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Next generation sequencing analysis in response to breast cancer therapy: study on canine models as platform for translational cancer research*

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Breast cancer represents one of the most common and deadly malignancy worldwide with concerning outcomes due to late diagnosis and lack of efficient therapies in advanced cases. In these terms, there is an urgent need for new therapeutic alternatives that have to advance rapidly in the clinic in order to timely impede the evolution of aggressive forms. One of the key features in the transition from the preclinical to clinical studies in terms of reliability, safety and efficiency is represented by the animal models used in the first phases of research. In these terms, the mice models with compromised immune system that allow the engraftment of human cells are failing in several aspects including non-spontaneous tumor formation, incomplete evaluation of the immune-tumor interplay and imbalance in the hormonal status reliability. Within the present study we evaluated through NGS the outcome of canine models of breast cancer after treatment with chemotherapy and DDW and also compared the initial mutational background found in canine tumor tissue and cell line with human *in vitro* models of breast cancer.

KEY WORDS: breast cancer / canine animal model / next generation sequencing

Breast cancer (BC) represents the most common form of malignancy in women worldwide associated also with concerning mortality rates. According to Globocan [2018], breast cancer represents 25% from all types of cancer [Bray *et al.* 2018]. The constant increase in mortality is partially caused by late diagnosis and unresponsive pathological phenotypes, where the current approved therapies are failing in eradicating all types of malignant mammary cells. In this sense, there is an urgent need of new therapeutic strategies that can be rapidly validated in preclinical context in order to benefit the clinical sector. A significant part of the current studies are using imunocompromised mice models that allow the artificial engraftment of human breast cancer (HBC) tissue or cells as *in vivo* validation of the proposed therapy [Huminiecki *et al.* 2017, Huminiecki and Horbańczuk 2018, Wang *et al*. 2018, Yeung *et al*. 2018, Yeung *et al*. 2019]. This approach has a number of disadvantages, including lack of a complete immune environment and spontaneous tumor installation, as also improper hormonal balance that can mimic the patient scenario. In order to fill the gap between preclinical and clinical studies, more reliable models of study have to be established by taking in consideration the anatomical and physiological resemblance, integrity of immune system, hormonal profile, cancer installation and development, therapeutic response and others. In this sense, researchers are proposing the canine model of breast cancer as a reliable platform of study due to the resemblance between the human and canine proposed pathology.

Along with the completion of the Human Genome Project together with the sequencing of genomes from other species, the field of comparative oncology based on physiological and also genetic resembling factors has greatly advanced [Raduly *et al*. 2018].

Canine breast cancer (CBC) mirrors the human one through the pathway of installation: spontaneous (and not artificially induced) that is also correlated with

global changes in the physiology of the organism, age of onset, hormonal background, course of the disease including the disease outcome, evolution of tumor in size, invasion at secondary spots and also lymphatic system and finally, proper interplay between the immune system and the malignant mass. Additionally, it has been demonstrated that the molecular characteristics between human and canine breast cancer are reliable in terms of mutations [Visan *et al*. 2016, Abdelmegeed and Mohammed 2018]. Specifically, BRCA1, BRCA2 and RAD51 are genes with role in DNA damage repair and are frequently lost in breast cancer, loss that is correlated also with an aggressive disease. Their role in malignant development has been attributed in the context of both canine and human breast cancer [Raduly *et al.* 2018]. Ki-67 and PCNA markers are proteins with role in cellular proliferation and DNA replication respectively; the presence of the molecules in canine and human breast cancer tissue samples were associated with poor prognosis and metastasis dissemination [Carvalho *et al.* 2016]. Mutations in the TP53 gene were observed in both species and overexpression of the mutant form was also associated with shorter survival time [Queiroga *et al*. 2011, Dolka *et al*. 2016]. Moreover, the similarities between canine and human TP53 exon organization and translated protein are promoting the sequence as a relevant therapeutic target for translational studies [Raduly *et al*. 2018]. A high degree of synonymy is observed also between the human and canine tumor associated antigens ErbB-1 and ErbB-2, with similar roles in disease development and progression [Singer *et al.* 2012, Burrai *et al.* 2015]. Meanwhile, there is currently a large study ongoing entitled The Dog Genome Project that investigates the connection between the canine genome and specific traits with further potential of data extrapolation for human diseases [Plassais *et al*. 2019].

Several studies demonstrated high histological similarities between HBC and CBC and the same histological grade classification in both species. The most frequent histological types in CBC are represents by simple carcinomas, complex carcinomas, adenocarcinomas and mixed tumors [Misdorp 1999, Weigelt *et al*. 2008, Malhotra *et al.* 2010, Im *et al.* 2014, Santos *et al*. 2015].

Breast papillary carcinoma represents 0.5% of diagnosed cases of mammary tumors in human. Both subtypes, invasive and *in situ* papillary carcinoma, show a higher prevalence in older postmenopausal women. Histological characteristics of this tumor types include cellular proliferations surrounding fibrovascular cores, with or without invasion [Pal *et al*. 2010]. In canines, papillary carcinoma represents one of the subtypes of malignant epithelial mammary tumors [Salas *et al*. 2015]. Inflammatory breast cancer in humans is an aggressive form of cancer with a poor outcome and characterized by inflammation, uncontrolled spreading, diffuse edema and erythema and a short overall survival [Anderson *et al*. 2005]. Inflammatory mammary carcinoma in canines, represent 7.6% of the mammary tumors, also associated with inflammatory reaction, erythema and generalized edema [de Toledo-Piza *et al*. 2009].

With a large range of applications, next generation sequencing, from whole genome to exome sequencing, targeted re-sequencing and functional studies, bring important results for development of personalized medicine [Harismendy *et al.* 2009,

Petric *et al*. 2015]. First of all the main purpose of these sequencing studies is to evaluate risk or prognosis in cancer, but, in the last years, with NGS platforms and reagents becoming more affordable, sequencing techniques are more frequently used in clinical applications for treatment decisions [Natrajan and Reis-Filho 2011].

In our study, we evaluated the mutational status of canine patients with mammary cancer before and after chemotherapy and deuterium-depleted water (DDW) intake. The aim of the study was to evaluate the overall mutational profile in untreated and treated canine patients and also highlight the resemblance with the human models of breast cancer. Depleted-deuterium water used in our study contains 25±5 ppm deuterium and was provided by MecroSystem SRL. Ion Torrent sequencing and the in-house bioinformatics algorithm were used for mutation analysis of tumor tissue and blood samples (before and after one-month treatment and DDW intake).

Globally, the study comprehensively presents the outcome of canine breast cancer patients with the intention of proving insights into the reliability of canine models for breast cancer studies.

Cases description

11 years Pekingese (EBC-7) and 12 years Dachshund (EBC-11) female canines with a tumor mass on mammary gland were recommended to surgery for excision of the tumor tissue. Tissue samples were stained for further immunohistochemistry and immunocytochemistry evaluation. The histological sections in EBC-7 presents a papillary carcinoma and in EBC-11 an inflammatory carcinoma (carcinomatous mastitis) pattern. Another 16 canine patients with different types of mammary tumors were also additionally included in our studies (Fig. 1 and Supplementary Tab. 1). All samples were collected from the canine patients after the owners signed the informed consent regarding the processing and the usage of data in experimental studies.

Material and methods

Patients and amples

For the study, we used fresh frozen tissue (tumor and normal) samples from 18 canine mammary tumors and whole blood samples from the two treated patients obtained before surgery and after a month of treatment with chemotherapy and DDW. Table 1 present the treatment followed by the two canine patients after tumor resection.

Cell lines

Two human breast cancer cell lines obtained from ATCC (HS578T and MCF-7) and two canine mammary cancer cell lines (CMT-U27 and P114) were used in the study. The CMT-U27 cell line was donated by Prof. Eva Hellmen (University of Agricultural Sciences, Swedish) and the P114 cell line by Prof. Gerard Rutteman (University of Utrecht - Netherlands). Every cell line was maintained in culture medium at 37˚C and 5%CO_2 incubators. For the HS578T cell line it was used DMEM medium

Table 1. Canine patients and treatment plan

Patient code	Breed	Age	Weight	Diagnosis	Treatment
EBC-7	Pekingese	11 years	6 kg	Papillary carcinoma	Endoxan (Cyclophosphamide), Doxorubicin and DDW
EBC-11	Dachshund	12 years	4 kg	Inflammatory mammary carcinoma (Carcinomatous mastitis)	Endoxan (Cyclophosphamide), and DDW

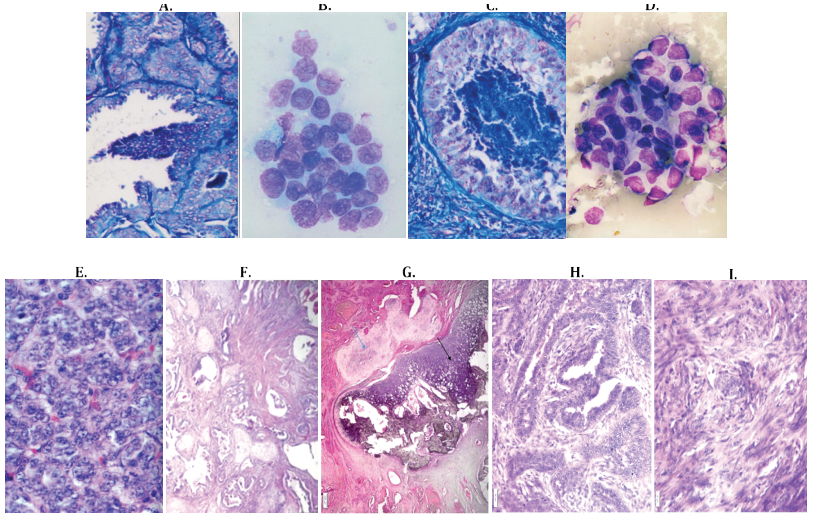

Fig. 1. Immunohistochemistry and immunocytochemistry images for tumor type classification. A/B. EBC-7 presents a papillary carcinoma histological type. C/D. EBC-11 presents an inflammatory carcinoma (carcinomatous mastitis) subtype. E. Simple carcinoma, F. Complex carcinoma, G. Mixed carcinoma, H. Papillary carcinoma, I. Carcinosarcoma.

with 4.5g/L G-glucose (Gibco) supplemented with 10% bovine serum (FBS), 2mM Glutamine, non-essential aminoacids (NEEA), 10% Peniciline/Streptomicine (Gibco) and insulin. MCF-7 cell line was maintained in MEM medium (Gibco) suplemented with 10% FBS, 2mM Glutamin and 10% Peniciline/Streptomicine (Gibco). For the CMT-U27 canine cell line it was used DMEM culture medium with 1g/L G-glucose supplemented with 10% of FBS and 10% Peniciline/Streptomicine (Gibco) and for the P114 cell line the used medium was RPMI 1640 1X (Gibco) supplemented with 10% FBS and 10% Peniciline/Streptomicine (Gibco).

DNA extraction

DNA extraction was performed using the Purelink Genomic Mini Kit (Invitrogen) according to the manufacturer's protocol from tumor tissue and healthy adjacent tissue and whole blood samples. Quantitative and qualitative control of DNA was checked by Nanodrop-1000 spectrophotometer (Thermo Scientific).

Ion Torrent PGM library preparation and sequencing

The DNA concentration used for library preparation was 20 ng/µl. An Ion Torrent adapter-ligated library was constructed with the Ion AmpliSeq Library Kit 2.0 (ThermoFicher Scientific) and a custom dog panel containing the primers equivalent to the human Ion AmpliSeq Cancer Panel Pool (ThermoFicher Scientific) according to manufacturer's protocol. The custom canine cancer panel primers were design using the algorithm developed by Petric *et al*. [2015] The amplicon libraries were barcoded using the Ion Express Barcode kit (ThermoFicher Scientific). The barcoded libraries were purified using the Ampure Reagent (Beckman Coulter) and quantified using the fluorometer Qubit 2.0 and the Qubit HS DNA kit. Sample emulsion PCR and enrichment were performed using the Ion PGM 200 Template Kit (ThermoFicher Scientific), according to the manufacturer's protocol. ISP enrichment was confirmed with The Qubit 2.0 fluorometer (ThermoFicher Scientific). Ion 316 chips were used to sequence four barcoded samples on the Ion Torrent PGM, and an Ion PGM 200 Sequencing Kit (ThermoFicher Scientific) was used for sequencing according to the recommended protocol.

Data analysis

The primary analysis was done on the Ion Torrent server using the Variant Caller software, where the secondary one was performed with an in-house algorithm. The sequencing data were aligning to the DogFam 3 genome.

Results and discussion

Analysis of DNA profile in canine mammary tumor tissue and canine and human cell lines. A comparative study

According to the data analysis obtained by NGS from the canine mammary cancer tissue samples we identified 175 different mutations within 29 genes (Fig. 2A). In the canine cell lines we found 45 mutations in 16 different genes (Fig. 2B), where in the human cell lines there were 17 mutations in 12 genes (Fig. 2C). Between canine tumor tissue and canine cell lines, the only mutated gene that was not found in the tissue samples is represented by SRC, gene that was previously proposed as therapeutic target in breast cancer [Finn 2008]. Even so, the tissue samples have a more heterogeneous mutational profile compared to the *in vitro* models, including alterations in the CDH1 gene that is responsible for the expression of E-cadherin and preservation of adherence between cells with involvement in the process of metastasis in non-functional or under-expressed forms, [Chiorean *et al*. 2013, Corso *et al*. 2014, Hu *et al*. 2016, Gulei *et al*. 2018]. Frequent altered genes in human breast cancer cell lines include PIK3CA and TP53, both also encountered in mutated forms in the canine malignant tissue; both genes are considered important therapeutic targets in breast cancer [Braicu *et al*. 2013, Braicu *et al.* 2015, Shimoi *et al.* 2018]. Within the total of 12 mutated genes, 9

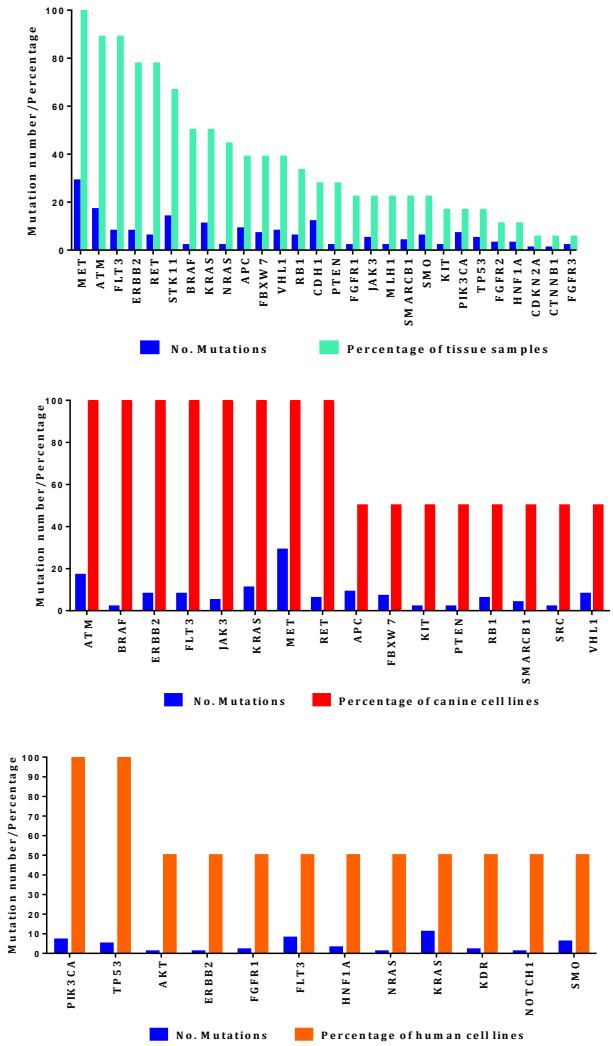

Fig. 2. Number of mutations, percentage of patients and number of cell lines which presents mutation in genes. A. Numbers of mutations in the canine tissue samples. B. Number of mutations in canine cell lines. C. Number of mutations in human cell lines.

are common between canine tumor tissue and human breast cancer cell line and 3 are encountered only in the human specimen: AKT, KDR and NOTCH1.

In canine patients and cell lines the majority of the identified mutations were exonic ones and their localization is presented in Figure 3. In the HS578T cell lines we identified 5 intronic mutations and 2 exonic ones. The MCF-7 cell lines presents 7 exonic mutations and 1 intronic mutation.

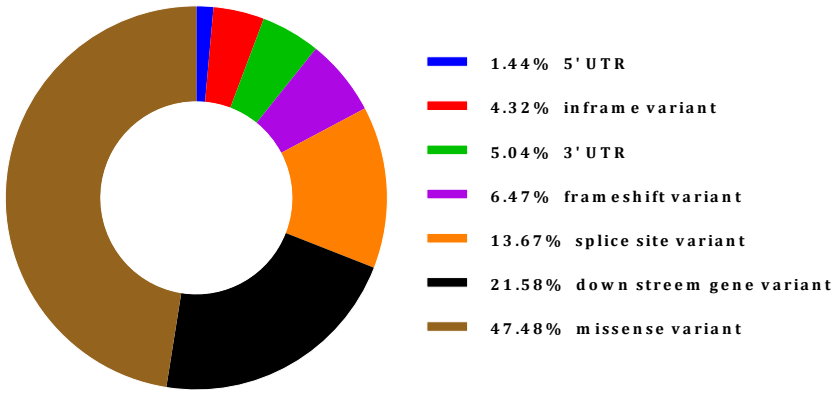

Fig. 3. The percentage of the mutations identified in the canine patients and cell lines.

Evaluation of the mutational profile in canine breast cancer patients before and after chemotherapy and DDW intake

To observe the dynamics of the circulating tumor cells mutations before and after standard treatment supplemented with adjuvant DDW, we analyzed using NGS the whole blood samples before and after 1 month of treatment.

Cancer panel sequencing and bioinformatics analysis results performed in whole blood samples before and after one-month treatment with chemotherapy and DDW revealed a number of eleven mutations common between EBC-7 without and with one month treatment, encountered in five different genes: EGFR, ERBB2, FGFR2 and STK11. For the case of EBC-11, three mutations were common between the two types of samples within the ERBB2, JAK3 and PTEN genes. Mutations in the coding regions are represented by synonymous variant, frameshift variant, missense variant and stop gained mutation types. The percentages of these mutations are presented in Figure 4. The complete and unprocessed data for the whole blood samples (before and after one month treatment) after sequencing is presented in Supplementary Table 2 for patient EBC-7-1 – before treatment, Supplementary Table 3 for patient EBC-7-2 – after one month treatment, Supplementary Table 4 for patient EBC-11-1 – before treatment, Supplementary Table 5 for patient EBC-11-2 – after one month treatment (code 1 is for samples before treatment and code 2 is for samples collected after one month treatment).

Common and different mutations between tumors and whole blood (before and after treatment) in EBC-7

The mutational profile and its dynamics can become reliable biomarkers for assessment of therapy response and decision upon further oncological management. Therefore, we comparatively analyzed the mutational profile in tumor tissue versus whole blood – before and after 1 month of therapy with Endoxan, Doxorubicin and DDW- in the canine patient with breast cancer – EBC-7.

When comparing all three scenarios: tumor tissue (EBC-7-TUMOR), blood

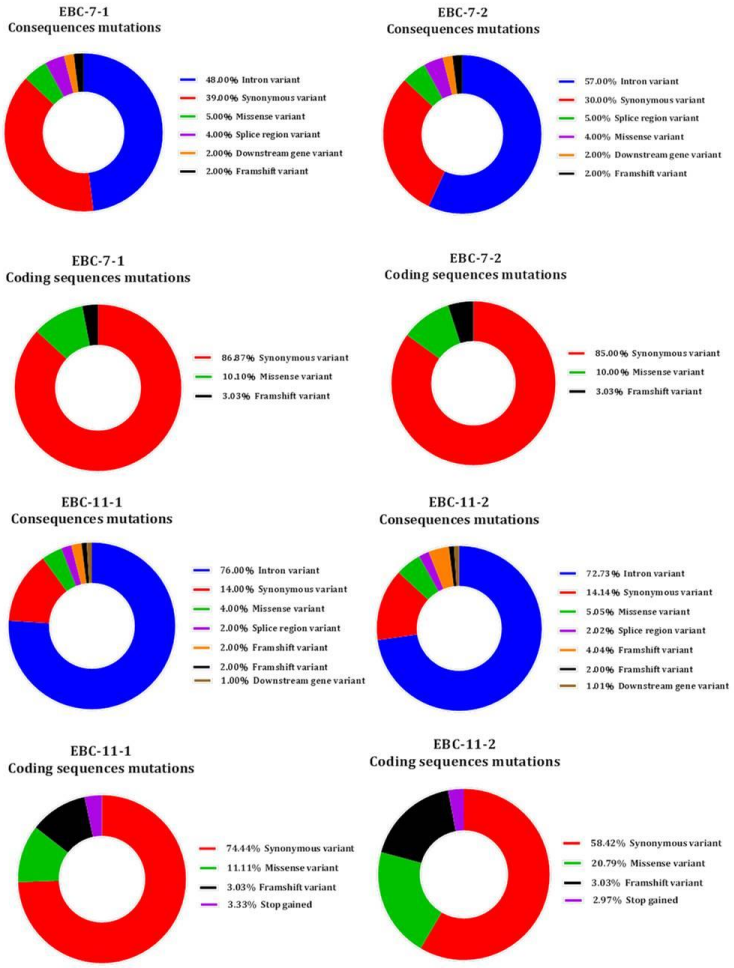

Fig. 4. The percentage of the main mutation types: coding consequence and consequence found in the two cases of canine patients treated. In the right column are the results before treatment and the left column after the treatment.

sample before surgery (EBC-7-1) and blood samples after one month of therapy (EBC-7-2), we found a number of five common mutations in ERBB2 (one mutation), ATM (three mutations) and JAK3 genes (one mutation). This mutational profile can become a reliable diagnostic tool for breast cancer that can be assessed only from blood samples considering the overlapping with the tumor tissue and also preservation after one month treatment; moreover, the constant mutational spectrum within the blood also after treatment can suggest that these mutations are widely encountered in circulating tumor cells and are reliable for diagnostic purposes. Specific mutations

for these genes are presented in Table 2.

Between whole blood samples before and after therapy, we highlighted the dynamics in 5 genes: KDR, MET, PIK3CA, PTPN11 and RET with 7 mutations that are found only in blood samples before therapy and not encountered after therapy (Fig. 5). Moreover, after therapy we observed 3 new mutations in 3 different genes: KDR, PIK3CA and PTEN that were not present before therapy (Fig. 5). These changes in the mutational profile can be considered for evaluation of the therapeutic response once validated within larger cohorts.

Tumor tissue presents eight distinctive mutations of the following genes: KRAS, MET, FLT3, and ATM. Between EBC-7-1 and EBC-7-2, results show eleven mutations in the genes: STK11, VHL, EGFR, PIK3CA and FGFR2.

Table 2. Common mutations found in samples EBC-7-TUMOR, EBC-7-1 and EBC-7-2 before and after treatment

Sample	Location	Mutation type	Symbol	Coding	Protein	Existing variation
EBC-7-T	20:8206760-8206770	3_prime_UTR_variant	ERBB2	c.*86_*95delTCACTTATTC	n/a	rs851634661
	5:24226831-24226831	missense_variant	ATM	c.5273T>C	p.Met1758Thr	rs24154550
EBC-7-1	5:24226831-24226831	missense_variant	ATM	c.5267T>C	p.Met1756Thr	rs24154550
EBC-7-2	5:24205430-24205430	splice_region_variant, intron_variant	ATM	c.7311-3A>C	n/a	rs24264483
	20:45060036-45060036	splice_region_variant, intron_variant	JAK3	c.1702-5C>T	n/a	rs850620825

Table 3. Common mutation types found in samples EBC-11-TUMOR, EBC-11-1 and EBC-11-2 before and after treatment

Sample	Location	Mutation type	Symbol	Coding	Protein	Existing variation
EBC-11-T	20:8206783-8206783	3_prime_UTR_variant	ERBB2	c.*73T>C	n/a	rs851541674
	5:24226831-24226831	missense_variant	ATM	c.5273T>C	p.Met1758Thr	rs24154550
EBC-11-1	5:24226831-24226831	missense_variant	ATM	c.5267T>C	p.Met1756Thr	rs24154550
EBC-11-2	5:24205430-24205430	splice_region_variant, intron_variant	ATM	c.7311-3A>C	n/a	rs24264483

Common and different mutations between tumors and whole blood (before and after treatment) in EBC-11

In the second case, EBC-11 we found four common mutations between the tumor tissue and the whole blood samples before and after one month therapy with Endoxan and DDW. These mutations appear in two genes, ATM and ERBB2 (Tab. 3). Importantly, the three mutations found in the ATM gene, besides the fact that are common between tissue and blood samples, are also common between the two canine patients. This fact underlines that these mutations are consistent between breast cancer cases (despite different histopathological diagnosis)

Table 4. The most important biological processes for the common identified genes - ATM, ERBB2 and JAK3

Function	FDR	Genes in network	Genes in genome
DNA integrity	9.842E-05	6	138
DNA damage	9.842E-05	6	132
Protein autophosphorylation	9.985E-05	6	148
Cell cycle	0.000401	6	209
Epidermal growth factor receptor signaling pathway	0.0068538	5	213
ERBB signaling pathway	0.0068538	5	216
Positive regulation of MAPK cascade	0.0113437	5	251
Regulation of mitotic cell cycle	0.0167789	5	290

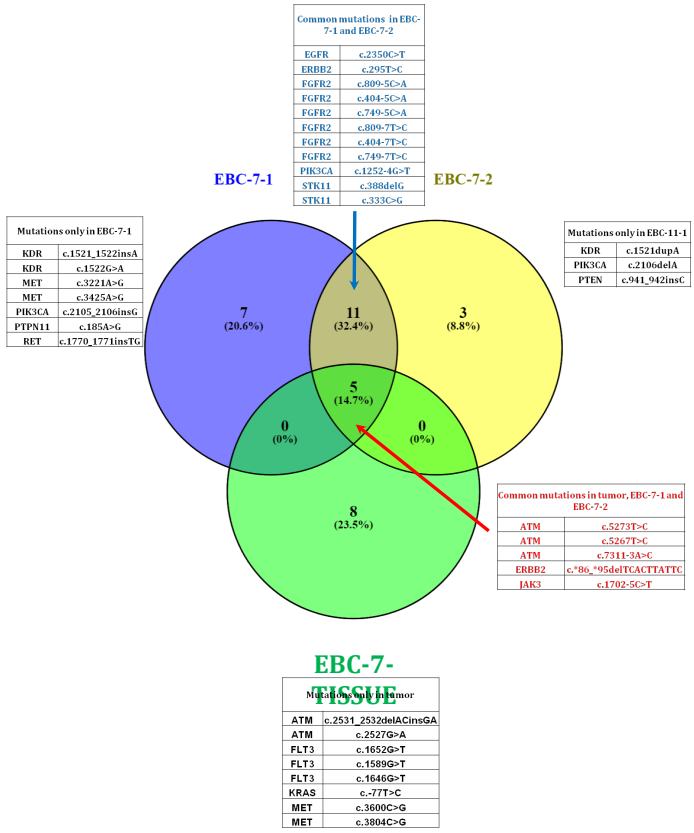

Fig. 5. Venn diagramm for common and specific mutations for EBC-7 before and after treatment with Endoxan, Doxorubicin and DDW.

and, after further validations on larger cohorts, can be used as a marker of minimally invasive disease diagnostic. For the case of ERBB2, although the gene is the same, the mutations are different between the two canine patients.

The mammary tumor tissue presents a number of 17 mutations in nine genes. The genes affected are ATM, FBXW7, FLT3, KRAS, MET and STK11. Only in EBC-11-1 (before treatment), we found a number of two mutations of the MET gene, that were no longer found in the treated samples. Three common mutations with EBC-11-2 (after treatment) were highlighted, mutation that affect ERBB2, JAK3, and PTEN genes. The abolishment of the mutations in the MET gene in the blood samples after one month treatment can be possibly associated with therapy response and used as a marker after long term follow up.

The mutations found only in EBC-11-2 sample obtained after treatment with Endoxan and DDW for one month were in a number of six for the FGFR3, PTEN and RET genes. The specific mutations can be found in Figure 6.

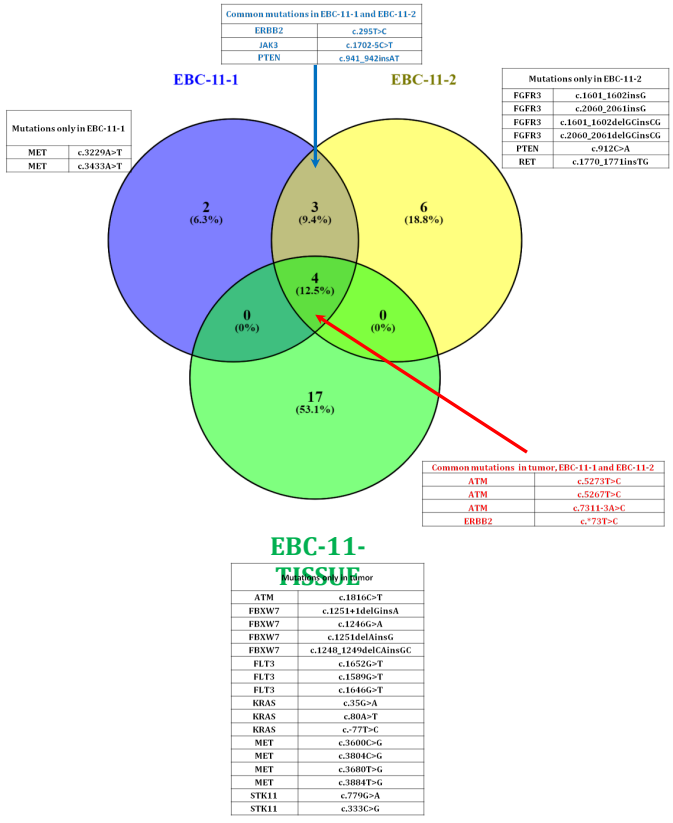

Fig. 6. Venn diagramm for common and specific mutations for EBC-11 before and after treatment with Endoxan and DDW.

Signaling pathways associated with the common mutations between tumor and whole blood samples before and after therapy

In a signaling pathway analysis of the ATM, ERBB2 and JAK3 mutated genes (found in our study as present in tumor and whole blood samples before and after chemotherapy and DDW for patient EBC-7) performed with GeneMANIA (www.genemania.org) we found that these mutations are involved in important processes like maintenance of DNA integrity, DNA damage protein autophosphorylation, cell cycle, ERBB signaling pathways and MAPK cascades. Specific data are presented in Figure 7 and Table 4.

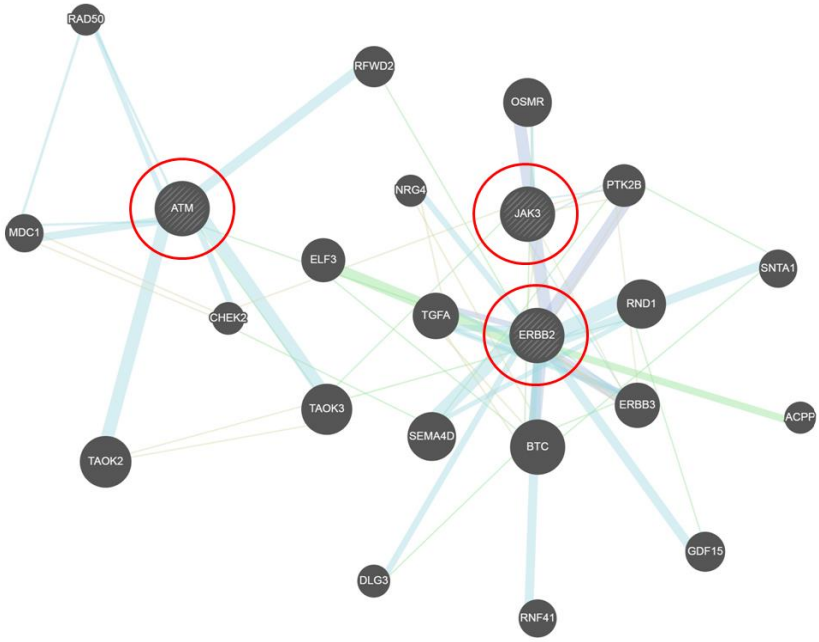

Fig. 7. Gene network analysis for the common identified genes in all sample types (tumor tissue and whole blood samples before and after chemotherapy and DDW intake) from patient EBC-7 – focus on ATM, ERBB2 and JAK3.

In the current study we evaluated the dynamics of the mutational profile in two canine mammary cancer patients followed by the same analysis for whole blood samples before and after one month of chemotherapy and adjuvant DDW with the purpose of identifying specific biomarkers and ease the gap between the canine model and human breast cancer.

Our results revealed that canine mammary tumor tissues present a specific molecular pattern with a high number of mutations in genes like: MET, ATM, STK11, JAK3, CDH1, KRAS, APC, ERBB2, FBXW7, PIK3CA and FLT3. In a comparative study between the canine cell lines, we identified common mutations in ATM, BRAF,

ERBB2, FLT3, KRAS, MET, RET and JAK3 genes. Also in canine cell lines we found mutations which appear in the tumor tissue samples to. Comparing the mutational profile of the canine samples and human samples we found some similarities regarding the mutations of FLT3 and KRAS genes.

Our results show that the ATM, ERBB2 and JAK3 genes presents the same mutations in the tumor and blood samples (also before and after the treatment), fact that suggests that ATM, ERBB2 and JAK3 DNA modifications are possible germline mutation in canine patients. Human breast cancer studies associate ATM related mutations with an increased risk for hereditary breast cancer development [Tan *et al*. 2008, van der Groep *et al*. 2011]. *ATM* encodes checkpoint kinases that play an important role in DNA repair processes. A heterozygous mutation of *ATM* carriers has an increased risk of breast cancer development [Feng *et al*. 2015]. Studies demonstrated that targeted therapy with ATM and DNA-PKs inhibitors in combination with Cisplatin induces DNA double strand breaks accumulation, cell cycle arrest and apoptosis in BRCA1 mutated breast cancer in women [Albarakati *et al*. 2015]. In canine breast cancer patients was observed that ATM gene and protein downregulation is involved in canine mammary gland tumorigenesis [Raposo-Ferreira *et al.* 2016].

The ERBB pathway has an important role in the breast cancer development, especially in patients with high expression of ERBB2 [Monteiro Ide *et al.* 2015]. ERBB2, beside to ERBB1, ERBB3, and ERBB4 make part of the human epidermal growth factor receptor family. Overexpression of ERBB2 was reported in 20-30% of breast cancers and a high percentage of these patients presents mutation of the ERBB2 [Bose *et al.* 2013]. The authors showed that the ERBB2 somatic mutations in breast cancer patients are involved in tumorigenesis [Bose *et al*. 2013]. Immunohistochemistry of canine tumors showed an overexpression of ERBB2 in 4 out of 10 patients. Also a 92% amino acid homology of ERBB2 between canine and human molecules was reported. A modeling analysis of the Trastuzumab binding site revealed a 100% identity in human and canine samples. Over-expression of ERBB2 was described in canine tumor cells including mammary carcinomas, but the specific expression pattern remain to be resolved [Peruzzi *et al.* 2010].

Studies revealed that JAK1 and JAK3 mutations are present in common human cancers, like breast and colon cancer with a higher frequency of the JAK1 mutations in leukemia patients. Several studies reported that phosphorylation of the kinases like JAK1, JAK2 and JAK3 may have a role in development of breast and prostate cancer [Babon, Lucet *et al*. 2014]. In canine patients with B-cell lymphoma it was described a common signaling pathway of the JAK/STAT with humans as a potential drug target [Mudaliar *et al*. 2013]. In canine patients with mammary tumor the role of the JAK3 genes remain to be elucidated.

Further analyses of different mutations type between tumor tissue and blood samples after treatment are necessary to identify the specific mutations, which remain after chemotherapy. Also, the study has to be extended on a larger cohort followed by a longer period of time. In addition, an extensive comparative analysis between

human and canines species is essential for a better extrapolation of the results obtained in canine models and possible implementation as reliable platforms of study.

Conclusions

nGS technology used in clinical studies represents an important tool for developing prognostic biomarkers and for adopting the optimal therapeutic strategy [Paolillo *et al.* 2017]. Our data represent a pilot study to optimize NGS sequencing method for canine samples using a specific canine cancer panel and find a better bioinformatics analysis for comparative studies in canine and human breast cancer. For comprehensive results, a larger number of patients have to be analyzed and also extended to a longer period of follow up. However, using spontaneous canine tumor models for breast cancer research represents a suitable experimental tool for new treatment strategy and personalized medicine development.

Competing interest

The authors have no competing interests to declare

Author contribution. *LR, IBN and IM designed the study and interpreted the data into the final context. LR prepared the samples for sequencing and LAP conducted the necessary steps for the NGS assay, while RC analyzed the data through the bioinformatics pipeline. OS and LB were responsible for the sample collection, preparation and short-term storage, where EB administrated the treatment of the patients and integrated the clinical data within the context of the study. NM provided valuable input regarding the possible translation of the canine genes within the sector of human oncology, being also involved in the writing of the introductive and discussion parts of the article. DG and CB helped with data interpretation and graphical representation, being also involved in the writing of the results sections. The final form of the article was revised and approved by all contributing authors.*

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