1   **Molecular Parallelisms and Divergences Between Human and Canine Cancers**

2   **Would a dog be an appropriate experimental model for human cancers?**

3   **Running title: Molecular Parallelisms and Divergences Between Human and Canine Mammary**

4   **Cancer**

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Abstract

Canine mammary cancer is poorly characterized at the genomic level. Dog really can be an appropriate experimental model for human cancers from the genomic and evolutionary perspective or not? Here, we perform a cross-species cancer genomics analysis, independent evolution of cancer from normal tissues, which provide us an excellent opportunity to address an evolutionary perspective of cancer. As, evolutionary theories are critical for understanding tumorigenesis at the level of species as well as at the level of cells and tissues, for the development of effective therapies. Analysis of canine mammary cancer reveals a diversity of histological types as compare to human breast cancer. Our systematic analysis of 24 canine mammary tumors with whole-genome sequences, reveals 185 protein-coding cancer genes carried exonic mutations. Cross-species comparative analysis of 1080 human breast cancers identifies higher median mutation frequency in human breast cancer and canine mammary cancer shows lower across exonic regions (2.67 and 0.187 average no. of mutations per tumor per megabase (Mb), respectively). A comparison of somatic mutations in the *PIK3CA* gene, reveals common recurrence of the conserved mutations, in both species. However, the Ka/Ks ratio in the human *PIK3CA* gene 2.37 is higher and 1.43 in dogs is lower. To address the mutation accumulation and antagonistic pleiotropy theory, we investigated Ka/Ks value 237 aging-related genes from human and canine, the aging-related genes do not show selection in canine mammary cancer. It demonstrates new aspects of cancer genes that are evolving in different species instantaneously. These findings may suggest, the same organs in different mammals impose different selective pressures on the same set of genes in cancer. In both species, some genes may experience strong selective pressures, but do not converge genetically or the conserved genes do not show the same selection pressure in both species. However, human breast cancer shows transcriptomic similarity with canine mammary carcinoma but the other subtypes are quite different. We found canine mammary tumor can be used as a model for inter and intra-tumor heterogeneity. These findings provided insight into mammary cancer across species and possessed potential clinical significance. Collectively, these studies suggest a convergence of some genetic changes in mammary cancer between species but also distinctly different paths to tumorigenesis.

Introduction

Tumorigenesis has been widely accepted as an evolutionary process that comprises two stages of evolution between tumors and normal tissues (Stage I) and within tumors (Stage II) [1]. Patterns of mutation and natural selection, the predominant evolutionary driver forces, vary at the two stages based on the evidence of low genetic convergence among different cancer cases revealed by The Cancer Genome Atlas (TCGA) data and of extremely high intra-tumor genetic diversity measured in high-density sampling studies [2,3]. At Stage, I, positive and negative selection may both exist but neatly counteract in absence of recombination, presenting a plausible neutrality 1, whereas non-Darwinian (neutral) selection was increasingly supported at Stage II by the high-density sampling studies [2,3] and comparatively genomic and transcriptional distances among distinct normal and cancerous cell populations [4]. Deciphering the evolutionary patterns during tumorigenesis such as selectivity or neutrality, adaptive convergence, or divergence is of both theoretical and clinical significance [5]. Cross-species cancer genomics, independent evolution from normal tissues, provide an excellent opportunity to address this long-standing issue: Does selection drive cancer evolution along with a relatively deterministic (selectivity) or contingent (neutrality) way across species? If there is the selection at Stage I, whether the same organs in different mammals impose similar selection pressures on the same set of genes? Is neutral selection at Stage II supported in different species? If there exists adaptive divergence among species, what are the causes and molecular mechanisms [6]?

The dog has experienced increased amounts of genetic diseases including cancers subject to human survival and aesthetic preferences, which is widely acknowledged as an increasingly powerful animal model to reveal the genomic signatures of cancer development and progression based on its desired characteristics [7]. Analogous to human, canine cancers are generally spontaneous and do not need to be induced by exogenous materials like murine cancer models. The spontaneous tumors in dogs share a wide variety of epidemiologic, biologic, and clinical features with human cancer [8]. Dogs are diagnosed with many of the same cancers as humans, with a similar prevalence of cancer types with few exceptions, and with a similar presentation, pathology, and treatment response [8]. Cancer biology studies using gene expression data in both human and dog indicate that the target genes and molecular signaling pathways involved in canine cancer development significantly resemble those found in human [9], supporting the use of canine cancer model as a homolog for what occurs in physiopathological aspects of cancer biology in human. Like other cancer types, concerning genetic, biological, anatomic, and clinical similarities are documented between canine mammary cancer and human breast cancer. Nevertheless, would dog can be an appropriate experimental model for human cancers from the genomic and evolutionary views?

It is acknowledged that cancer is a significant aging-related disease in both humans and dogs. Most canine cancers start to develop in later life with a sharp increase after the age of 6 years old and a peak at 10, which is similar to human [10]. Understanding the evolutionary origin of aging-related diseases like cancer and the underlying theory of aging evolution remains largely elusive. Mutation

90   accumulation[11] and antagonistic pleiotropy[12] are two prevailing theories proposed to address aging

91   evolution Mutation accumulation theory states that the effects of natural selection decrease as age

92   increases, resulting in maladaptive detrimental mutations that accumulate late in life. Antagonistic

93   pleiotropy theory hypothesizes a trade-off that evolutionary adaptations benefiting the fitness in early

94   life increase disease burden in later life after reproduction [13]. Both theories haven't been well verified

95   and exhibit setbacks. Increased incidence of cancer in both humans and dogs provides an opportunity

96   to investigate whether there exits evolutionary similarity or divergence between human and dog

97   cancer, if any, is the similarity attributed to mutation accumulation or antagonistic pleiotropy, and is

98   the divergence related to lineage-specific selective regime?[12]

99   In this study, we take breast cancer as an example to address the issues of adaptive convergence

100   or divergence in distinct species, because breast cancer is the most common malignancy in women

101   and is the third most common tumor in dogs. We performed whole-genome sequencing in 27 dog

102   mammary gland cancers and reanalyzed 560 WGS and 1080 WES human breast cancers in

103   comparison with their normal samples, profiling the genomic signatures of tumor development and

104   progression, and implemented the comparison of cross-species cancer genomics.

105

106   **RESULTS**

107   **Characterization of somatic mutations for canine malignant mammary cancers**

108   To address cross-species cancer genomics questions, we deeply sequenced whole-genomes of 27

109   malignant mammary tumors from 17 dogs that comprise 8 breeds with an average age of diagnosis of

110   12, along with the matched normal genomes from the same individuals. Cancers cover varieties of

111   histopathologic classifications, consisting of carcinoma (simple, solid, complex, special types

112   (squamous, adenosquamous, tubulopapillary, lipid)), sarcoma (osteosarcoma, rhabdosarcoma) and

113   carcinosarcoma according to the World Health Organization (WHO) criteria, and various grades

114   according to the Elston and Ellis scoring system, as presented in Fig.1 and Table S1, which exhibit

115   substantially morphological and biological heterogeneity compared with Human breast cancer.

116   Cancers were also classified as subtypes of luminal A, luminal B, human epidermal growth factor

117   receptor 2 (*HER2*)-overexpressed, and triple-negative using immunohistochemistry based on presence

118   of estrogen receptors (*ER*), progesterone receptors (*PR*), and *HER2*.

119   We performed single-nucleotide variation (SNV) calling in the WGS data from the canine

120   mammary cancers (see Materials and methods). A total of 47,715 somatic single nucleotide variants

121   (SNVs) comprising 180 exonic mutations (97 nonsynonymous, 69 synonymous and 11 stop gain)

122   (Table S4) were identified in the canine mammary neoplasms, with substantial variations in number

123   between and within histologic cancer types (Table S1, Figure 1). Although complex carcinoma

124   predisposes to carry fewest mutations whereas osteosarcoma exhibits the highest, the difference is

125   insignificant based on analysis of variance. 179 genes were identified with exonic mutations in at least

126   one sample, significantly regulating the biological functions of protein kinase activity and cellular

127   chloride ion homeostasis, as well as the pathways in cancer like *PI3K-Akt* signaling (*P*<0.05, Table S4,

128   S5). Only 4 genes (*PIK3CA*, *NEB*, *PCLO,* and *CKB*) recurrently mutated in at least 2 samples, with a

129   concurrency of 33.3%, 11.11%, 7.4%, respectively, which may involve mammary cancer

130   development in dog (Table S3).

131   In addition to our analysis of somatic single nucleotide variants(SNVs), we also looked for indels.

132   In total, we identified 16648 insertions and deletions (Indels) comprising 28 Indels in coding regions.

133   The three most frequently mutated genes (indels mutations) *ACTB, CHD2,* and *EFL1* with mutations

134   in the form of frameshifts indels are also prevalent in these genes **(Table S4).** According to Gene

135   Ontology (GO) analysis, these Indels are over-represented in 9 biological processes **(Table S4).**

136   Among these significant terms, most terms are highly associated with chromatin binding, DNA-

137   templated, and cell differentiation.

138   **Characterization of somatic copy number alterations for canine mammary cancers**

139   CNAs can affect gene dosage by altering the number of gene copies in the genome. Next, we analyzed

140   the copy number profiles of canine mammary cancer. We identified

141   recurrent large and whole-chromosome gains and losses in canine mammary cancer. We subsequently

142   estimated the cellularity for the canine mammary cancer samples by using the widely-used software

143   sequenza. The estimated cellularity range from (0.1-0.7) and the average sample cellularity is 37.2%,

144   indicating the low purity of canine mammary cancer samples. We identified the chromosomal ploidy

145   change for each chromosomal segment according to the read depth and B allele frequency revealed by

146   whole-genome sequencing. The ploidy of the chromosomes in canine mammary neoplasms is

147   generally diploid, with only three exceptions which have aneuploidy. Two solid carcinoma DB4-L4

148   (4), DB30-R3-R4 (3.2), and one osteosarcoma DB3L4-2 (3.7). It seems solid carcinoma can get

149   aneuploidy. We also characterized somatic copy number alterations (SCNAs) compared to normal

150   genomes, which is leading to 7762 chromosome segments ranging from 100bp to 1Mb including copy

151   number gain (5889) and loss (1874) or LOH (loss of heterozygosity) affecting hundreds of genes per

152   tumor. Five chromosomal CNAs are observed in three specimens on chromosomes 12, 16, 19, 22, and

153   29 **(Table S1) (Figure S2).**

154   We identified a total of 9136 CNAs at a median of 338 CNAs per sample **(Figure S6),** canine

155   mammary cancer, and almost all the gains and deletions were observed in at least four samples

156   **(Figure S2).** GISTIC 2.0 analysis (with a threshold of q < 0.25) revealed 3 focal amplifications and 4

157   focal deletions recurrently altered in canine mammary cancer along with 25 recurrently altered whole

158   arms **(Figure 2C and Table S6).** Among the recurrent focal regions, 26.26 , 27.27, 1.1, 3.3, 5.5, 8.8,

159   10.1,12.12, 16.16, 17.17, 18.18, 20.2 and 22.22 deletions have been previously found in canine

160   mammary cancers. Deletion events occurring at 26.26 and 2.2 encodes an oncogene *PTEN* and *CDK7*

161   which has been reported as a frequently amplified gene in canine mammary cancer[14].

162   To detect the potential functional effect of recurrently focal events, we performed Gene

163   Ontology (GO) analysis for the genes located in the amplified and deleted regions **(Table S7),**

164 respectively. Among the significant terms of amplified genes **(Table S7),** nucleoside triphosphate
165 catabolic process, cell cycle, and apoptotic processes were highly associated with tumorigenesis. On
166 the other hand, the deleted genes were enriched, cell cycle in some protein serine/threonine kinase
167 activity, and lipid catabolic process **(Table S7).** These results highlight the potential functional roles
168 of recurrent CNAs in the formation of canine mammary cancer.

169 Amplified and deleted genes the were enriched in **(Table S7)** polynucleotide 5'-phosphatase
170 activity, cell-cell adhesion, methylguanosine mRNA capping, transcription coactivator activity, and
171 neuropilin binding **(Table S7).** In this way, we identified several genes found in the Cancer Gene
172 Census (CGC) catalog that were focally amplified or deleted in canine mammary cancer. Specifically
173 deleted genes *PTEN, CDK7, SPP1, UBE4B,* and *ARG1* were enriched in the regulation of
174 transcription. These results highlight the potential functional roles of recurrent CNAs in canine
175 mammary cancer.

176 We also measured genomic instability index (GII, defined as the fraction of the genome altered by
177 CNAs, copy change≥1 relative to ploidy), and observed that the majority of tumors showed low
178 genomic instability (median of 2.1% per tumor, Figure 2A) except two samples display high genome
179 instability index DB30-R3-R4 (39.9%) and DB3L4-2 (18.9%) **(Table S1).** It depicts that the canine
180 mammary cancer shows genome stability. A median of only 0.15 % of the genome was affected by
181 high-copy gains and losses (copy change ≥2 relative to ploidy; defined as amplification and deletion-
182 based genomic instability index (adGII); **Figure 2B**).

183

184 **The landscape of mutational signatures in canine mammary cancers**

185 Mutational signatures are the particular patterns of mutations which is caused by mutagenesis
186 processes such as infidelity of DNA replication, repair deficiency, enzymatic DNA modification,
187 endogenous/exogenous mutagen exposures, provides insight into potential biological mechanisms of
188 carcinogenesis, which has exhibited applicability in cancer etiology, prevention, and therapy.
189 Mutational signatures in canine mammary cancer have not previously been formally investigated.
190 Here, we performed mutational signature analyses in canine mammary cancer and compared them
191 with human breast cancer.

192 The mutation spectrum in 6 base substitution catalogs revealed a significant dominance of
193 C→T/G→A transitions as in most tumors in humans **(Figure 3A),** indicating similar mutation
194 mechanisms (e.g., deamination of 5-methylcytosine) during tumorigenesis in both species. By
195 performing the non-negative matrix factorization (NMF) algorithm and model selection approach in
196 MutationalPatterns package **(see Materials and methods),** we reported four distinct mutational
197 signatures extracted from 27 canine mammary cancer specimens, comprising 3 previously observed

198 signatures in human breast cancer and a novel mutational profile uniquely occurred in dog mammary
199 cancer **(Figure 3B).**

200 Signatures 4 and 2 identified in dog cancer were analogous to human aging-related mutagenesis
201 (COSMIC signatures 1 and 5), with cosine similarity of 0.83 and 0.9, respectively. Signature 4
202 features a predominance of C>T transition in the context of NpCpG trinucleotide, whose analogy in
203 human displays etiology of spontaneous deamination of 5-methylcytosine, an epigenetic regulatory
204 mechanism with implications for aging, and correlates with the age of cancer diagnosis in a clock-like
205 manner [15] which may support the hypothesis of mutation accumulation theory [16], i.e., somatic
206 mutational signatures during tumorigenesis have been acquired over the lifetime of individuals in both
207 species. Based on the fact that the proportion of the enriched mutation types of NpCpG is much less in
208 signature 4 in dog compared with signature 1 in humans (4% vs 11%), implicating a potential
209 divergence in aging-related evolution during tumorigenesis. Signature 2 displays enrichment for T>C
210 and C>T substitutions, closely resembled with signature 5, which has a predominance of in the
211 ApTpN context with transcriptional strand bias.

212 Signature 1 in dog shows high similarity with Signature 17 with cosine similarity of 0.92, it is
213 characterized by the prominence of T>G mutations at NTT trinucleotides and was previously
214 identified in breast, stomach and oesophageal cancers but its aetiology is still unknown. Another
215 pervasive mutational signature identified, Signature 3 is unique in canine mammary cancer which is
216 primarily characterized by C>T has a high proportion of TpCpT trinucleotides substitution. Besides,
217 C>G substitution at NpTpA mutations is enriched as well as T>A substitution in the TpTpA context
218 shows the contribution to this novel mutational signature in canine mammary cancer. Signature 3
219 shows the contribution of almost all cancer samples. These results revealed that different mutational
220 processes were operative during the progression of canine mammary cancer.

221   **Identification transcriptome signatures and DEGs in canine mammary cancers**

222 RNA-seq analysis revealed total 1115 genes, of which 532 and 583 genes were up and
223 down-regulated, differentially expressed (p≥0.01 and fold change log2fc > 2) between all types of
224 canine carcinomas **(Supplementary Table9 and Fig. 4).** Strikingly, among these genes, negative
225 regulation of hormone secretion and tumor necrosis factor-activated receptor activity is the most
226 significantly enriched functions **(Supplementary Table10).** To understand the alteration in mammary
227 cancer, we first investigated canine four normal mammary glands where both luminal and
228 myoepithelial cells are visible. Additionally, 31 canine mammary cancer RNA sequences **(Table S8)**
229 were used to identify the number of transcripts were listed in **Table S8**.
230 For the differentially expressed gene (DEG) analysis, we performed a comparison between
231 thirty one including three subtypes of canine mammary cancer and matching adjacent four normal

232 tissues. DEGs with a p-value < 0.01 and changes greater than 2-fold were determined for

233 each comparison. Deseq2 analysis identified 1115, of which 532 and 583 genes were up- and

234 down-regulated, respectively, in a comparison of the thirty-one canine mammary cancers and four

235 matching adjacent normal tissues **(Table S9).** Hierarchical clustering with the Kendall correlation

236 matrix of the 1115 DEGs successfully distinguished canine mammary cancers and matching DEGs

237 adjacent normal in a heat map analysis **(Figure 4).** Our results indicated that transcriptomic signatures

238 for canine mammary cancer subtypes (carcinoma, sarcoma and carcinosarcoma) might represent

239 human breast cancer for few genes and provide new candidates for biomarkers. We did not observe a

240 significant correlation among human breast cancer and canine mammary cancer **(Table S9 and S10).**

241 We found the different profiles of differentially expressed genes across the species.

242

243 Many cell cycle, cell adhesion, T-cell proliferation, and B-cell receptor signaling pathway pathways

244 have been reported in canines **(Table S10).** To better understand canine mammary cancer and human

245 breast cancer, we performed KEGG pathway analysis using the web-based DAVID functional

246 annotation tool (https://david.ncifcrf.gov/summary.jsp). For the pathway analysis, we used a list of

247 DEGs genes from the overall canine mammary cancer comparison. Out of 2466 DEGs, 1587 up- and

248 879 down-regulated DEGs in human breast cancer were isolated and subjected to KEGG pathway

249 analysis **(Table S10).** Canine mammary cancer has been proposed as a comparative model for

250 spontaneous tumors of human breast cancer. However, some new Ensembl genes were identified in

251 canine mammary cancer and some of known cancer as a down-regulated in the dog but are known to

252 be up-regulated in human breast cancer. These results are not consistent with previous studies,

253 because we studied all subtypes of canine mammary cancer[17]. These results might represent

254 similarities and discrepancies that exist between human breast cancer and canine mammary cancer.

255

256 **Comparison between human and canine mammary cancer**

257 Following the analysis of canine mammary cancer, we proceeded to explore the catalog of mutated

258 genes in each species. We turned to comparative genomics, taking advantage of the observation that a

259 histologically similar malignant tumor, is common in dogs, which would enable discovery of

260 actionable targets by sequencing of paired tumor-normal tissue samples. To compare somatic

261 substitution of canine mammary cancer with human breast cancer, we profiled the mutational

262 spectrum of SNVs. We analyzed 1080 WES from human breast cancer (TCGA data). Human breast

263 cancer had in total 322,199 somatic mutations, consisting of 103,997 exonic mutations (68343

264 nonsynonymous, 24584 synonymous, and 5823 stop gain) **(Table S5).** Intriguingly, the average

265 number of coding region mutations per cancer case is approximately 9 in dogs, while 65 in human

266 mammary cancer **(Figure 5A).** After checking the average number of somatic mutations per tumor,

267 we checked the mutation per Mb of the genome, which represents the accumulation of somatic

268 mutations over the life of the tumor. Somatic mutation burden may influence tumor behavior and the

269　mechanisms underlying the initiation and progression of cancer. The prevalence of somatic mutations
270　was highly variable between and within cancer classes, ranging from about 0.001/Mb to more than
271　400/Mb. Breast in humans: average 1 mutation per megabase, ranging from 0.01~10. Intriguingly, the
272　average number of coding region mutations per cancer case per megabase (Mb) is approximately 10
273　in dogs. It was notable that canine mammary cancer shows lower (2.67 and 0.187 mutations per
274　megabase (Mb), respectively **(Figure 5B)** [18]. Furthermore, we compared the genome-wide average
275　mutation frequency per tumor per Mb in human and dog **(Figure 5C).** These results revealed a low
276　single nucleotide mutation burden in canine mammary cancer compare to human breast cancer which
277　depicts that dog may need fewer mutations for triggering carcinogenesis [19].
278　We have different DNA copy number profiles in dogs, it has fewer copy number changes compare to
279　human breast cancer. However, by comparing chromosomal ploidy results, we found human breast
280　cancer and canine mammary cancer show difference in ploidy. Chromosomal ploidy changes of the
281　chromosomes estimated by sequenza in canine mammary neoplasms is nearly diploid on average, few
282　tumors show the change in ploidy, significantly deviates from the findings of either aneuploidy or
283　tetraploidy frequently observed in cancerous cells in human including breast cancer [20]. Additionally,
284　amplification and deletion were also observed over cancer genes previously implicated in breast
285　cancer development including *PTEN, RB1, CDK6, AKT2, CCNE1, DNMT3A, MDM2, CCND1,*
286　*ZNF217, ESR1, ZNF703, EGFR, IGF1R, AKT1, CDKN2, NF1,* and *CDH1* **(Figure S1).**
287

Similarity and divergence between human and canine mammary cancer genes
289　To compare cancer genes in humans and dogs, we explored the catalog of mutated genes in both
290　species. A total of 179 genes from canine mammary cancer was compared with 1997 human breast
291　cancer genes having mutation frequency >1%. The four most significantly mutated cancer genes are
292　*PIK3CA* (32.2%) involve cancer initiation and progression, *TP53* (25.6%) regulate the cell cycle and
293　*TTN* (25.6%) regulate ATP binding to the kinase in human breast cancer and *MUC16* (13.9%) plasma
294　proteins protect from infectious agents **(Table S3),** seems quite different from the dog mammary
295　cancer as expected. The *PIK3CA* gene is one of the most frequently mutated common oncogene in
296　human breast cancer (32.22%) and canine mammary cancer (33.33%). By performing Fisher's exact
297　test, we found three significantly different genes both in human and dog mammary cancer (Fisher's
298　exact test, P-value <= 0.028). *TP53* and *TTN* mutations have relatively high mutation frequency in
299　human breast cancer compare to dog and *CKB* mutations predominate in canine mammary cancer.
300　*NEB*, *MUC16,* and *PCLO* mutations were found to co-occur. Since we have less number of samples
301　from dog mammary cancer compare to human breast cancer, we have limitation for this comparison.
302　Thus, for the further comparison based on significantly mutated genes, we have chosen 127 SMGs
303　(significantly mutated genes) from 20 cellular processes involved in cancer which already reported
304　across 12 major cancer types in humans [21]. These SMGs are involved in a wide range of cellular
305　processes, including histone modifiers, genome integrity, receptor tyrosine kinase signaling,

306 transcription factors/regulators, mitogen-activated protein kinases (*MAPK*) signaling, cell cycle,

307 phosphatidylinositol-3-OH kinase (*PI3K-Akt*) signaling, histones, ubiquitin-mediated proteolysis,

308 *Wnt/b-catenin* signaling, and splicing [21]. Comparison of SMG in human breast cancer and canine

309 mammary cancer **(Table 1)** shows the *PI3K-Akt* signaling pathway is significantly common in both

310 species, which play an essential role in the initiation and development of mammary cancer and is

311 considered to be a potential therapeutic target. We found many similarities, however, the genomes of

312 canine mammary cancer lacked mutations in other key human breast cancer drivers, such as *TP53* and

313 *CDH1*, etc. Thus, canine mammary cancer represents similarities and as well as a difference in

314 mutation profiles from human breast cancer.

315

316 Interestingly *PIK3CA* frequently mutated the gene in both species, human and dog. We revealed

317 a common occurrence of the same mutations in the *PIK3CA* gene, at position chr34, 12675674, which

318 is identical to the *PIK3CA* mutation reported with high frequency in human tumors (8% of all human

319 tumors). The mutation was present in 87.9% of canine mammary tumors and shows a protein

320 truncating effect **(Figure 6).** and shows the protein truncating effect, the amino acid substitutions at

321 conserved sites **(Figure 6).** The amino acid substitutions H1047R results in enzymatic

322 overactivation[22]. This conservation for both amino acid substitutions H1047R and H1047L, strongly

323 suggests that the same mutations in the canine cases also play a functional role in mammary cancer.

324 To investigate the selective pressure acting on the same tissue across human and canine

325 mammary cancer, we choose distributions of the evolutionary rate (*Ka/Ks*) to study this phenomenon.

326 The intensity of selection in cancer cases can be expressed as the K_a/K_s ratio, where K_a is the number

327 of nonsynonymous changes and K_s is the number of synonymous changes. We calculated the genomic

328 K_a/K_s ratio for both human and dog cancers and found that K_a/K_s in human equals to 1.12, a hallmark

329 of neutrality, indicating a generally neutral evolution. Whereas K_a/K_s in a dog is equivalent to 0.67,

330 implying a relatively strong negative selection in coding regions **(Figure 7)**. Most tumors have been

331 evolving neutrally in humans, but here negative selection in dog indicates, maybe coding point

332 mutations are lost through negative selection in canine mammary cancer or caused by the lineage-

333 specific characteristics [23,24]. Here, we found the evidence, which implies that different organisms have

334 different selective pressure on the same organ.

335

336 **Assumptions for varieties of mutations numbers between dog and human breast cancer**

337 **The antagonistic pleiotropy theory**

338 An evolutionary term (antagonistic pleiotropy theory) says benefits in early life increase the

339 organism's fitness, which may make it disease susceptible later in life. This explains the reason why

340 the degree of cancer susceptibility varies across mammals. As, humans live longer than dogs, they

341 have acquired the biological ways to slow down and regulate somatic evolution increase the cancer

342 risk in humans. Human life expectancy worldwide has increased dramatically due to improved food,

343　living conditions, hygiene, and medical care. The dog due to having a small life span cost of
344　maintaining reproductive capability, and of reproduction itself, has been demonstrated in a wide range
345　of animal species. However, little is understood about the mechanisms underlying this relationship.
346　We have explored the aging genes showing selection in humans compared to dogs[16]. We found that
347　sterilization was strongly associated with an increase in lifespan, and while it decreased the risk of
348　death from some causes, such as infectious disease, it increased the risk of death from others, such as
349　cancer. If the female dog is sterilized having less chance of mammary cancer, as we observed in our
350　datasets only two female dogs are sterilized and having mammary cancer. Our dataset includes 11
351　dogs having age 12 or >12. In general, smaller breeds of dogs tend to live longer and may age slower.
352　Dogs are considered old after they are about 8 years old and having chances to get cancer, hearing
353　loss, cataracts, and senile dementia are also observed. To address this question, we compared 237
354　aging-related genes both human and dog the aging-related genes do not show selection in dog
355　mammary cancer. As the previous analysis shows, Maximum lifespan potential (MLSP) estimations
356　show that longevity did not evolve in dog lineage[25]. Natural selection acts on species when longevity
357　evolves, give insights into adaptive genetic changes associated with the evolution of longevity in
358　mammals[13]. It seems by experiencing artificial selection for breeding dog trade-off its defense
359　mechanism DNA repair, immune function, and cell cycle genes. It is the evidence as certain
360　mechanisms that are beneficial for dogs in breeding reproduction and growth, early in life could lead
361　to bad health effects later similar to humans. Pleiotropic effects emphasizing the importance of life
362　span when studying cancer across species. The dog has undergone human-directed breeding and
363　artificial selection, which give rise to phenotypic variants many molecular mechanisms are involved
364　in dog mammary cancer progression as it shows a variety of cells. In addition, Dog develops some
365　horrible cancer that is absent or rare in humans for example transitional cell carcinoma of the bladder,
366　histiocytic sarcoma, squamous cell carcinoma, and canine venereal transmissible tumors. The
367　antagonistic pleiotropy theory shows that evolutionary adaptations maximizing fitness in dogs but
368　increase disease burden after reproduction.
369
370　**Aging-related genes**
371　To infer the antagonistic pleiotropy theory, we hypothesized that these genes can contribute to aging-
372　related disease. To test this, we downloaded aging-related genes from
373　http://genomics.senescence.info/download.html and calculated the Ka/Ks values for these mutated
374　genes in both humans and dogs. For dogs, only 5 genes have nonsynonymous mutations, while for
375　humans, the gene number increases up to 231. The 5 genes for dogs have Ka/Ks ranged from 0.64 to
376　1.43, where, only *PIK3CA* has Ka/Ks of 1.43 and the others have comparable Ka/Ks with the genomic
377　(background) Ka/Ks (0.64-0.83). Whereas, the same set of genes in human represent Ka/Ks ranging
378　from 1.64 to 12.96, far higher than the background Ka/Ks (1.16). Additionally, the Ka/Ks
379　distributions for these aging-related genes are dramatically different between humans and dogs.

380   Majority of the genes (221/237) didn't experience positive selection (maybe experience negative
381   selection) in the dog with all of the genes having Ka/Ks =0 or <1 with exception of *PIK3CA*, whereas,
382   inhuman, 3 genes have Ka/Ks >10; 30 genes have Ka/Ks of 3-10; 32 genes: 2-3; 50 genes: 1-2; 43:
383   <0.5, which indicated a positive and negative selection in human and dog, respectively **(Figure 8A).**
384   The *PTEN* is considered a gatekeeper tumor suppressor gene involved in multiple mechanisms that
385   lead to cellular defense against neoplastic transformation in human breast cancer. It shows the highest
386   Ka/Ks 12.96 as its occurrence is 3.3% in Human breast cancer **(Figure 8B).** *PIK3CA* converge
387   genetically between human and dog mammary cancer as its occurrence is high in both species. But, in
388   humans it experiences selective pressures Ka/Ks is 2.37 but in Dog Ka/Ks is 1.43 seems lower as
389   compared to humans, maybe neutral. So, it shows convergence and a lack of overall selection[23]. As,
390   in all cancer cases many genes experience strong selective pressures but do not converge genetically.
391   Alternatively, different cancer cases may be driven by weak selection, which may nevertheless
392   converge. Convergence and selective intensity are distinct phenomena[1]. These findings supported by,
393   antagonistic pleiotropy theory hypothesis, an evolutionary trade-off that evolutionary adaptations
394   benefiting the fitness in early life increase disease burden in later life after reproduction. Similarly, we
395   have found common genes in the same tissues in different species showing different selection
396   pressure. Ecological factors of the mammary glands across species show, same organs in different
397   mammals impose different selective pressures on the same set of genes[26].
398   As these aging-related genes involved in development in the early life of a human, the mutation in
399   these genes shows the selection in human breast cancer after reproduction. The modern human
400   populations have substantially increased lifespan over the last two centuries and limited purifying
401   selection, which has been weakened by the use of advanced medications. However, in the dog, it
402   sounds different, as trade-off did not observe.
403   Dog life span did not increase relative to human and the genes involve in development, did not show
404   selection in canine mammary cancer. Dog show different phenomena compare to humans may be in
405   dog's domestication trade-off the aging or cancer-related phenotypes[27]. Dogs have undergone
406   thousands of years of human-directed breeding and contribute to cancer risk rather than aging
407   selection. The present study represents the first report of testing the antagonistic pleiotropy theory
408   hypothesis. Although most wild animals, including dogs, develop cancer, the rates are typically
409   much lower than in domestic animals. Trade-off is important and helpful to study the origin of
410   aging-related diseases[12].
411
412   **Inter and Intra-heterogeneity of canine mammary cancers**
413   Little is known about tumor heterogeneity when it comes to canine mammary cancers. To examine
414   the functionally essential variations related to canine mammary cancer heterogeneity, we performed a
415   phylogenetic analysis of six cases of multi-tumor mammary carcinomas by using single nucleotide
416   variations (SNVs). The morphology and biological behavior of canine mammary cancer are quite

417   heterogeneous. This is 1st time we are going to report inter and intra-heterogeneity of canine
418   mammary cancers.

419

420   Phylogenetic relationships based on SNVs accurately reflected the clonal origin,
421   like our previous studies about human cancers shows that genetic variations can be used to reconstruct
422   clonal evolutionary relationships among different tumors [2,28,29]. Here, we found that inter and
423   intratumor heterogeneity in canine mammary cancer is very high. Each canine patient has a different
424   tumor evolution pattern, for example independently evolving, having a common origin, or genetically
425   identical or unique tumors. Since. high level of heterogeneity within and between patients or tumors is
426   a major obstacle to successful cancer therapy[30]. The histological and molecular heterogeneity,
427   together with evolutionary patterns, demonstrate distinct biological behavior of these tumors, different
428   clinical and therapeutic approaches are required for treatment[8]. In addition, dogs can help us to study
429   inter and intra tumor heterogeneity, as each female has eight to ten mammary glands. It is rare for
430   women to develop more than one tumor in the breast. In this study, we carry out whole-genome
431   sequencing on six cases of multi-tumor mammary carcinomas. Two tumors exhibited the intra-tumor
432   heterogeneity with multiple morphological patterns. Multiple tumors are evolutionarily more complex
433   than single tumors as they have divergent microenvironments and experience cell migration. The
434   cases of canine mammary cancer used in this study are summarized in **Table S12**. Further analysis
435   revealed, four cases are of independently originated because tumors from the same canine patient
436   share very few somatic mutations, for identical tumors >60% of mutations are shared. Independently
437   originated tumors, are interesting, as they offer no information on the evolution of inter-tumor
438   diversity within one canine patient[31].

439   **Phylogenetic relationship of DB3**

440   Dog3 showed two tumors, one on the left side fourth position (L4) and second on the right side
441   the same position (R4) with mixed morphology. Histopathology results display DB3-R4-1 as
442   carcinosarcoma, DB3-R4-2 as osteosarcoma, DB3L4-1 as lipid carcinoma and DB3L4-2 is
443   carcinosarcoma. We have sequenced four samples from Dog3 show only one common mutation
444   seems to evolve independently **(Figure 9A),** showing a different pattern from the human. It
445   demonstrates the heterogeneity of canine mammary cancer. The phylogenetic tree of Dog3 is
446   interesting, it displays the highly heterogeneous nature and independent origin of these tumors within
447   one canine patient. As, both tumors DB3L4 and DBR4 shows the carcinosarcoma, which contains two
448   types of cancer cells (epithelial and mesenchymal). Thus, this phenotypic diversity in one-tumor
449   shows may be epithelial to mesenchymal transition (EMT) that happens within the tumor in canine
450   mammary cancer.

451

Phylogenetic relationship of DB10

Dog 10 had one tumor one on the left side fourth position (L4) with diverse morphology (Figure 9B). Histopathology results show DB10L4-1 as carcinosarcoma and DB10L4-2 is adenocarcinoma. We have sequenced two samples from Dog10 tumor, shows 128 common SNVs (*NEB* gene shows exonic) mutations seem identical but the morphology is different. It seems epigenetics may also involve in this mixed morphology. Similar to dog3, may be epithelial to mesenchymal transition (EMT) happened within the tumor. Additionally, epigenetics changes may be contributing to the phenotypic diversity within the tumor rather than SNVs. We can assume from EMT the osteosarcoma originates, as shown in **(Figure 9B)** [32]. By this process, we can also say maybe the first tumor may be the cause of the second tumor within the same canine patient. Despite the intratumor heterogeneity, we study the inter mammary tumor heterogeneity within one canine patient, as a female dog has eight to ten mammary glands. Six canine patients have multiple tumors. However, DB3 shows inter and intratumor heterogeneity, as we have explained above. The other remaining five canine patients with multiple tumors are discussed here.

Phylogenetic relationship of DB4 and DB9

Dog 4 had two tumors on the left side, one on the left side fourth position (L4) and second on the left side fifth position (L5). This canine patient has two tumors with different histopathology and shared genome-wide 25 SNVs **(Figure S5A).** It shows different histopathology does not share the common mutations. These results show, genetically unique tumors are evolving separately within one canine patient. Dog 9 had two tumors on the right side, one on the right side fourth position (R2) and second on right side fifth position (R5). Analysis of this canine patient shows similar histopathology for both tumors, a subtype of carcinoma, and shared genome-wide 98 SNVs **(Figure S5B).** It demonstrates that carcinoma share genome-wide similar mutations. This canine patient shows both tumors are may be identical.

Phylogenetic relationship of DB12 and DB16

Dog 12 had two tumors on the left side, one on left side third position (L3) and second on left side fifth position (L5). Both tumors are a type of sarcoma and a shared genome-wide 15 SNVs **(Figure S6A).** It demonstrates that sarcoma may be shared genome-wide fewer mutations. These results indicate, genetically unique tumors are evolving separately within one canine patient. Dog 16 had two tumors same histopathology, one left side fourth position (L4), and the second tumor on the right-side third position (R3). Two tumors shared a few common genome-wide SNVs (16) **(Figure S5B).** It shows sarcomas also share less genome-wide mutations similar to dog12. These results show tumors are evolving separately, therefore genetically unique in one individual.

487 **Phylogenetic relationship of DB15**

488 Dog 15 had three tumors, one on the left side fourth position (L4)), second on right side fourth

489 position (R4), and third on right side fifth position (R5). This canine patient has three tumors with

490 different histopathology two tumors are the subtype of carcinoma (solid and simple carcinoma) and

491 one mixed tumor. Three tumors shared genome-wide 9 SNVs. DB15 share the exonic mutation

492 *PIK3CA* gene, which is a known driver gene, appear these tumors evolve from a common ancestor

493 cell **(Figure 10).** This result shows that mixed tumor which has epithelial and bone cells may be

494 originated from epithelial cells. Additionally, maybe epithelial to mesenchymal transition (EMT) is

495 cause diverse phenotypes in dog mammary cancer similar to dog3, dog4, and dog10. Canine

496 carcinosarcoma can be the best model to study epithelial to mesenchymal transition (EMT), it can be

497 assumed due to this process these tumors have different morphology. As, it is a natural behavior in

498 canine mammary cancer, it also happens in human breast cancer but very rare. However, further

499 studies are needed to explore the heterogeneity in canine mammary cancer by using multiple tumor

500 regions sampling and multi-omics analysis.

501

502 **DISCUSSION**

503

504 The recent development of sequencing technology has opened the door for detailed

505 profiling of canine mammary cancer genomes. Our study represents the first, as far as we are aware,

506 to examine somatic genomic alterations in all subtypes of canine mammary cancer. We have

507 performed a cross-species cancer genomics analysis, by using whole-genome and RNA sequencing a

508 cohort of 27 cases from canine mammary cancer. We identified recurrent mutations in more than half

509 of the cases whose human homologs are well-established cancer drivers, strongly suggesting that

510 these candidates indeed drive canine. Taken together, canine and human studies highlight the

511 benefits of comparative diseases genetics[5], the importance of selection for genome evolution, as

512 well as the extreme complexity of genome function and regulation. Nevertheless, the lack of a

513 deep understanding of the genetic causes of tumor variation in canine cancer limits the use of this

514 naturally occurring model for understanding human disease. Cancer in dogs also follows the general

515 epidemiology of human cancer occurrence[7]. The prevalence of different cancer types between

516 human and dog has provided some hints, the cross-species cancer genomics may offer new

517 insights into cancer evolution. Through 100X WGS, we have, for the first time, characterized the

518 genomic landscape of canine mammary cancer.

519 We identified and compared the mutation patterns of both human and dog, in terms of mutated

520 genes and the number of mutations that may influence tumor behavior and the mechanisms

521 underlying the initiation and progression of cancer. The average number of coding region

522 mutations per case of cancer is higher in humans than in dogs. Dogs may need fewer mutations for

523 triggering carcinogenesis[33]. As dogs have been undergone strong artificial selection within the last

524   hundreds of years with extremely high levels of morphological specificity based on strict breeding

525   programs, leading to a considerable genetic disease such as cancer as mutations accumulated

526   within the closed gene pool [7]. It depicts the side effect of domestication has been the inadvertent

527   enrichment for different types of diseases including cancer. Further, we reveal divergent indels and

528   DNA copy number profiles, as compare to human breast cancer, canine mammary cancer showing

529   fewer indels and copy number changes.

530   Importantly, we identified four mutational signature in canine mammary cancer. Three mutational

531   signatures show the similarity with human breast cancer signatures. We found signatures 4 and 2

532   in canine mammary cancer were analogous to human aging-related mutagenesis (COSMIC

533   signatures 1 and 5), respectively. Signature 4 analogy in human displays etiology of spontaneous

534   deamination of 5-methylcytosine, an epigenetic regulatory mechanism with implications for aging,

535   and correlates with the age of cancer diagnosis in a clock-like manner [15], which supports the

536   hypothesis of mutation accumulation theory, i.e., somatic mutational signatures during tumorigenesis

537   have been acquired over the lifetime of individuals in both species. Based on the fact that the

538   proportion of the enriched mutation types is much less in signature 4 in dog compared with signature

539   1 in humans (4% vs 11%), implicating a potential divergence in aging-related evolution during

540   tumorigenesis [34]. Additionally, we found a novel somatic mutational signature in canine mammary

541   cancer, previously not described in human cancers, these mutational signatures designate that dog

542   share same mutagenesis processes like the human, however still different at the mutational level that

543   might be the result of the difference in genetic background.

544   We identified the RNA expression, which suggests canine mammary cancer counterparts of human

545   breast cancer, can provide new clues for biomarker screening for human breast cancer. However,

546   some new Ensembl genes were identified in canine mammary cancer and some of known cancer

547   genes as a down-regulated in the dog but are known to be up-regulated in human breast cancer. As,

548   previous studies describe the canine carcinoma shows high similarity with human breast cancer but

549   sarcoma and carcinosarcoma not. We thus determined transcriptome level across the specie not

550   compatible at all. Since, many studies have been performed in comparison to human breast cancers

551   and canine mammary at the transcriptome level. Thus, our results reveal the similarities and

552   discrepancies between human breast cancer and canine mammary cancer[17].

553   Further, we reveal patterns of selection in cancer evolution by adapting methods from

554   evolutionary genomics and applying them to cancer genomes, which is characterized by the overall

555   representation close to neutrality in human breast cancer[23,35]. Genome-wide Ka/Ks ratio for

556   human's equals 1.19, a hallmark of neutrality, indicating a generally neutral evolution and in the

557   dog, Ka/Ks is 0.61, seems negative selection. As, most of the tumors in human have been evolving

558   neutrally, which does not mean the absence of selection[23]. It only reflects that the effect of positive

559   selection in accelerating evolution is exactly canceled out by the effect of negative selection [6,23].

560   Moreover, negative selection in canine mammary cancer indicates, maybe coding point mutations

561   are lost through negative selection or there are other unknown reasons. Purifying selection in the

562   evolution within tumors. Natural selection can be positive (for the beneficial mutations) or

563   negative/purifying (against the deleterious ones). Little attention has been paid to the power of

564   negative selection in cancer evolution[36]. Wu et al. (2016) reported the average Ka/Ks ratio of 1 on all

565   genes and the exceeding percentage of negative sites in comparison with the simulated distribution,

566   suggesting the possibility of negative selection operating on the genes with the essential function of

567   cells should not be ignored[26]. The operation results in stabilizing selection through the purging of

568   deleterious variations that arise, eventually reducing the variation in phenotypes in populations. In the

569   case of cancer cell population without recombination, the effect of negative selection could be high, as

570   the purging of deleterious variants will result in the removal of linked variation. The test on the

571   evolutionary forces and exploration of the effect and genes under selection remains required.

572

573   Further, we compared aging-related genes between human breast cancer and canine mammary

574   cancer. Our results demonstrate that aging genes with somatic substitutions are showing a clear

575   signal of positive selection, Ka/Ks is significantly high in human breast cancer as compared to the

576   dog. Here, we get the answer to our main question, whether the same organs in different mammals

577   impose different selective pressures on the same set of genes? These findings provide the answer

578   to our question, the same set of genes in the same organs in different mammals impose different

579   selective pressures[6]. Evolutionary constraints are a major theoretical challenge for explaining the

580   linkage of genes in aging and cancer [16]. Importantly, our findings with aging-related genes in

581   humans can be explained by widespread antagonistic pleiotropy hypothesis which suggests that the

582   same gene can be favorable for fitness but can confer the risk of traits later in life [13,37]. This theory

583   has not been rigorously tested previously.

584   But, in the dog, it looks different, as we did not observe selection on aging-related genes in canine

585   mammary cancer. Therefore, maybe in dog's domestication trade-off the aging or cancer-related

586   phenotypes of modern dog populations than at any time of macroevolution. The modern human

587   populations have substantially increased lifespan over the last two centuries [38] and limited purifying

588   selection, which has been weakened by the use of advanced medications [39]. A side effect of

589   domestication has been the inadvertent enrichment for different types of diseases. This interplay

590   between desired and deleterious traits, some of which are pleiotropic, has been particularly well

591   used in the study of both canine and feline genomics. As a consequence, ~50% of pet dogs develop

592   cancer in their lifetime. Presumably, as a consequence, advancing age is the major risk factor for

593   diverse types of loss of function, and highly prevalent chronic and killer diseases. Nevertheless,

594   important questions remain to be addressed.

595   So, maybe in dog's domestication trade-off the aging or cancer-related phenotypes of modern

596   dog populations than in any time of macroevolution. Dogs have undergone thousands of years of

597   human-directed breeding and selection, giving rise to the hypothesis that selection for desired

598 phenotypes may have led to piggybacking of deleterious alleles that contribute to cancer risk. Most

599 modern dog breeds only existed for 300 years and many are derived from small numbers of founders.

600 We can say by experiencing the same environment parallel evolution is most apparent in genes for

601 digestion and metabolism, neurological process, and cancer [7,40].

602 Further, we found that inter and intratumor heterogeneity in canine mammary cancer is very

603 high. Each canine patient has a different tumor evolution pattern, for example independently evolving,

604 having a common origin, or genetically identical or unique tumors. Dog9 shows genetically identical

605 tumors within one canine patient, other dogs show genetically unique tumors within one canine

606 patient. Four canine carcinosarcoma, maybe indicate epithelial to mesenchymal transition (EMT), it

607 can be assumed due to this process these tumors have different morphology[32]. The histological and

608 molecular heterogeneity, together with evolutionary patterns, demonstrate distinct biological behavior

609 of these tumors, different clinical and therapeutic approaches are required for treatment. To diversity

610 and heterogeneity of tumor cell types and states (such as metastasis and drug resistance) in both

611 species, one or multiple tumors from a single patient are of necessity. Thus, further exploration and

612 analysis of whole-genome sequences from dog mammary cancer will be required to complete our

613 understanding of the somatic mutational basis of the disease. Furthermore, multiple region sampling

614 can help us to investigate the heterogeneity and phenotypic diversity in canine mammary cancer.

615 Additionally, there is still a need to explore the epigenetic or regulatory cause of phenotypic diversity.

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626 **MATERIALS AND METHODS**

627 **Sample collection**

628 Twenty-four malignant tumor masses and adjacent non-tumor tissues were surgically resected by

629 simple or regional mastectomy from 17 female dogs with diagnosed mammary gland neoplasms in the

630 Veterinary Teaching Hospital of China Agriculture University and the Beijing Guanshang Animal

631 Hospital. Samples were collected under the guidelines of animal use protocols and informed consent

632   signed by dog owners. The 17 female dogs covered 8 breeds consisting of the Silver fox, Teddy,

633   Cocker, Poodle, Dachshund, Pomeranian, Shih Tzu, and Mixed, with a sterilization rate of 12% and

634   an average age of diagnosis of 12 (6-15) years old, 4 of which presented the clinical or radiological

635   evidence of spread to nearby lymph node or distant metastases in lungs. The 24 tumors varied in sizes

636   which ranged from 1.2 cm of diameter to 9.2 cm. According to the clinical TNM staging system of

637   canine mammary gland neoplasms (Table S2) that was developed by the World Health Organization

638   (WHO) and modified by Withrow and MacEwen (1996) [41], the tumors were staged as I-V based on

639   tumor size and metastasis status. The detailed information on the dog breed, age of diagnosis, and

640   tumor features such as anatomical sites, tumor sizes, metastasis status, and TNM staging were

641   summarized in **Table S1**.

642   For each specimen of mammary gland neoplasm, two sections were excised for histopathological

643   examination and Immunohistochemical (IHC) test of hormone receptors and growth factors,

644   respectively. The rest part of the specimen was stored at -80°C, until being processed for DNA

645   extraction. This study was approved by the Animal Research Ethics Committee of Beijing Institute of

646   Genomics, Chinese Academy of Science.

647

648   **Histopathological examination and grading**

649   Histopathological examination was performed by veterinary pathologists in the Veterinary Teaching

650   Hospital of China Agriculture University. A representative section of tumor specimen was fixed in 10%

651   formaldehyde, dehydrated in ethanol, and Xylene, embedded in paraffin wax, sectioned with 4 μm

652   thick and stained with haematoxylin and eosin (Beyotime, Shanghai, China). Histological

653   classification of the specimen was determined according to the WHO criteria for canine mammary

654   cancer [42]. According to the Elston and Ellis method [41], histological grading of the specimen was

655   assessed as well-differentiated (grade I), moderately differentiated (grade II), as well as poorly

656   differentiated (grade III). The histopathologic classifications and grading were described in Table S1.

657

658   **Immunohistochemical (IHC) analysis**

659   To determine the presence of hormone receptors consisting of ER and PR, and the epidermal growth

660   factor receptor 2 (HER2) in a 5μm-thick, formaldehyde-fixed, paraffin-embedded tumor section, IHC

661   experiment was performed by following the standard protocols [43]. The tumor section was incubated

662   with primary antibodies, including Anti-ERp57 antibody (Abcam, ab13506) for ER, Anti-PR antibody

663   - C-terminal (Abcam, ab191138) for PR, and human epidermal growth factor receptor 2 (HER2) Anti-

664   ErbB2 antibody (Abcam, ab16901) for HER2 were obtained from Abcam (Cambridge, MA, USA).

665   Images were taken with a CX31 optical microscope (Olympus, Japan). Immunohistochemical (IHC)

666   analysis was performed by veterinary pathologists in the Veterinary Teaching Hospital of China

667   Agriculture University. According to the hormone receptor status and HER2 status, we have

668   characterized the canine mammary tumors into four subtypes: Luminal A (ER+, PR+, and HER2-),

669 Luminal B (ER+, PR+, and HER2+), HER2-overexpressed (ER-, PR-, and HER2+), and Triple-
670 negative (ER-, PR-, and HER2-)[44] Table S1.
671
672 **DNA extraction, library construction, and WGS**
673 Genomic DNA was extracted from the dissected cancerous or adjacent normal tissues using the
674 QIAamp® DNA Mini Kit (QIAGEN, Duesseldorf, Germany). DNA quality and concentration were
675 monitored using agarose gel electrophoresis, Bioanalyzer 2100 system (Agilent Technologies, CA,
676 USA), and Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). High-quality sequencing libraries
677 were constructed by following the protocol of IlluminaTruSeq™ (cat. no. FC-121-2003) DNA
678 preparation kit (Illumina, CA, USA), then were sequenced on Hiseq2000 platform (Novogene,
679 Beijing, China). Approximately 114~150 millions of clean paired-end reads with a length of 150 bp
680 were generated per sample. The average sequence coverage was 90-fold for both tumor and normal
681 samples (Table S1B). Genome sequence data has been deposited in the genome sequence archive of
682 Beijing Institute of Genomics, Chinese Academy of Science with project accession number (GSA:
683 **CRA002536**) that are publicly accessible at http://bigd.big.ac.cn/gsa.
684
685 **SNV detection**
686 The paired-end reads of WGS for each sample were aligned to the dog reference genome (CanFam3.1)
687 using BWA-MEM (Version 0.7.17) with default parameters[45]. PCR (polymerase chain reaction)
688 duplication reads were marked with MarkDuplicates in Picard tools (Version 2.15.0) and filtered with
689 DuplicateReadFilter in GATK tools (Version 3.8-0). SNVs were called using MuTect2 for uniquely
690 mapped reads with default parameters, where local *de-novo* assembly of the haplotypes was
691 performed to increase the accuracy of mutation calling [46,47]. The variant sites identified in normal dog
692 populations that were considered as known SNPs (5,648,530 SNPs in total,
693 ftp://ftp.ncbi.nih.gov/snp/organisms/dog_9615/) were filtered out. The candidate SNVs were further
694 filtered using the following criteria: (1) the read depth of the SNV sites for both tumor and non-tumor
695 sections were at least 10, (2) at least three reads supporting mutant alleles were required in the tumor
696 sections, (3) only the SNVs supported by more than two reads in both forward and reverse strands
697 were retained, (4) the mutant allele fraction of SNVs in tumor sample was greater than 0.05. The final
698 SNVs were functionally annotated by both xenoRefGene using ANNOVA[48] and the Variant Effect
699 Predictor (VEP) (http://www.ensembl.org/vep).
700
701 **Calculation of Ka/Ks**
702 For each gene or the genome-wide genes as a supergene, we have counted the number of synonymous
703 (*s*) and non-synonymous substitutions (*n*) in coding regions, as well as the number of synonymous (*S*)
704 and nonsynonymous sites (*N*) in total. Based on these, we calculated the ratio of non-synonymous and
705 synonymous substitutions (K_a/K_s) using the following equation:

706   $K_a/K_s = (n/N)/(s/S)$

707   where, s for genes without synonymous substitutions was assumed to be 0.5 as suggested by Wang et

708   al. (2011)[1,49,50] to avoid calculation errors due to the few numbers of synonymous mutations detected

709   in genes.

710

711   **Mutation spectrum and signature analysis**

712   The MutationalPatterns package was utilized to extract and visualize mutational signatures in

713   base   substitution   catalogues   of   tumor   samples

714   (https://github.com/UMCUGenetics/MutationalPatterns). Non-negative matrix factorization (NMF)

715   was applied to extract *de novo* mutational signatures based on the mutation count matrix of 96

716   trinucleotide mutation classifications in canine mammary tumors [51,52]. The number of signatures was

717   determined according to the NMF quality measures of the cophenetic correlation coefficient and

718   residual sum of squares (RSS) as suggested by Gaujoux and Seoighe (2010) [53]. The optimal number

719   should be chosen as the smallest number for which the cophenetic correlation coefficient starts

720   decreasing and the number for which the plot of the RSS between the input matrix and its NMF

721   estimate shows an inflection point. As shown in **Figure 3B**, the number of mutational signatures of 4

722   was selected.

723   The mutation catalogues (mutation types and counts) of the discovered signatures in canine

724   mammary tumors were compared to those of the 30 standardized signatures in human cancer from the

725   COSMIC (https://cancer.sanger.ac.uk/cosmic/signatures), and the cosine similarity between them was

726   calculated with cos_sim_matrix[51]. Cosine similarity of 0.00 indicates completely different and

727   similarity of 1.00 indicates a perfect match.

728

729   **Estimation of tumor purity and copy number alterations**

730   We used the R package Sequenza to quantify cancer cellularity, ploidy, and chromosomal copy

731   number alteration [54]. The paired-end sequence reads of WGS for each sample were aligned to the dog

732   reference genome (CanFam3.1) using BWA-MEM (Version 0.7.17) with default parameters[45]. The

733   Pileup files of the tumor and normal specimens, as output by SAMtools, were processed by

734   'sequenza-utils' and binned by 1kb window size, calculating sequencing depth, homozygous and

735   heterozygous positions in the normal sample, and variant alleles and allelic frequency in the tumor

736   specimen. The *sequenza. the extract* was used to normalize the tumor versus normal depth ratios

737   based on GC-content and perform allele-specific segmentation using the 'copy number' package.

738   Based on the calculated B allele frequency and normalized depth ratio, the cellularity and ploidy were

739   inferred from the point estimates with the maximum log posterior probability by fitting a Sequenza

740   model using *sequenza.fit*. The allelic-specific copy numbers of chromosomal segments were further

741   inferred according to the estimated cellularity and ploidy. Summary of the aberrant cell fraction and

742   overall ploidy are provided in **Table S1**.

743 **Driver genes and regions analysis**

744 We defined potential canine mammary cancer driver genes as previously described [18]. The

745 significance of broad and focal CNAs was assessed from the segmented data using the GISTIC 2.0

746 algorithm [55]. We performed functional enrichment for these genes using DAVID [56].

747 **RNA Isolation and Total RNA Sequencing**

748 Total RNA was extracted from 27 mammary gland tumors tissues using the RNeasy Mini Plus kit

749 (Qiagen). Pulverization for sample homogenization was performed with liquid nitrogen before RNA

750 isolation according to the manufacturer's instructions. Cluster generation, followed by 2×100 cycle

751 sequencing reads, separated by the paired-end turnaround, was performed on the instrument using

752 HiSeq Rapid SBS Kit v2 (FC-402-4021) and HiSeq Rapid PE Cluster Kit v2 (PE-402-4002)

753 (Illumina). Image analysis was performed using the HiSeq Control Software version 2.2.58. The raw

754 data were processed, and base-calling was performed using the standard Illumina pipeline (CASAVA

755 version 1.8.2 and RTA version 1.18.64). A summary of the statistics of the RNA sequencing data is

756 listed in Table S8. RNA sequence data along genomic sequence data has been deposited in the

757 genome sequence archive of Beijing Institute of Genomics, Chinese Academy of Science

758 (http://gsa.big.ac.cn) with GSA accession number **CRA002536.**

759

760 **RNA-sequence analysis**

761 For dog mammary gland tumors, we downloaded the RNA sequencing data of 4 pairs of normal and 9

762 tumor specimens from the NCBI SRA database with accession number **SRP024250** [57]. Additionally,

763 RNA sequencing is done for 27 canine mammary cancers are **Table S8.** The numbers of transcripts

764 identified in the study were listed in Table S8. Paired-end reads of RNA-Seq were aligned to the dog

765 reference genome (CanFam3.1) using the STAR (Version 2.6.0)[58]. Next, we calculated the TPM

766 (transcripts per million) value for each gene using uniquely mapped reads with RSEM (RNA-

767 Sequence by Expectation-Maximization, Version 1.3.0) software in a single-stranded mode[59], which

768 quantified the gene expression level accurately. The uniquely mapped paired (~70-79%) was used to

769 quantify a gene's expression level by calculating its value using RSEM with the default parameters

770 and the canine RefGene and Ensembl annotation downloaded from the NCBI and Ensembl genome

771 site. Raw RSEM expected counts for all samples were normalized to the overall upper quartile. To

772 identify the differentially expressed genes (DEGs) between different sample groups and calculate the

773 significance level of expression differences, we performed the DESeq2 package (Version 1.14.0) with

774 a negative binomial test based on the raw read depth [60].

775 Heatmaps were then generated by differentially expressed genes using their expected count via

776 hierarchical clustering. Furthermore, gene functional annotation and enrichment analyses were

777 achieved using the DAVID Functional Annotation tool (david.abcc.ncifcrf.gov)[56].

778 **Construction of phylogenetic trees**

779 We constructed a phylogenetic tree of the canine patients and tumors using the Maximum parsimony

780 method in the PHYLIP package [61]. After excluding SNVs located in LOH regions, the remaining

781 SNVs were used to construct the phylogenetic tree. SNVs called from

782 WGS was used for tree construction.

783 **Human cancer datasets**

784 All VCF (Variant Call Format (Version 4.2)) files (called by Mutect2) for the human breast

785 carcinoma (BRCA) were downloaded from the TCGA data coordinating centre

786 (https://portal.gdc.cancer.gov/), each being reprocessed to eliminate known germline single nucleotide

787 polymorphisms (SNP) present in the dbSNP database. Additionally, for a comprehensive

788 understanding of somatic mutations in breast cancer across the genome, previously reported SNVs

789 analyzed by using the WGS dataset, were downloaded from ftp://ftp.sanger.ac.uk/pub/cancer/Nik-

790 ZainalEtAl-560BreastGenomes[20]. All variants coordinate mapped to the GRCh37, re-annotated using

791 the ANNOVAR[48], as it would have required a uniform variant calling and filtering workflow. RNA-

792 seq dataset downloaded from publicly available cancer transcriptome data (TCGA and MET500

793 transcriptome sequencing), http://ualcan.path.uab.edu/index.html [62].

794

795 **Author contributions**

796 SA, XL, and YH conceived and designed the experiments, SA and WD performed the DNA and RNA

797 extraction, library construction, SA, LG, XB, and YH performed the data analysis, SA, YH and XL

798 wrote the manuscript, D.L, G.B, Y.S, and YH provided and collected the samples.

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964
965

966 **FIGURE LEGENDS**

967 **Figure 1** Canine mammary cancer A. Carcinoma, Low power lens: The gland epithelial cells are
968 arranged in the island, High power lens: Glandular epithelial cells are arranged closely, with unclear
969 boundaries, **B.** Sarcoma, Low power lens: Local areas showed cartilage-like, bone-like structure, most
970 of them as an island. High power lens: The cells that in the outer edge of the cartilage-like, bone-like
971 structure **C.** Carcinosarcoma, Low power lens：The tumor is not a complete capsule, composed of a
972 large number of cell components, High power lens：Tumor cells varied. Glandular epithelial cells
973 arranged in a group like, the cartilage-like structure can be seen. Scale bar = 100 and 20 μm. The top
974 panel shows the enlarged view of the areas directed.
975 **Figure 2** Genomic instability and copy number landscape of canine mammary cancer.
976 A bar plot representing the fraction of the total genome altered with copy change ≥1 relative to
977 median integer ploidy, which is termed as the genomic instability index (GII). B. A bar plot
978 representing the fraction of the total genome affected by high-copy gains and losses (amplification
979 and deletions with copy change ≥2 relative to ploidy), which is termed as the amplification- and
980 deletion-based genomic instability index (adGII). **C.** Amplification (Left) and deletion (Right) plots
981 using all data and amplitude threshold. The green line represents the significance threshold (q-value =
982 0.25).
983
984 **Figure 3 A.** Mutation spectrum across human and canine mammary cancer. Mutation spectrum six
985 base substitution types. Bars represent the mean relative contribution of each mutation type and error
986 bars depict standard deviation.

987 **B.** Comparison of mutational signatures in human and canine mammary cancer. Three mutational
988 signatures in canine mammary cancer show similarity with human breast cancer (a): Signature 1 in
989 dog shows 0.92 similarities with signature 17 in humans. (b): signature 2 dog shows 0.9 similarities
990 with signature 5 in humans. (c): The signature 4 in dog shows 0.83 similarity with, signature 1in
991 human.

992 **Figure 4** Cross-species comparison of the heatmap and hierarchical clustering of top 50 genes
993 differentially expressed transcripts (p≥0.01, 2.0 FC) in human breast cancer and canine mammary
994 cancer (red: upregulation; blue: downregulation) from the RNA-seq data. The left panel illustrates the
995 human breast cancer and the right panel illustrates the canine mammary cancer. Each column
996 represents a sample and each row represents a transcript. Expression level of each gene in a single
997 sample is depicted according color key.
998 **Figure 5 A. Average number of somatic mutations per tumor (Exonic region).**

999 Bars represent the average number of somatic mutations per tumor is 9 and 65 in the exonic region in

1000 human and canine mammary cancer, respectively.

1001 **B.** Cross-species comparison of average no. of mutations per tumor per Mb across the exonic region.

1002 Bars represent average no. of mutations per tumor per Mb across exonic regions in human and dog

1003 and error bars depict standard deviation.

1004 **C.** Cross-species comparison of genome-wide average no. of mutations per tumor per Mb

1005 Bars represent average no. of mutations per tumor per Mb across all classes of somatic mutation

1006 exonic, Intronic, intergenic UTR3, and UTR5 regions, and error bars depict standard deviation.

1007 **Figure 6** Alterations in the *PIK3CA* gene. The mutations needle plot shows the distribution of the

1008 observed cancer mutations along the protein sequence. The needles' height and head size represent

1009 mutational recurrence. PIK3CA is frequently mutated and shows a protein-truncating effect in humans

1010 and dogs.

1011 **Figure 7** Genome-wide Ka/Ks value in human breast cancer and canine mammary cancer.

1012 Distributions of the genome-wide evolutionary rate (Ka/Ks) dog versus human mammary cancer. Y-

1013 axis represents the Ka/Ks value and the X-axis represents the organism name.

1014 **Figure 8 A.** Ka/Ks of aging-related genes in mammary cancer

1015 Distributions of the evolutionary rate (Ka/Ks) of aging-related genes in mammary cancer (human

1016 versus dog). Genes without Ka/Ks (the genes are not mutated or have the only synonymous mutation)

1017 are 97% and 3.3% in dogs and humans, respectively. The observations show much higher percentages

1018 of genes with Ka/Ks in human breast cancer.

1019 **B.** Ka/Ks for Genome-wide and five common aging-related genes in mammary cancer

1020 Although the genome-wide average of Ka/Ks is very close to 1, the Ka/Ks ratio for individual genes

1021 deviates from 1 in human breast cancer. The genome-wide and the Ka/Ks ratio for individual genes is

1022 less than one in canine mammary cancer except for *PIK3CA*, which has Ka/Ks=1.43.

1023 **Figure 9 A. Phylogenetic relationship between the tumors in Dog 3 between four samples**. The

1024 lengths of branches are proportional to mutation numbers. The tree was anchored using a DNA

1025 sequence from the normal tissue of a canine patient. **B. Phylogenetic relationship between two**

1026 **samples in Dog10.** The tree was anchored using a DNA sequence from the normal tissue of the

1027 canine patient. The lengths of branches are proportional to mutation numbers. The numbers

1028 correspond to the number of common mutations and specific mutations in the samples.

1029 **Figure 10 Phylogenetic trees based on SNVs of Dog15.** The phylogenetic tree has been constructed

1030 using somatic mutations from three tumors (simple carcinoma, solid carcinoma, and carcinosarcoma).

1031 The lengths of branches are proportional to mutation numbers.

1032 **Table 1. The 127 SMGs from 20 cellular processes in mammary cancer**

1033

1034   **Figure S1 The heatmap showing the segmented copy-number profiles in Canine mammary**

1035   **cancer.** The chromosomes are arranged vertically from top to bottom and samples are arranged from

1036   left to right. Red and blue represent gain and loss, respectively.

1037   **Figure S2**. **Tumor copy number profile of tetraploidy DB3L4-2, DB4-L4 and DB3-R2-R3.**

1038   X-axis represents each chromosome, and Y-axis represents copy number in the tumor.

1039

1040   **Figure S3 Sampling strategy of Dog 3 and Dog10 intratumor heterogeneity.** The blue dots show

1041   one sample from each part within tumor. Each circle shows one tumor.

1042

1043   **Figure S4** Sampling **strategy of Dog 4, 9, 12, 15 and Dog16 inter-tumor heterogeneity.** The blue

1044   dots show one sample from each tumor. Each circle shows one tumor.

1045

1046

1047

1048   **Figure S5 A. Phylogenetic relationship between the tumors in Dog 4 between four samples**. The

1049   lengths of branches are proportional to mutation numbers. The tree was anchored using a DNA

1050   sequence from the normal tissue of a canine patient. **B. Phylogenetic relationship between two**

1051   **samples in Dog9.** The tree was anchored using a DNA sequence from the normal tissue of the canine

1052   patient. The lengths of branches are proportional to mutation numbers. The numbers correspond to the

1053   number of common mutations and specific mutations in the samples.

1054

1055

1056

1057   **Figure S6 A. Phylogenetic relationship between two samples in Dog12**. The lengths of branches

1058   are proportional to mutation numbers. The tree was anchored using a DNA sequence from the normal

1059   tissue of a canine patient. **B. Phylogenetic relationship between two samples in Dog16.** The tree

1060   was anchored using a DNA sequence from the normal tissue of the canine patient. The lengths of

1061   branches are proportional to mutation numbers. The numbers correspond to the number of common

1062   mutations and specific mutations in the samples.

1063

1064

1065   **Tables Provided as Excel files.**

1066 **Table S1.** Supplementary data for canine mammary case information, including histopathological
1067 description, ER, PR and HER status, age, breed, and sex. This table provides supplementary data for
1068 Figure 1.
1069
1070 **Table S2.** Supplementary data for the TNM staging system for canine mammary carcinomas.
1071
1072 **Table S3.** Supplementary data mutation discovery in canine mammary cancer and human breast
1073 cancer by WGS and WES, total SNVs listed across the genome.
1074
1075 **Table S4.** Supplementary data for mutated genes with indels, GO for indels. And SNVs analysis
1076 Fisher test.
1077
1078
1079 **Table S5**. Supplementary data for the percentage of a mutated gene listed SNVs base substitution
1080 type occurrence in human and dog. The supporting data for Table 1.
1081 .
1082
1083 **Table S6.** Supplementary data for somatic copy number variation from GISTIC 2.0 analysis.
1084
1085
1086 **Table S7.** Supplementary data, GO for somatic copy number variation.
1087
1088
1089 **Table S8.** Supplementary data for RNA-seq sequencing and mapping.
1090 **Table S9.** Supplementary data for differentially expressed genes among canine carcinomas and
1091 Human breast cancer, including the gene list and p-value and log fold change among the genes.
1092
1093
1094 **Table S10.** Supplementary data for GO of differentially expressed genes among canine carcinomas
1095 and Human breast cancer, including the gene list and enriched functions among the genes.
1096
1097 **Table S11.** Supplementary data for data for Ka/Ks for aging-related genes in mammary cancer in
1098 both species. The supporting data for Figure 7.
1099
1100 **Table S12.** Supplementary data for inter and intra tumor heterogeneity.
1101
1102
1103
1104

Carcinoma	Sarcoma	Carcinosarcoma
(A)	(B)	(C)

Figure 1

A

Genomic instability index (GII)

B

Amplification and deletion based genomic instability index (adGII)

C

Figure 2

A

Human Breast cancer

Dog Mammary cancer

B

Signature 1

GpTpT

Dog

Human

Signature 17

Signature_1 shows 0.92 similarity with Signature17 in Human cancer

Signature 2

ApTpG

Dog

Human

Signature 5

Signature_2 shows 0.9 similarity Signature5 in Human cancer

Signature 4

ApCpG

Dog

Human

Signature 1

Signature_4 shows 0.83 similarity with Signature1 in Human cancer

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

A Ka/Ks: Aging-related genes
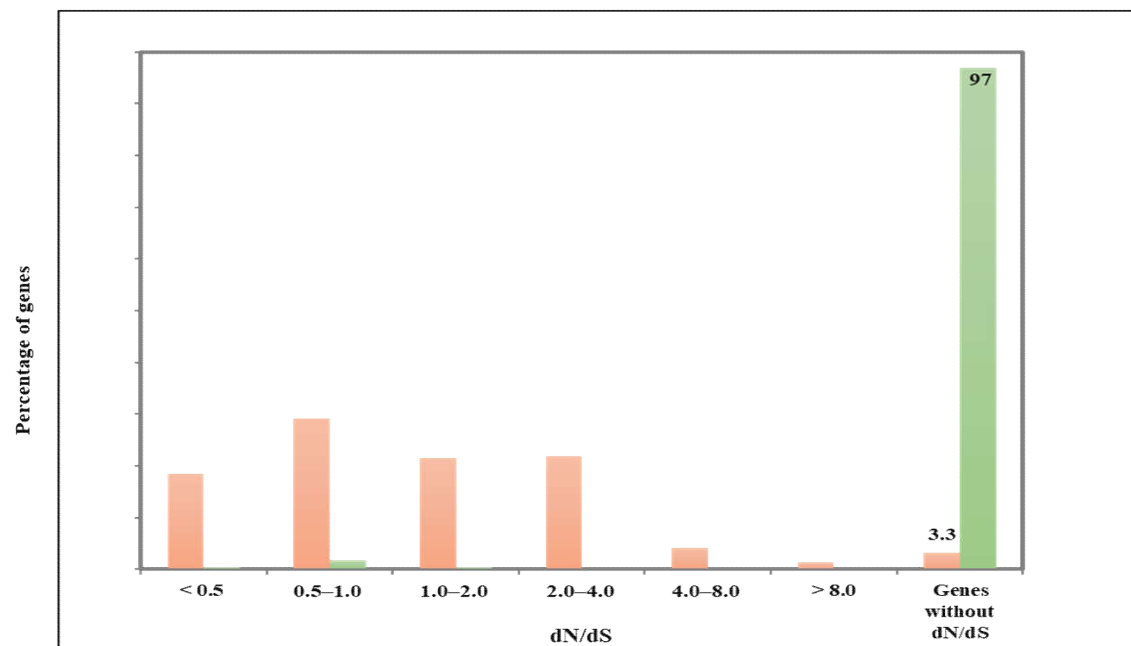
B (Ka/Ks) of Five Aging related genes in Mammary cancer

Figure 8

Figure 9

A

DB3

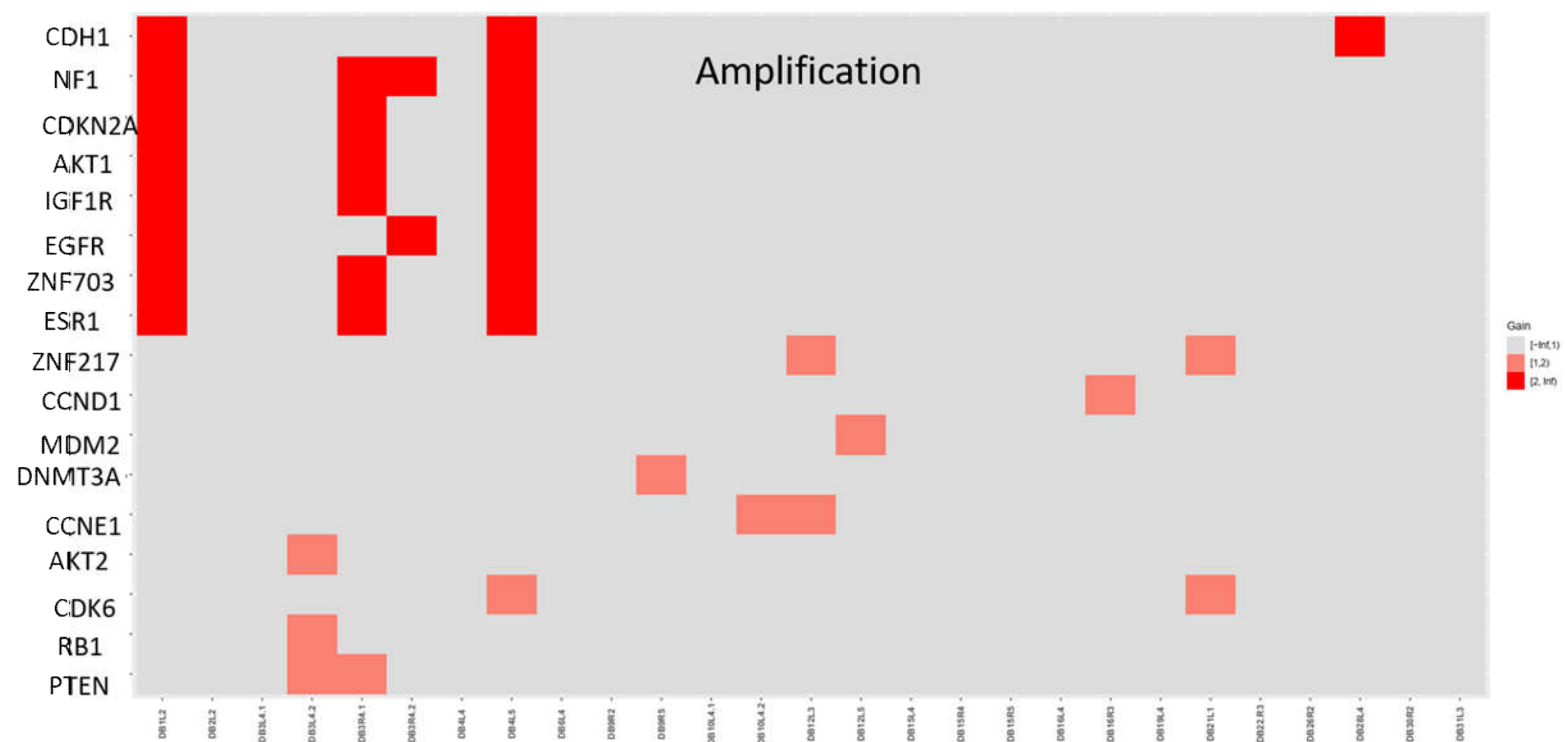1467 — DB3R4.1 (dog3, right-4, Carcinosarcoma, segment 1)

1074 — DB3L4.2 (dog3, left-4, Carcinosarcoma, segment 2)

N**1**

2419 — DB3R4.2 (dog3, right-4, Osteosarcoma, segment 2)

13

575 — DB3L4.1 (dog3, left-4, lipid carcinoma, segment 1)

300.0

DB3-R4.1   DB3-R4.2

Carcinosarcoma   Osteosarcoma

DB3L4.1   DB3L4.2

Lipid carcinoma   Carcinosarcoma

B

DB10

NEB

1520 — DB10L4-2 (dog10, left4, Adenocarcinoma)

N **128**

827 — DB10L4-1 (dog10, left4, Carcinosarcoma)

200.0

DB10-L4-1: Carcinosarcoma

DB10-L4-2:Adenocarcinoma

DB10L4

Epithelial cells

EMT

Carcinosarcoma (Mixed tumor)

DB15

1038

PIK3CA ——— DB15R5 **(dog15, right4, Simple carcinoma)**

9

N —

248

DB15R4 **(dog15, right5, Solid carcinoma)**

16

649

DB15L4 **(dog15, left4, Carcinosarcoma)**

200.0

DB15L4 DB15R4 DB15R5

Figure 10

Figure S1

DB3L4-2:Carcinosarcoma   Cellularity=0.7; Ploidy=4 Lumina B

DB4-L4:Solid Carcinoma   Cellularity=0.6; Ploidy=4 Lumina A

DB30-R2-R3:Solid carcinoma   Cellularity=0.52; Ploidy=3 Lumina B

Position (Mb)   ■ Tumor copy number

Figure S2

Intra-Tumor Heterogeneity

2 Dogs: DB3, DB10

DB3-R4-1: Carsinosarcoma
DB3-R4-2: Osteosarcoma
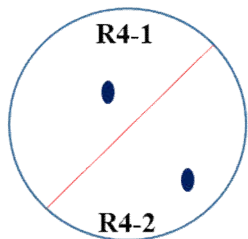
DB3
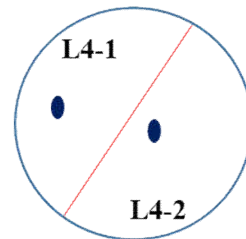
DB3-L4-1:Lipid tumor
DB3-L4-2:Carsinosarcoma
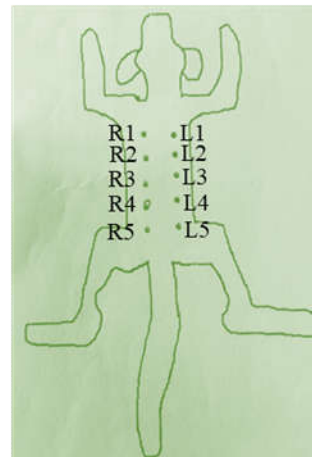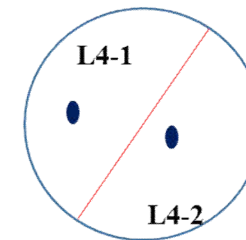
DB10

DB10-L4-1:Adenocarcinoma
DB10-L4-2:Carsinosarcoma

Figure S3

Figure S4
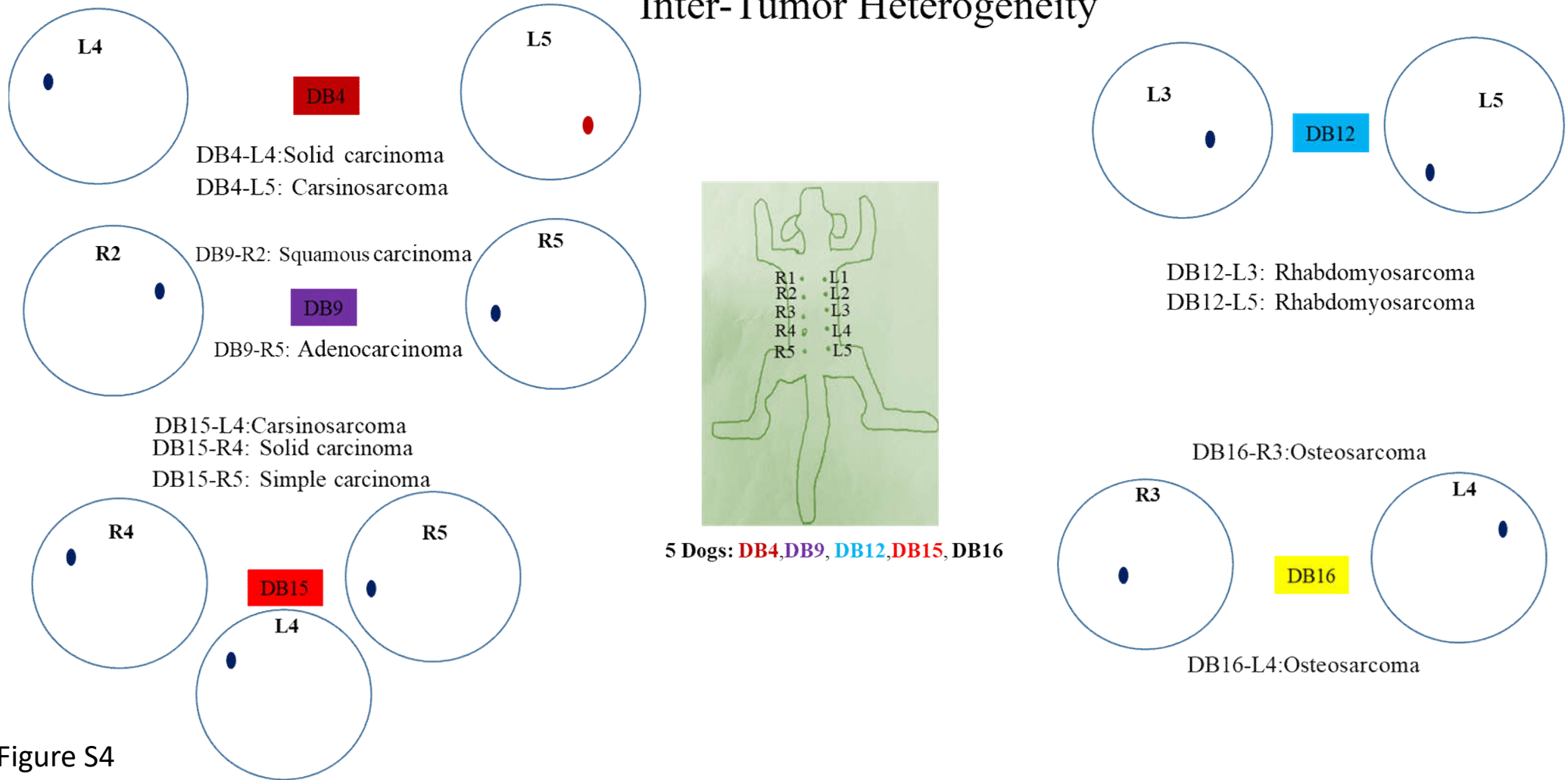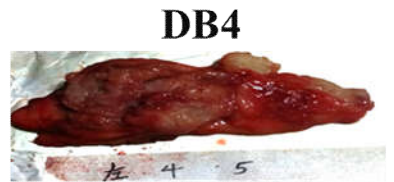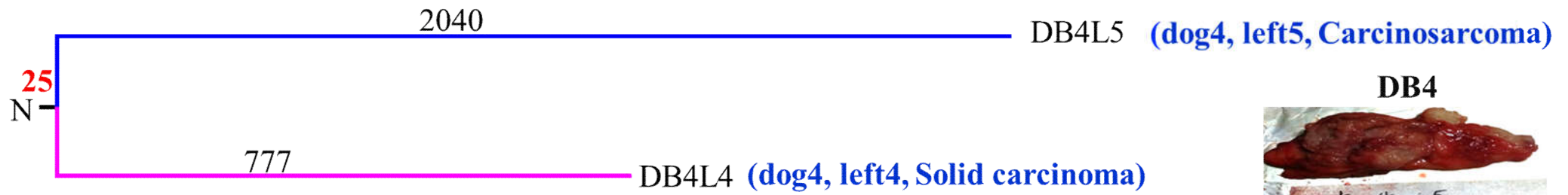
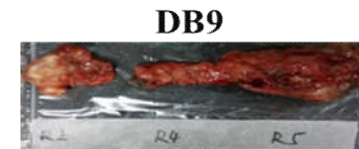# Sampling of multiple tumors

Inter-Tumor Heterogeneity

DB4-L4: Solid carcinoma
DB4-L5: Carsinosarcoma

DB9-R2: Squamous carcinoma

DB9-R5: Adenocarcinoma

DB15-L4: Carsinosarcoma
DB15-R4: Solid carcinoma
DB15-R5: Simple carcinoma

DB12-L3: Rhabdomyosarcoma
DB12-L5: Rhabdomyosarcoma

DB16-R3: Osteosarcoma

DB16-L4: Osteosarcoma

5 Dogs: DB4, DB9, DB12, DB15, DB16

A

DB4

2040 — DB4L5 **(dog4, left5, Carcinosarcoma)**

25

N

DB4

777 — DB4L4 **(dog4, left4, Solid carcinoma)**

200.0

B

DB9

1186 — DB9R5 **(dog9, right5, Squamous carcinoma)**

98

N

959 — DB9R2 **(dog9, right2, Adenocarcinoma)**

DB9

200.0

Figure S5

A

DB12

1406 — DB12L5 (dog12, left5, Rhabdomyosarcoma)

15
N

214 — DB12L3 (dog12, left3, Rhabdomyosarcoma)

DB12L3   DB12L5

200.0

B

DB16

308 — DB16R3 (dog16, right3, Osteosarcoma)

16
N

1095 — DB16L4 (dog16, left4, Osteosarcoma)

200.0

Figure S6