

Relationship Between Plasma Cell-Free DNA Changes and Lysyl Oxidase During the Treatment and Prognosis of Canine Transmissible Venereal Tumor

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Abstract

Background

Transmissible Venereal Tumor (TVT) is a wide tumor of canine, there are no effective markers to monitor the therapeutic response in real-time. Circulating biomarkers may be valuable for the early diagnosis and prognosis of cancers, so in this study, we aimed to investigate the significance of the cell-free DNA (cfDNA) and cfDNA integrity index to monitor the response of TVT to vincristine and compare them with lysyl oxidase activity. Plasma and sera were collected before drug administration within four weeks from fifteen male dogs. The analytical method was mainly based on quantitative polymerase chain reaction for short and long cfDNA, and lysyl oxidase activity was measured in serum.

Results

The results of cfDNA integrity index showed significant ($p < 0.05$) difference in a baseline to 2nd and 3rd week (with cut-off value 1.118 and 93.33% specificity). We found that the cfDNA integrity index was increased during weeks due to the reduction of shorts cfDNA in the 1st week after treatment. Lysyl Oxidase activity was increased during the 4th week ($p < 0.001$) but there were no significant differences in the other weeks compared to baseline. ROC analysis of Lysyl Oxidase revealed high sensitivity (100%) and specificity (93%) 2nd, 3rd weeks on comparison between Baseline. Multivariate analysis between cfDNA integrity index and Lysyl Oxidase showed significant correlation ($p < 0.05$) only baseline results.

Conclusions

Taken together, we propose short cfDNA, cfDNA integrity index, and Lysyl Oxidase activity as a diagnostic biomarker and a putative prognostic candidate in TVT patients. These biomarkers could be used simultaneously for quickly diagnose TVT in combination with cytology.

Background

Transmissible venereal tumor (TVT), also known as infectious sarcoma, sticker tumor or transmissible lymphosarcoma and has been recorded in all continents during the 20th century (1, 2). They are naturally occurring tumors transmitted between animals during copulation through viable tumor cells. The tumor cells primarily affect external genitalia and occasionally the internal genitalia, although some exceptions of additional engaged sites have been observed. The masses tend to bleed easily due to extensive ulceration of the epithelial surface lining (3).

Dogs with TVT experience pain, hemorrhages and exhibit serosanguinous discharge in the external genitalia; Usually cauliflower-like and red-colored. Hemorrhagic discharge is strongly associated with mucosal membrane-based TVTs, which can engage genitalia, oral and nasal cavities (3, 4). The host's immune system plays a vital role in inhibiting tumor growth and metastasis in TVTs; Younger dogs or with a compromised immune system have shown higher tendencies for metastasis (5). The tumor

analysis is primarily based totally on a physical exam and a cytological analysis. Several treatments, which include surgery, radiotherapy, immunotherapy, and chemotherapy, had been implemented for TVT (6). Anyway, chemotherapy is taken into consideration because the best and practical approach for TVT treatment, and vincristine sulfate is mostly selected for many patients (7, 8).

Liquid biopsy checking out can assist a couple of programs throughout the continuum of canine most cancers care, which includes screening (for early detection) in excessive-threat populations, resources withinside the diagnosis, selection of targeted therapies, and tracking for most cancers recurrence or for remedy reaction through serial checking out; and guarantees to bring the power of precision oncology to veterinary exercise thru an easy blood draw that doesn't require adjustments to the clinical routine (9). Finally, a liquid biopsy checks overlaying cancer-related regions of the genome which have excessive homology among puppies and human beings can allow the identity of somatic changes in puppies which have clinically actionable human homologs. Such insights received from liquid biopsy-primarily based totally genomic profiling might be used to hurry the adoption of targeted human most cancers therapeutics for the remedy of dog most cancers (10).

Cell-free DNA (cfDNA) has been suggested to be a promising tumor marker. However, its level is also elevated in various non-malignant disorders. Therefore, more specific approaches such as measuring the integrity of DNA have been proposed (11). Pervious study showed that circulating biomarkers are advantageous over tissue biopsies due to the higher concentration availability in invasive procedures and the accessibility of withdrawing frequent samples in a period (9, 12). Furthermore, in cancer cfDNA does not originate only from tumor cells (13). It also originates from cells of the tumor microenvironment, as well as other non-cancer cells (e.g., endothelial or immune cells) from various parts of the body (14). It seems to be the case that all cells are capable of, and are likely, continuously releasing cell-specific DNA into the extracellular environment. For diagnosis it may, therefore, be sufficient to look only at apoptosis-derived cfDNA originating from cancer cells. However, to better estimate tumor dynamics, mutation load, progression or assess the efficacy of treatment, the best approach may be to determine the proportion of aberrant vs wild-type DNA, including all forms of cfDNA (10).

The extracellular matrix provides essential structural and biochemical support to cancer cells. LOX is a secreted copper-dependent amine oxidase family member that plays a fundamental role in extracellular matrix remodeling and maturation (15, 16). LOX is best known for initiating the crosslinking of collagen and elastin, which stabilize fibrous deposits. LOX has also been known to enhance cancer cell proliferation, metastasis, and angiogenesis. However, mature active LOX and LOX-PP play opposing roles in cancer progression. LOX-PP has been found to have tumor suppressor functions. The actions of LOX-PP have been proved to inhibit cell transformation, proliferation, growth and induce apoptosis in various tumor cells (16, 17).

This study aimed to conduct a qualitative and quantitative assessment of cfDNA fragments and the cfDNA integrity index in canine transmissible venereal tumors (CTVTs) to assess its potential as a diagnostic/prognostic marker and monitoring therapeutic response in real time. Lastly, this study uses a

comprehensive analysis of serum LOX and investigate its relationship with a CTVT's prognosis to assess its eligibility as a liquid prognostic biomarker.

Results

Circulating cfDNA in plasma and c fDNA Integrity Index

Circulating cfDNA in plasma of dogs with TVT was assessed for short and long cfDNA fragments concentration by real-time PCR and then the integrity index was calculated (long/short) for each week of treatment. When analyzing short cfDNA values from neoplastic dogs between weeks of treatment, a significant outlier was found (Fig. 1-A) which presenting short fragments = 720.02 ± 167.49 ng/ml plasma (Mean \pm SD) before prescribe of vincristine and 487.38 ± 139.80 , 465.11 ± 97.50 , 227.17 ± 37.28 ng/ml for 1st to 3rd week, respectively. Long cfDNA amount (n = 15) after treatment in 1st week showed significantly lower than neoplastic dogs before treatment, but comparing results of long cfDNA in other weeks with baseline didn't show significance (Fig. 1-B). The long fragments result of the baseline to the last weeks of treatment were as follows, 380.89 ± 102.34 , 229.02 ± 69.74 , 282.23 ± 116.55 and 388.06 ± 80.35 , respectively.

Differentiation between short and long cfDNA in each week was shown in figure 2-A Comparison of short and long results in subjects all weeks of treatment reveal significant changes in the concentration of short and long fragments. Also, the comparison of the last weeks of treatment shows that the amount of long cfDNAs has increased compared to the previous weeks.

Pre- and post-Integrity of circulating cfDNA Integrity Index results were shown in figure 2-B. A higher DNA Integrity Index is seen in 3rd week and in compare with Other weeks, $p < 0.0001$. Comparison of the DNA Integrity Index in other groups weren't shown to be significantly change ($p > 0.05$).

Lysyl oxidase activity

Results in figure 3 show the LOX activity of dogs with TVT in 4 weeks which revealed that 2nd and 3rd week had higher LOX activity in compare with other weeks. The highest amount (1.431 nmol/ml) of activity is related to the 3rd week after treatment, while in the first week there is no difference ($p > 0.05$).

ROC curves analysis for cfDNA Integrity Index

ROC curve was plotted to determine the cfDNA Integrity Index in discriminating effect of treatment between each week. The best cut-off for results of ROC curves which is related to the comparison of the Baseline with 3rd week was 1.118 with a diagnostic sensitivity of 100%, specificity of 93.33%, positive predictive value of 99.66% and negative predictive value of 70.18%. In addition, the results of the ROC curve of cfDNA integrity evaluations of the other weeks together are shown in figure 4. The area under curve was respectively 0.5956 for Baseline-1st week in canine with TVT, and 0.5556 and 0.9867 for Baseline-2nd and 3rd weeks respectively. For 1st week between 2nd week-3rd weeks, the ROC was respectively 0.6333 and 0.9867 and 0.9733 for 2nd week-with 3rd week.

Cut off point, sensitivity and specificity of cfDNA integrity and LOX activity

We expanded on these observations by calculating sensitivity and specificity data (Table 1); obtained by receiver operative characteristics (ROC) curves using 95% confidence intervals (Fig. 4). The cut-off point higher than 0.5985 for Baseline-3rd week with an AUC equal to 55.56%. Otherwise, cut-off point of 1.118 (AUC: 0.97) was obtained for Baseline-3rd week providing high levels of sensitivity with an AUC equal to 100%. There was strong sensitivity and specificity in cases to before treatment, and 3rd week (Sensitivity 100%, Specificity 93.33%) which is consistent with the cfDNA integrity Index. Poorer sensitivity results were seen in 1st -2nd and Baseline-1st weeks (Sensitivity 26,67%) (Table 1).

Table 1

Cut-off points, sensitivity, specificity and area under the curve (AUC) of ROC for cfDNA Integrity Index and LOX activity during treatment of canine Transmissible venereal tumor.

	Baseline-1st week	Baseline-2nd week	Baseline-3rd week	1st week-2nd week	1st week-3rd week	2nd week-3rd week
cfDNA integrity index						
Sensitivity %	26.67	46.67	100.00	26.67	100.00	80.00
Specificity %	93.33	80.00	93.33	93.33	93.33	86.67
Cut off point	< 0.3485	> 0.5985	> 1.118	> 0.9070	> 1.022	> 1.329
AUC of ROC %	59.56	55.56	93.33	63.33	98.67	97.33
LOX activity						
Sensitivity %	60.00	100.00	100.00	100.00	100.00	62.50
Specificity %	70.00	90.00	90.00	90.00	90.00	87.50
Cut off point	< 0.6960	> 0.8648	> 0.8648	> 0.7396	> 0.7396	> 1.332
AUC of ROC %	58	100	100	100	100	81

In addition to the results of cfDNA, also ROC analysis for LOX enzymes activity was showed in Table 1. The cut-off point lower than 0.6960 for Baseline-1st week with an AUC equal to 56% revealed that case has disease or inflammation and in cases with treatment could be use as marker for choose best way of treatment. A cut-off point of 0.8645 was obtained for comparing Baseline with two other weeks. There

was strong sensitivity and specificity in cases baseline-3rd week, 1st -2nd and 1st -3rd weeks (Sensitivity 100%, Specificity 93.33%) which is consistent with the LOX activity in serum of animals with TVT.

ROC curves analysis for LOX activity

ROC curve analysis was also used to investigate the diagnostic performance of the LOX marker under study by comparing during treatment with Vincristine. Among the weeks of treatment, except for the Baseline-1st week (the ROC was 0.5644), the rest of the weeks have acceptable sensitivity and specificity for using this marker in deciding for improvement or treatment process (Fig. 4).

Correlation and Multivariate analysis for the determinants of cfDNA integrity index and LOX activity

We evaluated the cfDNA integrity and LOX activity every week of treatment in the TVT dogs through correlation analysis (Table 2). CfDNA Data shown that 1st week ($r = 0.3541, p = 0.1954$), 2nd week ($r = -0.1051, p = 0.7094$) and 3rd week ($r = -0.0023, p = 0.9933$) were not significantly correlated with LOX activity. But the remarkable thing about these results is that if the alpha is considered less than 0.05, the results show that the comparison between baseline will be significant ($p = 0.0329$), which may be applied in the clinic or decided to continue treatment. Also, Table 2 presents multivariable regression analysis in TVT patients, for the associations between cfDNA integrity levels and the LOX activity in univariable analysis which there was only independent determinant for the plasma level of cfDNA integrity index with serum LOX activity before treatment.

Table 2
Relationship between the cfDNA integrity and LOX activity each week of treatment.

	<i>Pearson's analysis</i>		<i>Multivariate regression analysis</i>			<i>P-value</i>
	<i>r</i>	<i>95% confidence interval</i>	<i>R squared</i>	<i>β</i>	<i>t-value</i>	
Baseline	-0.5521	-0.07991 to -0.1455	0.3852	-1.684	2.387	0.0329
1st week	0.3541	-0.1043 to 0.6884	0.1254	1.959	1.365	0.1954
2nd week	-0.1051	-0.5229 to 0.3535	0.0110	-0.2576	0.3809	0.7094
3rd week	-0.0023	-0.4440 to 0.4402	0.0001	-0.0052	0.0085	0.9933

Discussion

Assays performed on neoplasm surrogate samples are engaging in the main because of the restricted invasiveness of sample collection. CfDNA determination might represent an inexpensive thanks to impacting on an attainable diagnostic, prognostic and observance tool in the medical specialty (14). The rise of cfDNA has been reported in various diseases particularly inflammation situations, and it's been found also in human patients with various forms of cancer (18). As yet, current cfDNA in blood has been thought about as a promising biomarker in various human tumors and, today, cfDNA fragmentation

diagrammatic by the integrity index is studied for its ability to discriminate cancer patients from healthy (19). Few knowledge are accessible for the canine neoplasms (20, 21). During this study, we have a tendency to evaluate the extent of Line-99 and 218 cfDNA fragments by qPCR of the two amplicons with completely different lengths (short and long), within the plasma of Transmissible venereal tumor dogs.

By tracking the cfDNA concentration and cfDNA integrity index at several weeks in 15 Dogs with TVT, we found that cfDNA parameters correlated well with the clinical stage and tended to increase during or before periods of disease progression, suggesting comparability in monitoring the clinical stage, such that our statistics indicated that short cfDNA lower in a time-dependent manner, which reaches minimal concentration in the third week of remedy. Dogs with a cfDNA of higher than 1.118 value had a 98% probability of being dealt with due to the cfDNA integrity index. Circulating cfDNA are short fragmented DNA (22) that can be detectable in serum or plasma in order to be purified, quantified, and ultimately specially amplified via way of means of polymerase chain reaction (PCR) (23). On the other hand, way to an upward push in cell death leading to an extended quantity of DNA fragments diffusing into a move in which acute ailment tactics had notably better elevations in brief cfDNA concentrations than continual ones (24). Several studies decided on the patient-particular mutations diagnosed within the tumor tissues for cfDNA evaluation of plasma and proved the employment for postoperative surveillance with unique sequencing technology in animals (25–27) which the consequences from those research evaluated that analyses of mutations in cfDNA changed into strongly associated with ailment severity in a clinical setting. In agreement with formerly published data specifically in humans, in our study, the neoplastic puppies earlier than treatment confirmed a better quantity of each short and long cfDNA fragment than treatment weeks. Only a few studies (12, 28, 29), compared circulating DNA in non-neoplastic with neoplastic dogs (lymphoma, mammary tumors, and different cancers) and discovered comparable results.

Serum cfDNA integrity index (Long/Short cfDNA) is a potential molecular biomarker in diagnosis and prognosis of cancers which may be a beneficial indicator of disease status or discriminate most cancers from healthy Patients for physicians and veterinarians (12, 30, 31). Increased necrosis inside the tumor led to noticeably fragmented DNA copies released into circulation, so Index ought to be lower in cancers (32). Our Data indicated that this index sharply increased after three weeks of treatment in assessment with other weeks ($p < 0.0001$), consequently this increase (decrease DNA fragmentation) turned into determined to be good after an anticancer treatment and the endurance of such modifications have been related to accurate prognosis (33). Similar outcomes have been acquired in Dogs with mammary tumors in evaluation with healthful puppies (12). That look affords evidence that cfDNA integrity index will be a diagnostic marker in puppies carrying mammary nodules suggesting that its ability application in early diagnostic procedures ought to be similarly investigated. Contrary to our results and the study noted above, a look at turned into performed on Feline diffuse iris melanoma especially which indicates cfDNA concentration and integrity evaluation found out no significant variations among the cats with iris melanoma and Healthy group (27). The comparable cfDNA integrity outcomes have been Located in dogs with lymphoma or leukemia, hemangiosarcoma, and remote metastasis; cfDNA stages correlated properly with a medical stage and tended to increase in the course of or earlier than intervals of disease

progression, suggesting ability efficacy of cfDNA for the detection of the remote. However, it additionally increases some other problems that cfDNA integrity won't be taken as a biomarker especially for some tumors however be more appropriate for treatment surveillance or prognosis in dog tumors (21, 26).

Moreover, we discovered a higher AUC value of cfDNA integrity in Baseline-third week results, it'd be rash to conclude cfDNA integrity is now no longer the suitable biomarker for the first weeks of treatment until it ought to be evaluated with a short cfDNA level. Nevertheless, we additionally executed ROC curve evaluation for every week and it'd be impractical to apply cfDNA integrity alone to the assessment of response to treatment in the first week of TVT. A value lower than the cutoff value (1.118) turned into taken into consideration awful prognosis, and smaller values have seemed like successful treatment. Interestingly, Akter et al. (23) has already reported that cfDNA in blood will be an ability screening marker for figuring out parasite variety in dogs.

In agreement with previously published data in particular in humans (34, 35), identity and acquiring sensitivity/ specificity of biomarkers is critical to be covered with inside the listing of exams which required to adjust to the treatment of patients, and therefore the initial diagnosis and selection to start remedy is also based totally at the results of the cfDNA index, so ROC curve evaluation became finished and located out maximum accuracy AUC of ROC for Baseline-third week 98.67% which gave a corresponding 100% sensitivity and 93.33% specificity. On the opposite hand, in line with the outcomes of the present study and based totally on the cut-off value, we recommend that dogs with distant TVT are probably discriminated from other puppies with an excessive percent of sensitivity while the usage of LINE-99 primers for qPCR evaluation. Of course, similar research is desired to check this phenomenon. Additionally, evaluation of cfDNA integrity results confirmed that the amount of this index decrease than 0.3485 suggests the case has an ailment or confirmed a loss of a successful treatment for the duration of the weeks and it is able to be vital in clinician decisions. A preceding study mentioned that the cutoff point for cfDNA integrity in canine mammary tumors becomes 0.62 (21).

Lysyl oxidase (LOX) may be a cell-secreted amine oxidase which differentially regulated by status of disease or cancers (36) and plays a pivotal role in cancer progression, including metastasis, and is therefore is an attractive therapeutic target (15). It's been used differently in animals and has also been reported in cardiovascular disease of dogs (37) or copper deficiency in cattle (38). Results of this study, investigated effects of treatment with Vincristine on LOX secretion by tumor cells which could promote treatment resistance. a major increase in lysyl oxidase activity was observed within the second and third weeks after treatment, which investigated LOX secretion by tumor cells which could promote treatment resistance (39). Studies have shown that LOX leading to cancer niches where tumors can develop and metastasize. In other hand it has been shown to have an inhibitory effect in the development of cancer tumors (40, 41). Pervious study for characterizing time0dependently of LOX activity showed that Type I Collagen and lysyl oxidase mRNA expression peaked in samples collected after 14 days of study (42). Due to our results, LOX activity start increase after 7 days of first dose of vincristine, which because of histological changes to mediate crosslinking of collagen and elastin (43).

Unlike cfDNA, the quantity of enzyme activity within the first weeks is helpful in helping to diagnose or evaluate the response to treatment, in order that within the first week, with 66.67% sensitivity and 66.67% specificity, it can substrate animals that haven't received treatment with treated animals. within the comparison second week with baseline (100% sensitivity, 93.33% specificity and AUC 100%), we found that if the LOX activity is a smaller amount than 0.8645, it are often concluded that the treated animals don't respond well to vincristine and may be dosed or variety of treatment should be reviewed by a veterinarian. within the study of Saleam et al., the results of LOX evaluation with a high percentage for the choice of this enzyme in breast cancer were. Their report about LOX expression in canine mammary tumors (CMT), revealed that lysyl oxidase expressed as His-tagged fusion protein in prokaryotic expression vector was used for detection of circulating protein LOX in serum of CMT subjects. Their ROC results showed high sensitivity (90%) and specificity (85%) with histopathology because the reference standard and that they proposed LOX as a diagnostic biomarker and a putative prognostic candidate in CMT cases (28). Examination of the relationship between cfDNA index value and enzyme activity showed that only baseline results had a major difference between the 2 markers if the 95% significance level is taken into account.

Conclusion

The results of this study demonstrate which case we discover that these two markers had significant difference (Inverse relationship) which could help in quickly diagnose of TVT cases together with cytology. These markers within the diagnosis of TVT with using as liquid biopsy which might change in diagnosis of suspected patients in early-stages. Further studies should be completed in tumor subtype or other treatments to raised test a prognostic role and to verify applicability of thresholds of cfDNA integrity or LOX activities values.

Materials And Methods

Samples

The 60 samples were specifically obtained and followed from 15 male dogs (range 5-12 years) in Iran between Jun 2020 and January 2021 which intravenously administered vincristine sulfate at a dose of 0.7 mg/m² of body area (44). Samples were obtained on the day of admission immediately before chemotherapy. Blood was drawn into EDTA-containing tubes and centrifuged at 1000g for 10 min at 4°C. Plasma was carefully removed, leaving 3-5 millimeter above the buffy coat, added to a tube and re-centrifuged at 1000g for 10 min at 4°C. Plasma was carefully removed and stored at -80°C in 1 ml aliquots for cfDNA assay.

Exclusion criteria were a history of current disease, abnormalities detected on routine clinical examination, or significant abnormalities on a complete blood count.

Extraction of circulating free nucleic acids and QC checks

For cfDNA analysis, DNA isolated from 0.5ml citrated plasma using silica membrane-based DNA purification spin columns according to the manufacturer's protocol (DNP™ Kit High yield DNA Purification Kit, CinnaGen, Iran). Concentrations of cfDNA on purified samples were measured in duplicate with UV absorbance at 260 nm employing a UV-spectrophotometer (JENWAY, UK). Real-time PCR was performed using YTA SYBR Green qPCR MasterMix2x. Briefly, the reaction was performed in 20 µL reaction volumes containing Master Mix, 0.5 µl of every primer, and 1 µL of the sample. The real-time PCR conditions consisted of an initial denaturation step for five minutes at 95°C followed by 40 cycles for 15 seconds at 95°C and annealing/extension for 1 minute at 60°C. A negative control (without the template) was performed on each plate. The sequences for RT-PCR primers were as follows: Line-99 forward, 5'-AAATGCAATGAAACGCCGGG-3'; Line-218 forward, 5'-TGGGAATGTGAACTGGTGCA-3' and reverse 5'-TCTTTCGTTGGACACCGAGG-3' (21). Serial dilutions of (from 1–10,000 ng/mL) of genomic DNA obtained from the peripheral blood leukocytes of a healthy dog were analyzed, and also the resulting standard curves were used to calculate the DNA concentrations of cfDNA in each sample. All samples were evaluated in triplicate, and a negative control (without template) was included in each plate(21).

Lysyl Oxidase Activity

We comprehensively analyzed lysyl oxidase activity to measure LOX related to the disease. Blood samples were obtained on the day of admission immediately before chemotherapy in TVTs patients in sterile tubes, centrifuged at 2000 × RPM for 10 min, aliquoted into 1.5-mL tubes, and sera were preserved at – 80 °C until analysis (15). Serum samples were diluted with an equal volume of phosphate-buffered saline and duplicate diluted samples were assayed for serum LOX concentration using a commercial kit (Kiazist Co, Iran). Briefly, 20 µL of the undiluted sample, 250 µL of the LOX Substrate Buffer, 5 µL of the horseradish peroxidase, and 1 µL of the LOX Probe was added to each well for every 5 samples and incubated for 1 h at room temperature. Then after adding LOX Lysis, the absorbance's of samples and standards at 570 nm were measured immediately using Plate Readers (Biotech Instruments, INC, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 9.0. Comparisons between every week for cfDNA and LOX Data were analyzed by the T-test. For comparisons among over two continuous variables, an ANOVA test was performed. Pearson's correlation coefficient analysis was accustomed assess bivariate correlations between all the variables. The receiver operator characteristics (ROC) plot was obtained by calculating the sensitivity and specificity for each distinct observed data value and plotting sensitivity against 100%-specificity. The ROC curve was used to evaluate the optimal cut-off values for mortality prediction. A cutoff point on the curves was chosen to achieve the best compromise between sensitivity and specificity for fatal outcome.

Abbreviations

TVT: Transmissible venereal tumors; CTVT: Canine Transmissible venereal tumors; cfDNA: cell-free DNA; LOX: Lysyl Oxidase; ROC: Receiver operator characteristics; ANOVA: Analysis of variance.

Declarations

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None. No funding to declare.

Authors' contributions

Conceptualization and Data curation: HC, DSh. Funding acquisition: MM, HC. Investigation: MM, HC, AH. Methodology: HC, DSh, AH. Performed the statistical analysis: HC, Supervision: HC, DSh. Writing: HC, MM.

all authors have read and approved the manuscript

Ethics statement

The study protocol was approved by the Research Ethics Committees of Razi University, Kermanshah, Iran (REC reference number: IR.RAZI.REC.1400.016). Client-owned animals gave written informed consent before participation.

All methods were carried out in accordance with relevant guidelines and regulations.

No humans were involved in this study.

No agents were administered to dogs in any way in this study

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interests regarding the publication of this article, financial and/or otherwise.

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Availability of data and Materials

All data generated or analysed during this study are included in this published article and its supplementary information file (with RAW DATA name file).

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Figures

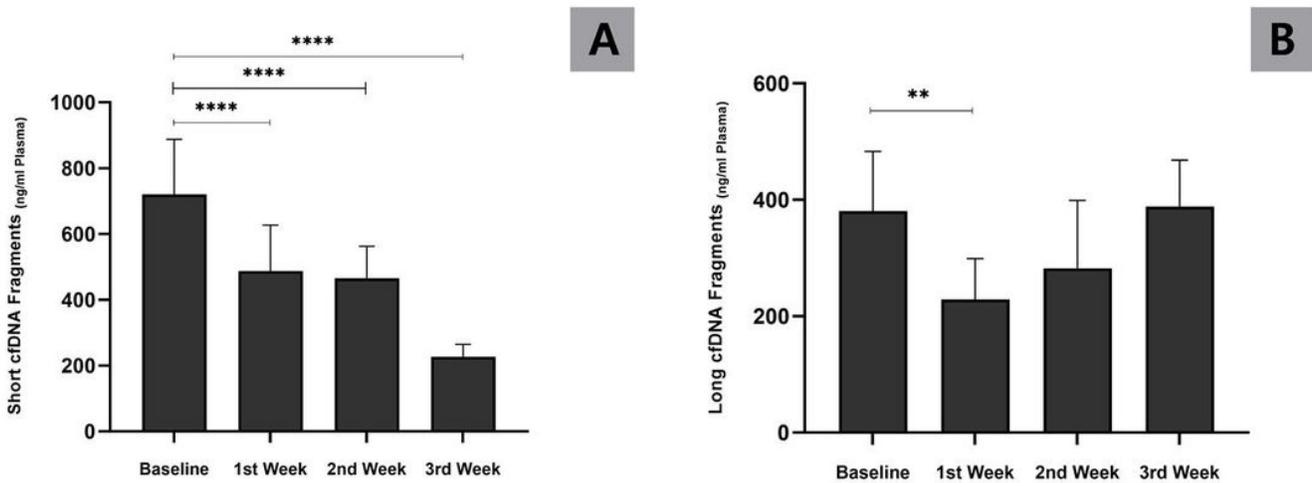


Figure 1

A. Results for Short (Line-99) and B. Long (Line-218) of plasma circulating cfDNA concentrations in dogs with four weeks of treatment. **P < 0.01 and ****P < 0.0001 vs. Baseline.

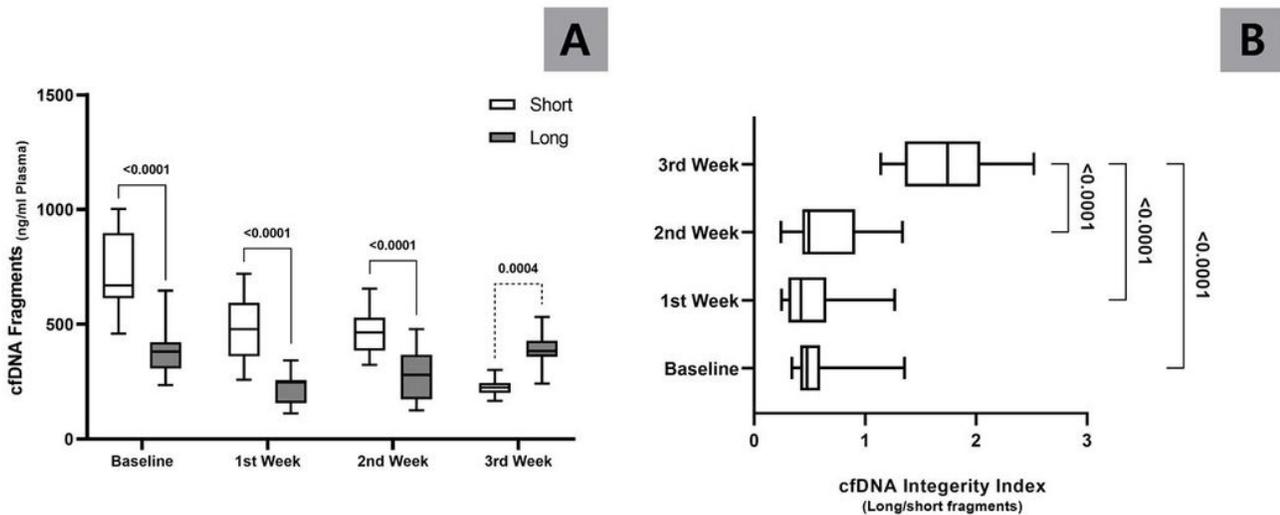


Figure 2

A. Box-plot of short and long cfDNA for each week was shown. B. Box plots with whiskers of comparing cfDNA integrity Index (Long/Short) of the effect of Vincristine treatment of the Dogs in the fourth week. Each box indicates the 25th and 75th percentiles. The horizontal line inside the box indicates the median, and the whiskers indicate the extreme measured values.

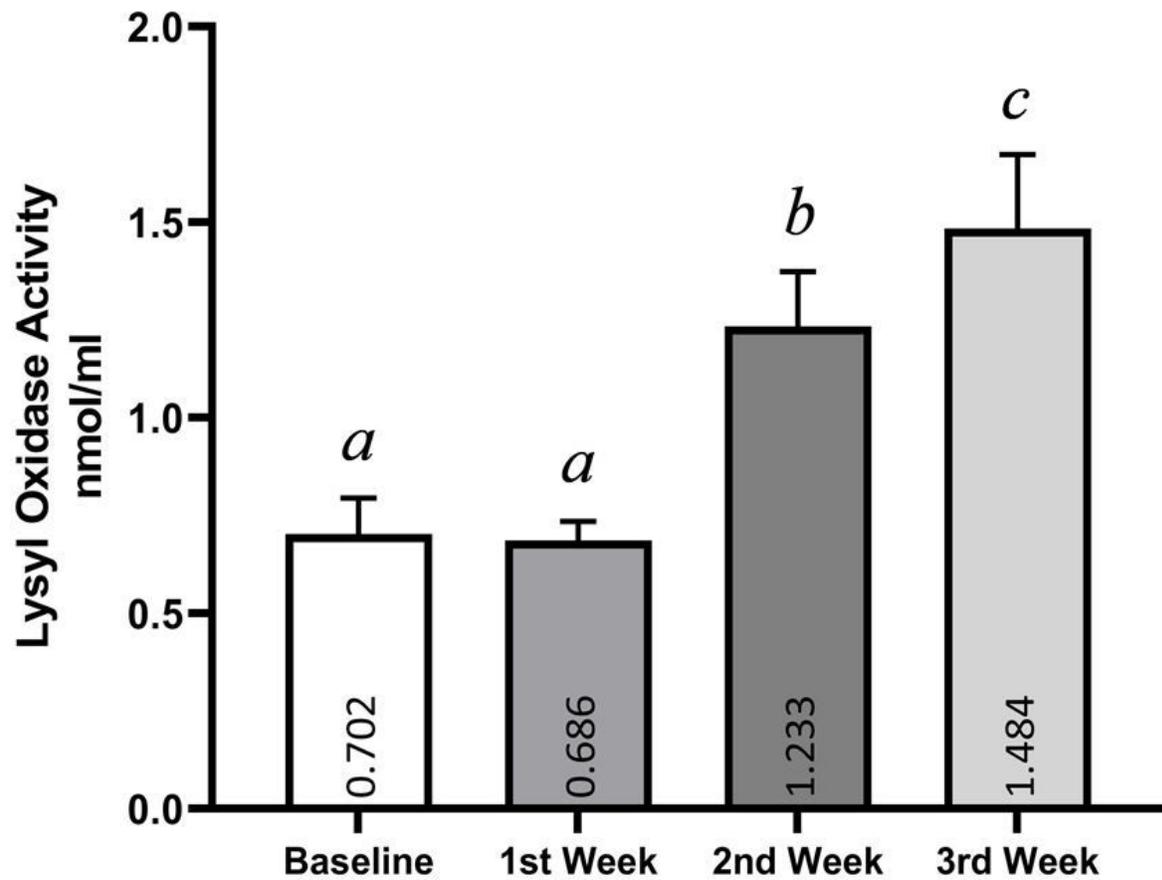


Figure 3

Lysyl oxidase activity for each week of Vincristine treatment. Data are expressed as Mean \pm SD (Lysyl oxidase activity is expressed as nmol/ml). Values with non-identical letters (a, b and c) are significantly different (ANOVA, $p < 0.05$).

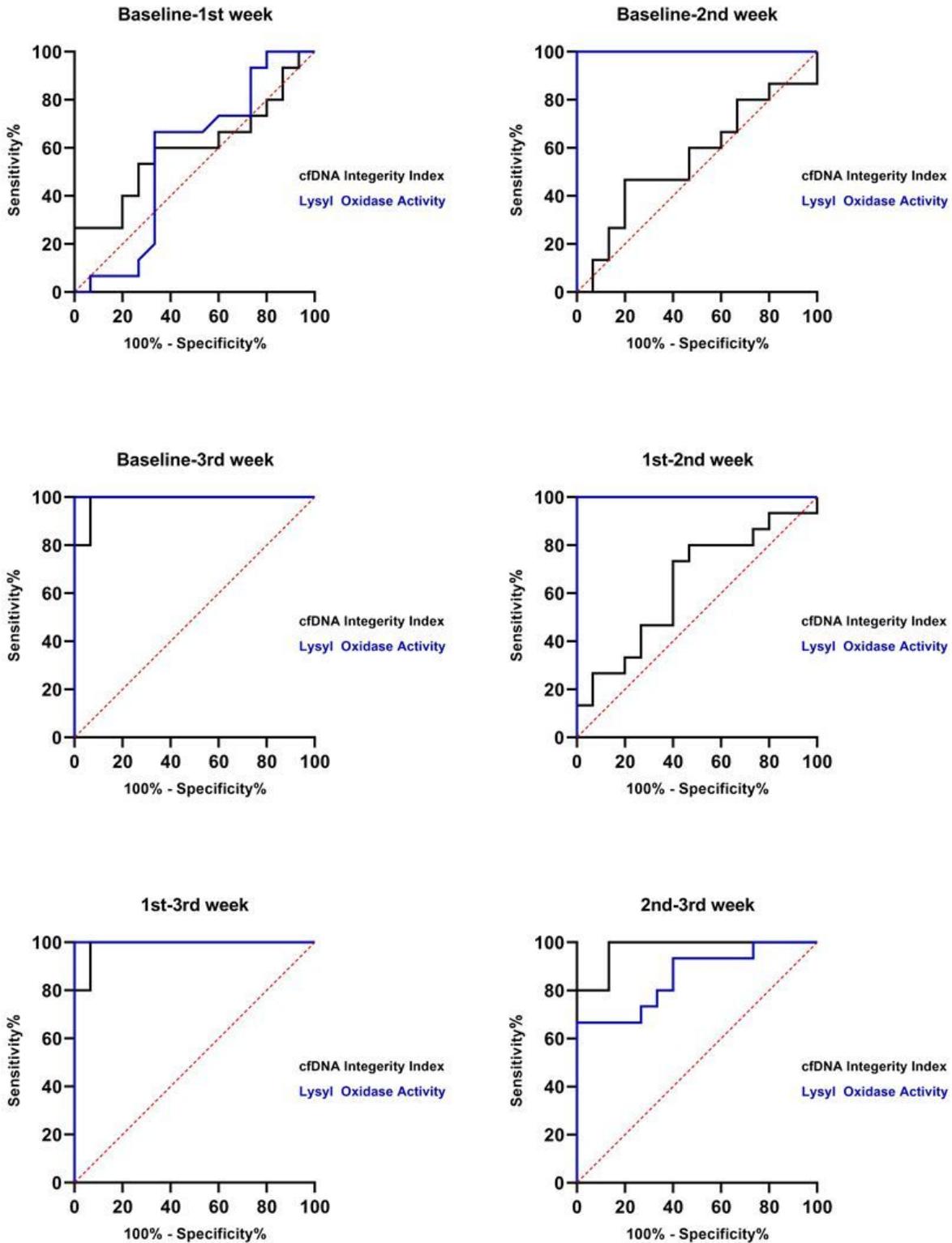


Figure 4

ROC curve of cfDNA integrity index LINE218/99 (Black lines) and Lysyl oxidase activity (Blue lines) in dogs with cytological diagnosis of TVT within the treatment.

Supplementary Files

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- [RawData.xlsx](#)