. The sections were deparaffinized and then rehydrated with descending alcohol concentrations to buffer. For VEGFA sections, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 10 min at room temperature. Antigen retrieval was performed by immersion in Tris-EDTA buffer (pH 9) using a water bath under high-temperature antigen unmasking 98°C for 20 min. For MVD slides, heat-induced epitope retrieval with citrate buffer (pH 6.0) for 30 min was followed by endogenous peroxidase blocking with 0.3% hydrogen peroxide for 30 min and incubation with the primary CD31 antibody, overnight at 4°C. The antibodies used were a mouse monoclonal anti VEGFA antibody (Santa Cruz Biotechnology, Inc, Dallas, TX) at a dilution of 1:25, and a mouse monoclonal anti CD31 antibody (DAKO Omnis). The immunoreactive complexes were detected using the avidin-biotin peroxidase complex technique with the Vectastain Elite ABC Kit Universal and the diaminobenzidine (DAB) Substrate kit (Vector Laboratories). Canine mammary carcinoma was used as positive control for both CD31 and VEGFA. For this latter, endothelial cells were used as an internal positive control. For negative controls, the sections were incubated without the primary antibodies.

Specimen's evaluation

Immunostaining results were independently evaluated with no knowledge of the clinicopathological features. In the event of differing evaluations, a final decision was made by consensus. For the VEGFA staining, immunoreactivity was evaluated using only intensity scoring as follows: 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive) and for statistical analysis, tumours scored 1 and 2 were classified as "Low" expression and tumours scored 3 were classified as "High" expression.

MVD was determined according to the method of Weidner [16]. Sections were scanned at a microscope magnification of 20x or 80x to identify heavily stained vascular "hot spots" within the tumour [10]. Counts of the 3 most vascular areas for each specimen were made at microscope magnification of 200x (field area 0.74 mm²), and the mean of the 3 areas was used for further analysis. Positively stained endothelial cells in microvessels were clearly seen in all specimens. A visible vascular lumen was not required for a vessel to be included in the count. Very tortuous microvessels were seen especially in highly vascularised areas. In such cases, each profile was counted as an independent vessel [16]. Large and small arteries and veins were very rare and not included in the counts [2].

Statistical analysis

The relation between MVD and VEGFA expression and clinicopathological findings were grouped into contingency tables for statistical analysis (Table 1) and analysed using Fisher's exact test. Overall survival (OS) and disease free interval (DFI) were determined for survival analysis. OS was defined from the day of surgery until death of the patient. Survival or absence of tumour recurrence until the end of the monitoring period or death from a cause other than OSA was considered a censoring event. DFI, which is related to patients who showed no evidence of disease after primary therapy, was defined from the end of primary therapy until first evidence of local, regional

Case Number	Age (Months)	Weight (Kg)	Gender	Breed	Localisation	Diagnostic	Status	Lung Metastasis	Dfi (Days)	Os (Days)	Grade	Mvd/0.74 Mm ²	VEGFA Expression
1	10	43	M	Crossbreed	Tibia distal	Osteosarcoma Osteoblastic	Dead	1	242	252	3	38	Low
2	7	75	M	Great dane	Tibia distal	Osteosarcoma Osteoblastic	Dead	1	94	152	3	66	High
3	8	43	M	Crossbreed	Humerus proximal	Osteosarcoma Osteoblastic	Dead	1	168	277	3	66.33	High
4	10	45	F	Rottweiler	Humerus proximal	Osteosarcoma Osteoblastic	Dead	1	137	151	3	69	Low
5	13	41	F	Crossbreed	Humerus proximal	Osteosarcoma Osteoblastic	Dead	0	156	194	3	100	High
6	6	37	F	German shepherd	Femur distal	Osteosarcoma Fibroblastic	Dead	1	697	727	2	16.33	Low
7	10.5	36	M	Labrador	Tibia distal	Osteosarcoma Osteoblastic	Dead	0	981	981	2	34	Low
8	7	46	F	Cane Corso	Femur distal	Osteosarcoma Cndroblastic	Dead	0	171	203	2	35.66	Low
9	6	32	M	Boxer	Carp distal	Osteosarcoma Osteoblastic	Dead	1	138	175	2	37.66	Low
10	10	34	F	Greyhound	Humerus proximal	Osteosarcoma Osteoblastic	Dead	0	34	36	2	94	High
11	5	39	M	Cross-breed	Femur Proximal	Osteosarcoma Cndroblastic	Dead	1	48	56	1	32	Low
12	1.11	38	F	Golden retriever	Tibia Proximal	Osteosarcoma Osteoblastic	Dead	0	282	304	1	56.33	High

M: Male, F: Female, DFI: Disease Free Interval, OS: Overall Survival

Table 1: Clinicopathological data with MVD and VEGFA expression in canine appendicular OSA specimens included in the study.

or distant tumour progression of disease. Survival curves were plotted according to the Kaplan–Meier method, and the Log – rank *p*-values were calculated, considering all known prognostic factors for canine OSA, to compare groups for DFI and OS. All statistical analyses were performed using the Statistical Package for Social Sciences software (SPSS, Inc., USA) and $P < 0.05$ was considered statistically significant.

Results

Case characteristics

Of the 12 cases examined in this study, equal numbers were male and female. The mean age of the dogs was 7.8 years, with a range of 1 – 10 years. Four of the dogs were mixed breed, while eight were pure breeds one each: Boxer, Rottweiler, German Shepherds, Great Dane, Cane Corso, Golden Retriever, Greyhound, Labrador.

The median DFI was 262 days with a range of 34 – 981 days. The median ST was 292 days, with a range of 36 – 981 days. 51% of the dogs that were included in the study died of metastatic disease, 19% died of unrelated causes, while the remaining dogs were still alive at the end of the monitoring period and were all censored from survival analysis (Table 1).

Association of MVD with clinicopathological finding

The vascular density of canine OSA “hot spots” seen in this study ranged from 16.33 to 100 mean profiles of three fields at 200x magnification. No significant differences in mean vessel density were found when comparing with age, gender, grade, histological subtype, anatomic location and lung metastasis (Data not shown).

Association of VEGFA Expression with Local MVD

To investigate the relationship between VEGFA expression and MVD in canine appendicular OSA, we immunohistologically stained the specimens of 12 primary canine OSA using antibodies specific to VEGFA and CD31. As representatively shown in Figure 1, VEGFA staining was defined as positive in all OSA tumours with different intensity (weak, moderate, strong) (Figures 1A-1C). The staining pattern of the tumour was uniform, and VEGFA was localized in the cytoplasm of OSA cells. Immunostaining with the anti-CD31 antibody clearly defined endothelial cells in the microvessels (Figures 2A-2C). Notably, the number of vessels counted in VEGFA-High expression (76.5) (Figure 2C) was significantly greater than those in the VEGFA-low expression (37.5) ($p < 0.05$) (Figures 2A and 2B).

Association of MVD with OSA cases grouped by metastatic status and prognosis

Cases were divided into groups based on the metastatic status of the animal. Group 1 consisted of dogs that had pulmonary metastases at the time of diagnosis, $n=2$. Dogs in Group 2 were apparently metastasis free at diagnosis, but later developed pulmonary metastases, $n=4$. The earliest development of metastatic disease in this group occurred within 3 months of surgery. The group 3 consists of cases without pulmonary metastasis at death, $n=4$. The last group 4 consisted of dogs with distant recurrence, $n=1$. The recurrence occurred one month from the time of surgery.

Cases were divided based on metastatic status and no significant differences in MVD were seen (Table 2). The group presenting distant recurrence had higher MVD in the primary tumour than animals with pulmonary metastasis at the time of diagnosis and those which later developed metastasis. Moreover, this group had lower DFI and OS than

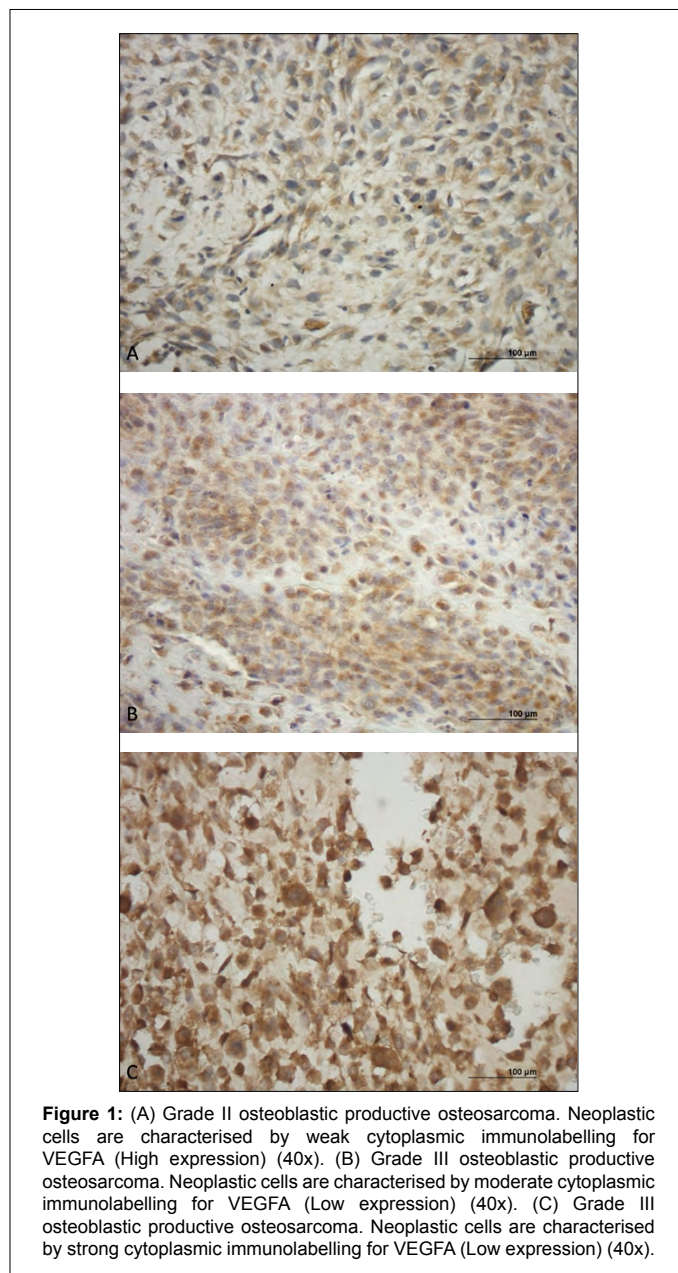
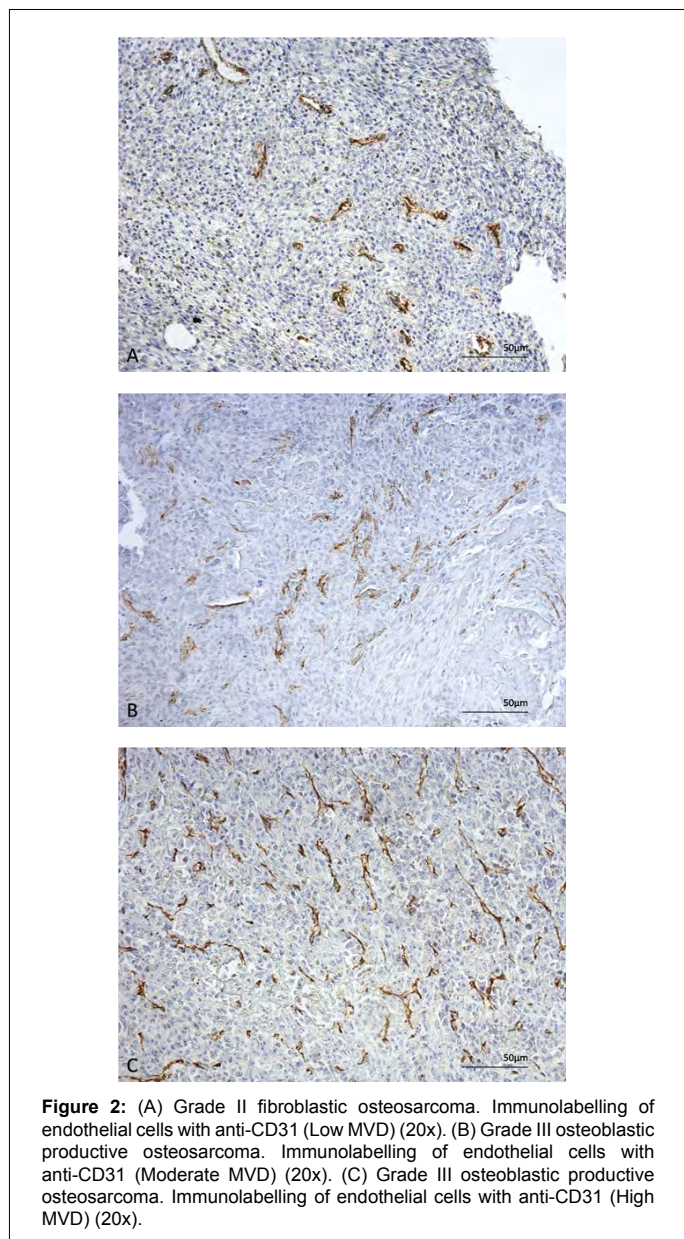


Figure 1: (A) Grade II osteoblastic productive osteosarcoma. Neoplastic cells are characterised by weak cytoplasmic immunolabelling for VEGFA (High expression) (40x). (B) Grade III osteoblastic productive osteosarcoma. Neoplastic cells are characterised by moderate cytoplasmic immunolabelling for VEGFA (Low expression) (40x). (C) Grade III osteoblastic productive osteosarcoma. Neoplastic cells are characterised by strong cytoplasmic immunolabelling for VEGFA (Low expression) (40x).

the other groups. Regarding prognosis, cases were then divided into three equivalently sized groups, with tumours containing low (≤ 38), intermediate (39-69), or high (≥ 70) numbers of microvessels. MVD was not significantly associated with DFI ($P=0.648$) and OS ($P=0.766$) (Figure 3).

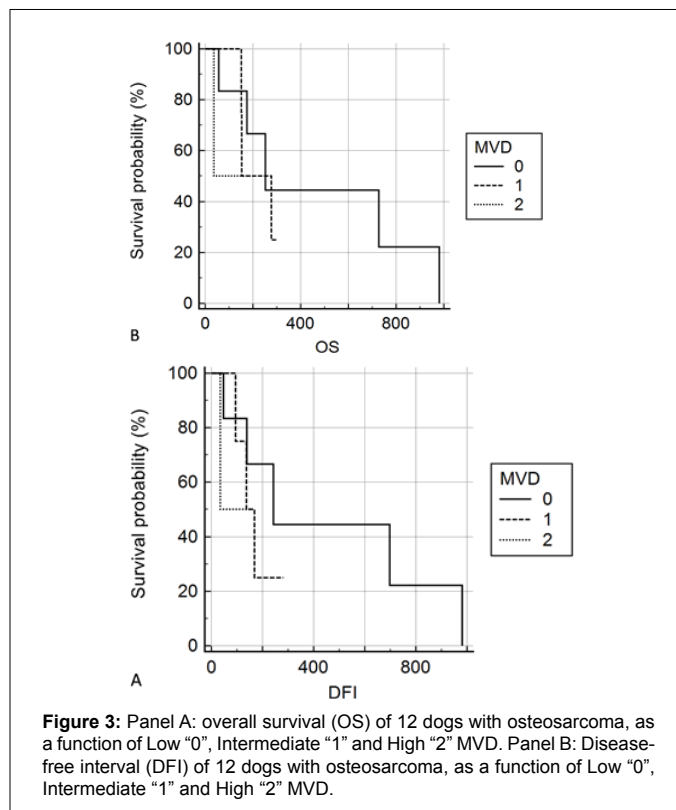
Discussion

In the present immunohistochemical study, we were able to demonstrate that canine appendicular OSA is a relatively vascular tumour, supported by the fact that a majority of cases revealed different levels of tumour microvessels per 0.74 mm^2 . Moreover, in all tumour specimens, various positive intensity levels of VEGFA expression were evident within the cytoplasm of the OSA cells and were displayed in all tumour “hot spots”. We have also demonstrated that the local MVD in primary canine appendicular OSA is significantly correlated



with VEGFA immunostaining intensity in the tumour tissue. Our results suggest that VEGFA secreted by OSA cells elicits angiogenesis as this marker is considered as a potent pro-angiogenic factor by activating endothelial cells via interaction with its receptors, Flk-1 and Flt-1, which are selectively expressed in the endothelium [11]. This association is further echoed in OSA with positive VEGFA expression correlating with increased local MVD [3,12].

Although we failed to demonstrate any correlation between the MVD and clinicopathological features, development of pulmonary metastasis and prognosis, this result has been further echoed in other human cancer studies. It is becoming increasingly apparent in the literature that MVD may not be as relevant a prognostic marker in sarcomas as it is in carcinomas [13,14]. The assumption is that this may be because of different patterns of blood vessel distribution in sarcomas as opposed to blood vessel distribution in carcinomas. The latter cluster in 'bursts', the former, sarcoma vessels are more homogenous and



Variables	Number of cases	MVD	DFI	OS
Pulmonary metastasis at the time of diagnosis (Group 1)	2	50.5	92.5	103.5
Later development of pulmonary metastasis (Group 2)	5	44.9	267.8	316.6
No pulmonary metastasis at death (Group 3)	4	56.5	397.5	420.5
Distant recurrence (Group 4)	1	94	34	36

MVD: Microvessel Density, DFI: Disease Free Interval, OS: Overall Survival.

Table 2: MVD of OSA specimens with known outcomes grouped by metastatic status.

diffusely distributed. This leads to a single compartment of neoplastic cells that present uniformly as angiogenic cytokines [13]. Further studies also failed to show prognostic significance of MVD in various soft tissue sarcomas [13,17,18]. Moreover, a study by Mantadakis et al. that used CD34 as an endothelial marker failed to demonstrate any correlation between intratumoral MVD and long-term outcome in patients with non-metastatic OSA [3,21-23]. Regarding canine OSA, a preliminary work made by Brenda et al. found significant relationships between MVD and pulmonary metastatic status on initial presentation. Their results suggest that for animals treated only with surgical removal of the tumour, and who have had no adjunct therapy, might show that MVD is an important prognostic indicator. Very high vessel density, therefore, suggesting a short survival, is caused perhaps by prior existence of metastatic disease [2]. In light of the findings of our present study, further research is required, with a sufficient number of cases, regarding the possible role of MVD as a prognostic indicator in canine appendicular OSA.

The only patient in the fourth group (Table 2) with distant recurrence presented a highly angiogenic primary tumour and several

studies have now shown that highly vascularised tumours are more likely to have significantly high chance to develop distant recurrences than those that are poorly vascularised [8,13]. Then it might follow that this group of patients may benefit most from anti-angiogenic adjuvant therapy [13].

Conclusion

From the results of our study, MVD did not correlate with clinicopathological features or disease outcome in canine appendicular OSA. However, although angiogenesis may not be of prognostic significance, it is likely that it has a key functional role in tumour progression and spread. Therefore, it may be a potential target for novel anti-angiogenic therapies to enhance the effects of chemotherapy and potentially reduce tumour malignancy. Further investigation is needed in canine OSA to better understand the pattern of microvessel progression and expression of factors regulating angiogenesis process in mesenchymal tumours and its role in tumourigenesis. In conclusion, the present study provided evidence for the significant association between VEGFA expression and MVD, and also that angiogenesis may not be as a prognostic indicator in canine appendicular OSA. Considering this as a pilot study with a small number of cases, our findings should be further verified in a larger number of cases.

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