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# Exome sequencing and SDH (A, B) immunohistochemistry of canine Pheochromocytomas

**F.M. Abed[1] and M.J. Dark[2]**

[1]Department of Pathology and poultry disease, College of Veterinary Medicine, University of Mosul, Mosul, Iraq, [2]Department of Comparative, Diagnostic and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA

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***Correspondence:***
F.M. Abed
firasabed@uomosul.edu.iq

**Abstract**

Pheochromocytomas (PCs) are tumors originating from the chromaffin cells of the adrenal medulla. In people, there are highly correlated to inherited gene mutations in the succinate dehydrogenase (SDH) pathway; however, to date, little work has been done on the genetic basis of these tumors in animals. Out of the total of 2.203 Gb of canine DNA sequenced, 88.35% of bases mapped to exons and 11.65% mapped to introns. Out of 26 genes of interest containing 404 exons, 278 exons were sequenced 68.81%. Sequencing was considered successful when the average read depth was 3x and the entire exon was covered. Coverage ranged from 30% to 100%. Both SDHA and SDHB had exon mapped 46.6% and 62.5% respectively. Additionally, out of 45 known canine variants, exome technique able to detect 36 variants (80%). We performed SDHA and SDHB immunohistochemistry on 35 canine formalin-fixed, paraffin embedded. Interestingly, we had loss of immunoreactivity for both SDHA and SDHB in four samples, suggesting a mutation in SDHx including SDHA. Out of 35 samples, 6 had immunoreactivity for SDHA and 25 lacked immunoreactivities for SDHB. 29 out of the 35 (82%) may have an SDH family mutation other than SDHA. Exome sequencing and immunohistochemistry are able to predict malignant behaviour and likelihood of reduction of PCC/PGLs in humans. This can be used to determine whether there are similar mutations in the pseudo-hypoxic, kinase signalling, and other genes of interest exist in dogs, as well as finding novel genes involved in canine Pheochromocytomas oncogenesis.

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## Introduction

Pheochromocytomas (PCs) are catecholamine-secreting neuroendocrine tumors arising from the chromaffin cells of the neural crest (1,2). PCs are more often seen in dogs and cattle (3). And can be unilateral or bilateral and functional or nonfunctional. While canine pheochromocytomas are usually benign, they can invade adjacent tissues and may be malignant, with metastasis to distant tissues. The behavior of Pheochromocytomas is difficult to predict based on histologic findings (4,5). Immunohistochemistry (IHC) data has found that canine and human PCs are highly similar, as neoplastic cells in both share the expression of numerous antigens, including S100, synaptophysin (SYN), chromogranin A (CGA), and substance P (SP) (6). Recent technological advancements have made possible through genetic analysis have considerably improved understanding of human pathogenesis cancers. There are several molecular targets known. via genetic research and the development of drugs that target mutated molecules and pathways have

transformed cancer treatment (7). Use of the human exon-capture methods provides an attractive, cost-effective approach for the comparative analysis of non-human primate genomes, including gene-based DNA variant discovery (8). It is Cost-effective whole exome sequencing approach frequently employed in research on illness associations (9). The use of Next Generation Sequencing (NGS) technology in basic and translational. However, the limited supply of fresh frozen (FF) tumor tissues and the subpar quality of Formalin-fixed, paraffin-embedded (FFPE) DNA extraction has greatly hampered this procedure in the solid tumor field (10). SDH is an enzyme that is a component of both the citric acid cycle (Krebs or tricarboxylic cycle) and the mitochondrial electron transport chain. It is a heterotetramer complex with four subunits (SDHA, SDHB, SDHC, and SDHD). It catalyzes the oxidation of succinate to fumarate and is highly conserved between species. The enzyme is located inside the mitochondrial inner membrane but is a nuclear protein (11,12). Subunits A and B form the SDH complex, while the C and D subunits anchor the complex to the membrane and bind the b-type cytochrome (12). Pheochromocytoma and paraganglioma familial syndromes result from the accumulation of succinate due to SDHB, C, and D mutations. SDHA does not appear to have tumor suppressor activity (13). In addition, succinate dehydrogenase complex assembly factor 2 (SDH5/SDHAF2) is essential for normal functioning of the SDH complex. Any defect will cause loss of function of the SDH complex by affecting the stability of the enzyme complex by decreasing subunit synthesis and assembly (14). Next Generation Sequencing (NGS) is the best of choice for large scale data production (15).

In particular, exome sequencing is a method for quickly examining protein-coding sequence to identify mutations. This can be used to determine whether there are similar mutations in the pseudo-hypoxic, kinase signaling, and other genes of interest exist in dogs, as well as finding novel genes involved in canine Pheochromocytoma oncogenesis.

## Materials and methods

### Case material

Paraffin embedded tissue blocks were obtained from dogs in the University of Florida, College of Veterinary Medicine, Anatomic Pathology Archive. These were selected from cases with a diagnosis of PCs (16-31). Representative sections of each tumor were evaluated by a board-certified veterinary pathologist to confirm the diagnosis. When available, samples from normal tissues were also included for genetic comparison with tumor samples. Genomic DNA from 66 humans (Human TAQ MAN Genomic DNA [Biosystem]) and dogs (Dog ZYAGEN Dog Kidney, Genomic DNA DG-901 [Fisher]) were used as controls.

### Immunohistochemistry

Sections from each block were cut at 4µm and stained using commercially available antibodies (SDHA Mouse Monoclonal Antibody [2E3GC12FB2AE2, ThermoFisher Scientific catalog #459200] at 1:100 dilution and SDHB Rabbit Polyclonal Antibody [ThermoFisher Scientific catalog #PA5-23079] at 1:75 dilution). Staining was performed on a Leica Bond immunostainer per the manufacturer's directions. Sections from 20 canine adrenal glands and 20 cardiac samples were used to verify immunoreactivity of the antibody with canine antigens. Pheochromocytoma samples from dogs were listed as positive when there was granular intracytoplasmic immunoreactivity with a similar intensity as the internal positive controls (endothelial cells, sustentacular cells, and/or lymphocytes). Negative samples lacked immunoreactivity in cells of the mass with immunoreactivity in internal positive controls (32,33).

### DNA extraction

The QIAampR DNA FFPE Tissue Kit (Qiagen Inc., Valencia, CA, USA) was used for DNA extraction per the manufacturer's protocol. All samples were eluted using 50 ml of buffer ATE with 5 min of incubation at room temperature**.**

### TruSeq exome library preparation

Library Construction: The Illumina TruSeq Exome Library Prep kit was used to process extracted DNA. This was performed as described in the manufacturer's directions with some modifications; briefly, 300ng of DNA in 50µL was used for shearing using an S220 Covaris to 150bp. Freshly isolated human and dog control DNA were at 100ng each in 50 µL solution. Ends were repaired followed by adenylation of the 3' ends for ligation with unique base molecular barcode adapters. PCR cycles for the enrichment steps were increased for all samples from 8 to 12 cycles to simplify downstream pooling. Library validation using the High Sensitivity D1000 Screen Tape® (Table 1) was performed after each enrichment to check the quality and quantity of the library.

### Processing and data analysis

Sequences analyzed in CLC Genomics Workbench on the departmental genomics workstation, normal and tumor samples will be compared to each other and the published canine genome. Variations, including single nucleotide polymorphisms (SNPs) and large-scale polymorphisms (LSPs), will be detected using the variant caller in CLC genomics.

Table 1: Summarize total input and library concentrations in each step before pooling

| Samples | | N7-S17-196 | N9-S17-196 | N10-S17-97 | N12-S17-197 | N13+Ctr | N14+Ctrl |
|---|---|---|---|---|---|---|---|
| Tape station 1 conc. genomic DNA ng/ul | | 7.98 | 15.40 | 7.02 | 10.9 | 10 | 10 |
| Tape station 1 genomic DIN | | 3.2 | 5.9 | 3.3 | 5.8 | ----- | ------ |
| Normalizing g DNA (FFPE) ng/ul | | 300 | 300 | 300 | 300 | 100 | 100 |
| Not exceed 50ul each in fragment DNA step | | 37.6 | 19.5 | 43 | 27.6 | 10 | 10 |
| DNA adapter index | | A002 | A005 | A006 | A007 | A0012 | A0019 |
| No. of PCR cycles | | 12 | | | | | |
| Qubit concentration | Quantify Libraries | 75.2 | 46.1 | 61.2 | 38 | 71.5 | 64.8 |
| | Recalibration | 74 | 26.58 | 60.41 | 27.47 | 69.62 | 44.66 |
| Input into Enrichment ng/ul not exceed 40ul | | 6.75 | 18.8 | 8.27 | 18.20 | 2.87 | 2.23 |

**Results**

Out of the total of 2.203 Gb of canine DNA sequenced, 88.35% of bases mapped to exons and 11.65% mapped to introns (Table 2). Out of 26 genes of interest containing 404 exons, 278 exons were sequenced 68.81%. Genes that have 50% mapped exons are SDHC, SDHD, EGLN2, TMEM127, P16, and GDNF (Figure 1). MAX and IDH1 had 71.1% and 72.2% of exons mapped, respectively (Figure 2). Both SDHAF2 and EPAS1 had 75% of exons mapped, while SDHA and SDHB had 46.6% and 62.5% respectively Figures 1 and 2. Other genes with a high percent of exons mapped include BAP1, KIF1B, KMT2D, FH, GNAS, and NF1 at 82.35, 85.41, 87.27, 90, 90.9, and 98.2%, respectively (Figure 3). Some genes like BRACA1 and BRACA2 had 52 and 34.48% mapped, while HRAS had 40% and RET had 45.45% of exons mapped. Additionally, out of 45 known canine mutations, Nextera human enrichment kit was able to sequence 36 mutations 80% of Cluster I genes (Figure 4). Only 9 variants missed to be sequenced, which represent 20%. Four genes (SDHC, SDHAF2, IDH1, and VHL) have 100% sequenced variants, variant detection in seven canine pheochromocytomas sequenced samples. Out of 35 sections examined, there was a lack of both SDHA and SDHB immunoreactivity in four samples 11.4% and 6; 25 samples 71.4% lacked SDHB immunoreactivity but had SDHA immunoreactivity. Out of the 12 samples with no reported invasion, 10 34.84% lacked immunoreactivities to SDHB (Figures 5 and 6). Two samples out of these 12 described adhesions to the omentum, splenic vessels, apex of the left pancreatic limb, and/or the left kidney. Out of the five samples that had both SDHA and SDHB immunoreactivity, 3 (60%) also had an invasion. Immunohistochemical summarized the findings and clinical information for all cases (Table 3).

Table 2: Showed the bases mapped

| Mapped | Total Mapped |
|---|---|
| Total exons | 88.35 |
| Total introns | 11.65 |

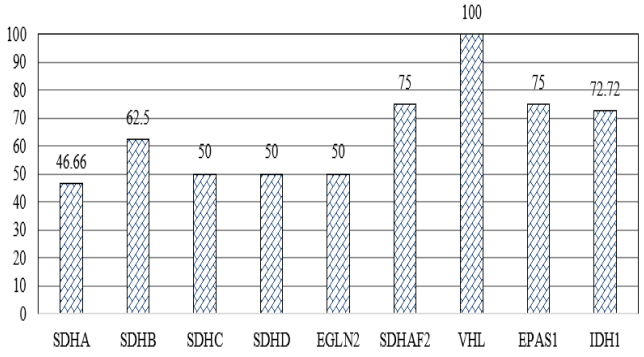


Figure 1: Exon capture percentages for canine genes of interest cluster I pathway

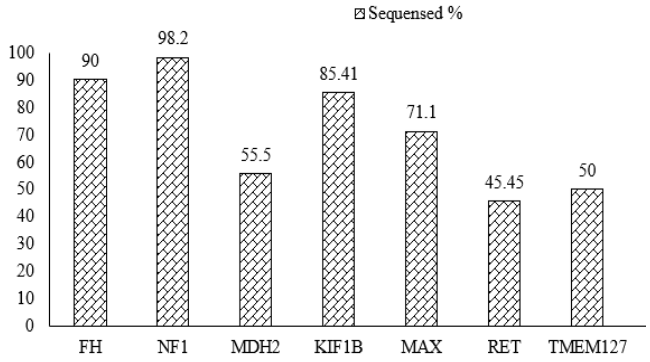


Figure 2: Exon capture percentages for canine genes of interest cluster II pathway.

Table 3: Clinical and immunohistochemical data

| n | M/F | Mean Age | IHC (SDHA/SDHB) | Invaded/non-Invaded |
|---|---|---|---|---|
| 4 | 4/0 | 10 | -/- | 3/1 |
| 5 | 1/4 | 11.5 | +/+ | 3/2 |
| 25 | 17/8 | 9.9 | +/- | 14/3 |
| 1 | 0/1 | 15 | -/+ | 1/0 |

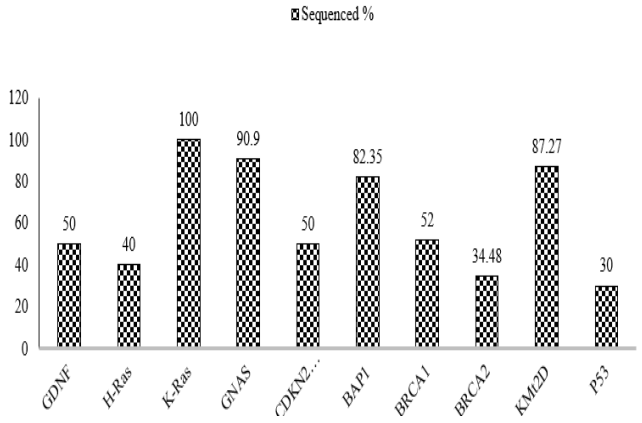


Figure 3: Exon capture percentages for canine genes of interest.

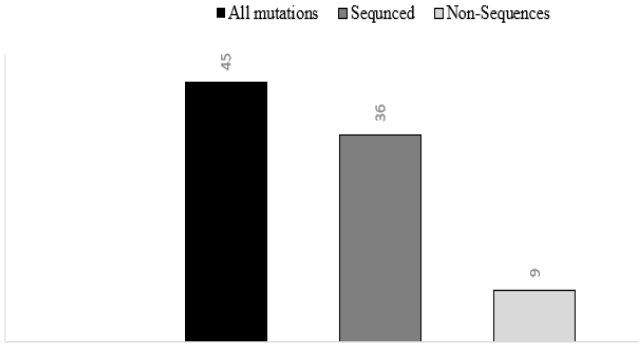


Figure 4: Known mutations in canine pheochromocytomas genes.

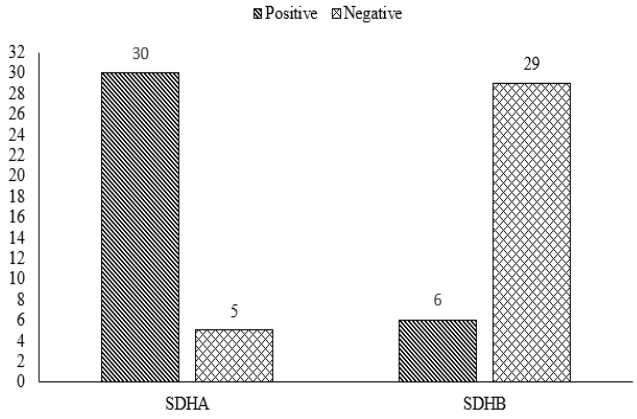


Figure 5: SDHA/SDHB IHC immunoreactivity summary.

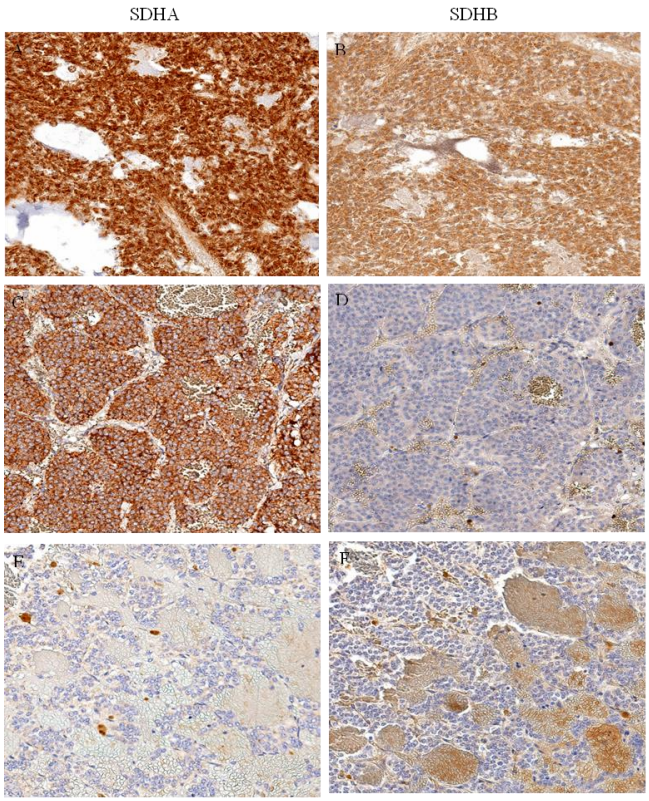


Figure 6: A, C, E - SDHA immunohistochemistry. B, D, F - SDHB immunoreactivity. A and B represent a case with both SDHA and SDHB immunoreactivity, C and D have SDHA but not SDHB immunoreactivity, and E and F represent a case lacking both SDHA and SDHB immunoreactivity. 40X objective

**Discussion**

The use of 250 ng of a fresh commercial ZYAGEN DNA Dog DNA and 50 ng human DNA in hybridizing probes step (pool library) of these two samples for Illumina MiSeq were compared. The output sequenced on an Illumina MiSeq; sequences analyzed in CLC Genomics workbench on the departmental genomics workstation. Out of the total of 2.203 Gb of canine DNA sequenced, 88.35% of bases mapped to exons and 11.65% mapped to introns, which agrees with Mannelli M (33,34) in the results he showed with Nextera enrichment kit techniques. Out of 26 genes of interest containing 404 exons, 278 (68.81%) exons were sequenced. Sequencing was considered successful when the average read depth was 3x and the entire exon was covered (35). Coverage ranged from Canine pheochromocytomas (PCs) were used as an example of a specific disease where veterinary samples could be useful as a model, these results proof the same data that revealed by Pillai (36) mentioning that it is possible to successfully discover coding-region variation in non-human primates using current whole exome

resequencing technology. Whole exome resequencing can help improve the annotation of non-human primate genomes in addition to revealing variance (37). In our study, canine PCs have similar immunohistochemical characteristics to those previously reported in human tumors, with approximately 82% having immunohistochemical evidence of a succinate dehydrogenase SDH family mutation. In addition, genetic screening of extracted dog DNA from FFPE samples from pheochromocytomas cases was performed using the Nextera human Exome Enrichment Kit to capture mutations in canine DNA. This found that the human exome enrichment kit can be applied to studies of tumors in dogs by determining which genes of interest can be enriched as mentioned by Salajegheh *et al.* (38). Genes of interest had between 46.6-100% of exomes sequenced after enrichment. Finally, evidence of mutations in the Cluster I gene family were found after examination of genetic sequencing data. SDH subunits are conserved through evolution in contrast to other respiratory chain complexes, making these attractive targets for immunohistochemistry. As expected, all controls had an intracytoplasmic immunoreactivity for SDHA and SDHB similar to that found in human tissues. In the 35 sections examined, there was a lack of both SDHA and SDHB immunoreactivity in four samples 11.4%; 25 (71.4%) samples lacked SDHB immunoreactivity but had SDHA immunoreactivity. The lack of SDHB immunoreactivity correlated with both age (with younger animals redisposed (10 years old: 62.07%,> 10 years old: 37.93%; $P < 0.0421$) and sex (male: 75.86%, female: 24.14%; $P < 0.05$). Out of the 35 sections examined, there was a lack of both SDHA and SDHB immunoreactivity in 4 (11.4%) samples. Based on human studies, this suggests a mutation in SDHA. We found that 25 (71.4%) samples lacked SDHB immunoreactivity but had SDHA immunoreactivity. Studies in humans suggest this pattern indicates a mutation in the SDHB, C, D, and/or SDHAF2 genes (39,40). Together, these data suggest that 29 out of the 35 (82.8%) samples have a mutation in at least one of the SDH family genes. Human studies found that 75% of samples with SDHA mutations lacked immunoreactivity for SHDA and SHDB and 90% of samples with SDHB, C, D, or SDHAF2 mutations had immunoreactivity for SDHA and lacked immunoreactivity for SDHB.

Out of 23 samples that had clinical evidence of invasion, 19 (82.6%) lacked SDHB immunoreactivity. This agrees with several human studies (41-43). Out of these 19.14 samples (73.6%) had vascular invasion (caudal vena cava, phrenicoabdominal vein, and/or renal vein) while two out of these 19 (10.53%) metastasized to the renal vein or regional lymph node, which agrees with findings in a human study (44). Only one sample lacked SDHA immunoreactivity but maintained SDHB immunoreactivity; this sample showed signs of invasion and metastasis to the renal vein. However, as inactivation of SDHA does not lead to tumorigenesis (45), this might represent a somatic hypermethylation of the promoter region (46), leading to accumulation of succinate and later inhibition of demethylase enzymes. This can lead to promoter hypermethylation and tumor suppressor gene inactivation (47-51); in humans, this syndrome is known as Leigh syndrome. Out of the five samples that had SDHA and SDHB immunoreactivity, 3 (60%) also had invasion. There was no significant association between immunohistochemistry findings and tissue invasion; however, this may be due to limited numbers of cases with both SDHA and SDHB immunoreactivity.

Finally, we have begun sequencing several canine pheochromocytomas to determine if the immunohistochemistry results found correlate with mutation status. Out of 6 PCs lacking both SDHA and SDHB immunoreactivity sequenced, four sample had a total of 36 non-synonymous mutations in SDH family genes. While this is at an early stage, it suggests that IHC status correlates with genomic changes. Further work is needed to determine the utility of immunohistochemistry as a prognostic factor for PCs in dogs.

## Conclusions

This study will increase the understanding of the molecular pathology of PCs in dogs. Ultimately, this may allow for the development of new successful, molecular-targeted therapies. Most of our recent knowledge on the molecular defects in PCs has developed via the progression of knowledge of an increasing number of germline mutations in many patients, even in those with apparently sporadic disease. Applying this information to dogs will aid in diagnosis, treatment, and prevention of disease. These studies were able to show the utility of dogs as a model for human pheochromocytoma. Because the rarity of this disease in people, utilizing the greater frequency of these masses in dogs would be helpful in examining the efficacy of future treatments. The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers): University of Florida Institutional Animal Care and Use Committee approved IACUC Protocol 201408639.

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## Conflicts of interest

The authors declare no conflict of interest.

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# التسلسل الاكسومي والكيمياء المناعية النسجية لإنزيم هيدروجيناز سكسينات للنوعين أ و ب في ورم القوائم في الكلاب

**فراس محمد عبد[١] و مايكل جيمس دارك[٢]**

[١]فرع الأمراض وأمراض الدواجن، كلية الطب البيطري، جامعة الموصل، الموصل، العراق، [٢]قسم الطب التشخيصي والمقارن وطب المجتمع، كلية الطب البيطري، جامعة فلوريدا، غينزفيل، الولايات المتحدة

**الخلاصة**

أورام القوائم هي أورام تنشأ من خلايا الكرومافين في اللب الكظري. رغم أن القليل من الدراسات الجينية قد أجريت في الحيوانات، ففي الإنسان هناك ارتباط كبير بالطفرات الجينية الموروثة في مسار أنزيم هيدروجيناز سكسينات. تم تشفير ٨٨,٣٥٪ من القواعد في الموروثة المشفرة و ١١,٦٥٪ تم تعيينها من الحمض النووي الرابط بين الموروثات المشفرة من إجمالي ٢,٢٠٣ غيغابايت من بيانات الحمض النووي. احتوى ٢٦ جينا على ٤٠٤ مورثة مشفرة، تم تحليل ٢٧٨ (٦٨,٨١٪)، وتم اعتبار قراءة التسلسل ناجحا بمتوسط عمق ٣× وتراوحت شمول المورثة المشفرة بين ٣٠ الى ١٠٠%، حيث أن شمول المورثة لأنزيم هيدروجيناز سكسينات للنوعين أ و ب كانت ٤٦,٦ و ٦٢,٥٪ على التوالي من بين ٤٥ طفرة. وجد أن ٣٦ (٨٠%) من عينات الدراسة الحالية تحتوي على طفرات، تسعة من هذه الطفرات فقط تعذر احتواءها والتي تمثل ٢٠%. أجريت تقنية كيمياء الأنسجة المناعية لكل من أنزيم هيدروجيناز سكسينات للنوعين أ و ب في ٣٥ عينة من ورم القوائم، وتعذر الحصول على تفاعل مناعي لإنزيم هيدروجيناز سكسينات للنوعين أ و ب في أربع عينات، لوجود طفرة في جين أنزيم هيدروجيناز سكسينات بما في ذلك أنزيم هيدروجيناز سكسينات للنوع أ. من بين ٣٥

عينة أظهرت ست عينات تفاعلات مناعية موجبة الى أنزيم هيدروجيناز سكسينات للنوع أ بينما لم يلاحظ أي تفاعل مناعي في خمسة وعشرون عينة لأنزيم هيدروجيناز سكسينات للنوع ب، كما لوحظ أن ٨٠% (٣٥/٢٩ عينة) أظهرت طفرات في عموم أنزيم هيدروجيناز سكسينات غير تلك الموجودة في النوع أ. إن تقنية التسلسل الاكسومي والكيمياء النسيجية المناعية قادرة على التنبؤ بالسلوك الخبيث لأورام القواتم في الإنسان. تستنتج الدراسة أن هذه التقانات تحدد ما إذا كانت هناك طفرات مماثلة في نقص الأكسجة الزائف، وإشارات الكيناز، والجينات الأخرى والتي تتواجد في الكلاب في العثور على جينات جديدة تشارك لتكوين هذه الأورام في الكلاب.