รายงานวิจัยฉบับสมบูรณ์

โครงการการวิเคราะห์โปรตีโอมิกของน้ำลายและซีรั่มในโรคมะเร็งช่องปากเมลาโนมา ในสุนัขเพื่อติดตามการตอบสนองต่อการรักษาและการวินิจฉัยระยะแรก ก่อนการเกิดโรคซ้ำและ/หรือการแพร่กระจาย

โดย รศ.สพ.ญ.ดร.กรรณาภรณ์ สุริยผล

พฤศจิกายน 2562

สัญญาเลขที่ RSA5980053

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการนี้ได้รับอนุมัติเงินทุนพัฒนานักวิจัยประจำปีงบประมาณ 2559 ของสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ผู้วิจัยขอขอบคุณ น.สพ.เศกรินทร์ พลอยเพ็ชร์ รศ.น.สพ.ดร.อนุเทพ รังสีพิพัฒน์ รศ.น.สพ.ชนินทร์ กัลล์ประวิทธ์ ดร.สิทธิรักษ์ รอยตระกูล คุณจันทิมา จเรสิทธิกุลชัย คุณนฤมล เผ่านักรบ คุณสุชีวิน กรอบทอง น.สพ.ภัทรกฤช ธีวสุตระกูล สพ.ญ.วีจาริน ปภุสสโร สพ.ญ.วรพรรณ ธาดาดลทิพย์ และคุณสุพรรณษา วันวัฒนกุล รวมถึงเจ้าของสุนัขทุกท่าน ที่ให้ความช่วยเหลือในงานวิจัย

ผู้วิจัย

พฤศจิกายน 2562

บทคัดย่อ

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โรคมะเร็งช่องปากเมลาโนมาเป็นโรคที่พบได้บ่อยในสุนัข เป็นมะเร็งที่มีความรุนแรง แพร่กระจายได้รวดเร็วและมักตรวจพบในระยะท้าย การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาเปรียบเทียบโปรตีโอมในน้ำลายและซีรั่มที่เกี่ยวข้องกับโรคมะเร็งช่องปากเมลาโนมาในระยะคลินิกระยะแรก (Early-stage oral melanoma, EOM) ระยะคลินิกระยะท้าย (Late-stage oral melanoma, LOM) LOM ที่ได้รับการรักษาด้วยการผ่าตัดและเคมีบำบัด โรคมะเร็งช่องปากสแควมัสเซลล์คาร์ซิโนมา (Oral squamous cell carcinoma, OSCC) โรคเนื้องอกช่องปากชนิดไม่ร้าย (Benign tumors, BN) โรคปริทนต์เรื้อรัง (Chronic periodontitis, P) และสุนัขปกติ (Control, C) โดยใช้เทคนิค Matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF MS), Liquid chromatography-tandem MS (LC-MS/MS) และIn-gel digestion coupled with mass spectrometry (GeLC-MS/MS) ผลการศึกษาในน้ำลายโดยใช้เทคนิค MALDI-TOF MS ควบคู่กับ LC-MS/MS พบ peptide mass fingerprint (PMF) ที่เป็นเอกลักษณ์เฉพาะกลุ่มและพบการเกาะกลุ่มของโปรตีน (clustering) เฉพาะกลุ่มใน EOM LOM OSCC และ BN ทั้งนี้พบการเกาะกลุ่มของโปรตีนใกล้กันใน LOM และ OSCC นอกจากนี้พบการเกาะกลุ่มของโปรตีนในกลุ่ม P และ C (CP group) จากการใช้ Western blot ในการยืนยันผลโปรตีโอมิกส์ พบการแสดงออกของ Sentrin-specific protease 7 (SENP7) ใน OSCC การแสดงออกของ Toll-like receptor 4 (TLR4) ใน LOM และ OSCC เมื่อเทียบกับกลุ่ม CP และการแสดงออกของ Nuclear factor kappa B (NF-**K**B) ซึ่งเป็น TLR4 partner ใน OSCC เมื่อเทียบกับ CP, BN and EOM และใน LOM เมื่อเทียบกับ EOM ทำการยืนยันลำดับกรดอะมิโนของแบนโปรตีนจาก Western blot ด้วย LC-MS/MS ผลการศึกษาในน้ำลายโดยใช้เทคนิค GeLC-MS/MS และยืนยันผลด้วย Western blot พบการแสดงออกของ Tyrosine phosphatase non-receptor type 5 (PTPN5) มากในกลุ่มเนื้องอกทุกกลุ่มเมื่อเทียบกับ CP และพบการแสดงออกมากใน LOM และ OSCC เมื่อเทียบกับ BN และ EOM นอกจากนี้ยังพบการแสดงออกของ Tumor protein p53 (p53) ซึ่งพบในวิถีปฏิสัมพันธ์ระหว่าง

PTPN5 และยาเคมีบำบัด มากในกลุ่มเนื้องอกทุกกลุ่มเมื่อเทียบกับ CP ผลการศึกษาโปรตีนในน้ำลายในกลุ่ม LOM ก่อนการผ่าตัด (Pre-surgery, PreS) หลังการผ่าตัด (Post-surgery, PostS) หลังการทำเคมีบำบัดด้วยยา Carboplatin 1-7 ครั้ง (AT1-AT7) โดยใช้เทคนิค LC-MS/MS และยืนยันผลด้วย Western blot พบว่าในกลุ่มที่มีอัตราการอยู่รอดสั้นกว่า 1 ปี (Short-term survival, STS) สัดส่วนของ Free ubiquitin D (fUBD) ต่อ Conjugated ubiquitin D (cUBD) ใน PreS มีมากกว่า AT2, AT4 and AT5 และพบว่าสัดส่วนของ fUBD ต่อ cUBD ใน PreS ในกลุ่ม STS มีมากกว่าสัดส่วนใน PreS ในกลุ่มที่มีอัตราการอยู่รอดยาวกว่า 1 ปี (Long-term survival, LTS) นอกจากนี้ยังพบการแสดงออกของ fUBD ใน PreS ในกลุ่ม STS มากกว่าใน AT2 สำหรับการศึกษาโปรตีโอมในซีรั่มโดยใช้เทคนิค MALDI-TOF MS ควบคู่กับ LC-MS/MS พบ peptide mass fingerprint (PMF) ที่เป็นเอกลักษณ์เฉพาะกลุ่มในกลุ่มรวม LOM-OSCC และ กลุ่ม P และพบการเกาะกลุ่มของโปรตีน (clustering) เฉพาะกลุ่มใน EOM LOM OSCC BN P และ C จากการใช้ Western blot ยืนยันผลไม่พบความแตกต่างอย่างมีนัยสำคัญของ Immunoglobulin superfamily member 10 (IgSF10) ผลการศึกษาโดยใช้เทคนิค GeLC-MS/MS และยืนยันผลด้วย Western blot ไม่พบความแตกต่างอย่างมีนัยสำคัญของ Tumor necrosis factor receptor associated factor 3 interacting protein 1 (TRAF3IP1) สรุปในการศึกษาครั้งนี้ พบ PMF ที่มีเอกลักษณ์ในน้ำลายกลุ่ม EOM LOM OSCC BN และ CP และในซีรั่มกลุ่มรวม LOM-OSCC และ กลุ่ม P ในตัวอย่างน้ำลายพบการเกาะกลุ่มโปรตีนรวมกันใน P และ C และพบความใกล้ชิดของกลุ่ม LOM และ OSCC แสดงถึงความเป็นไปได้ในการเฝ้าระวังโรคโดยใช้ PMF และการเกาะกลุ่มโปรตีน นอกจากนี้ยังพบความเป็นไปได้ของโปรตีนที่อาจใช้เป็นตัวบ่งชี้ทางชีวภาพของโรคได้แก่ SENP7, TLR4, NF-**K**B, PTPN5, p53 ส่วนสัดส่วน fUBD ต่อ cUBD อาจใช้แสดงการพยากรณ์อัตราการรอดชีวิตในสัตว์ป่วยเมื่อทำการรักษาด้วยเคมีบำบัด carboplatin งานในอนาคตควรทำการศึกษาการแสดงออกของโปรตีนดังกล่าวและโปรตีนที่เกี่ยวข้องในกลุ่มประชากรที่มากขึ้นเพื่อเก็บข้อมูลยืนยันผลดังกล่าว และศึกษาความเป็นไปได้ในการใช้สัดส่วน fUBD ต่อ cUBD ในการหาแนวทางการทำเคมีบำบัดที่เหมาะสมกับสัตว์แต่ละตัวตามแนวทางของหลักการแพทย์แม่นยำ (Precision medicine)

คำสำคัญ: โรคมะเร็งช่องปากเมลาโนมา โรคเนื้องอกช่องปาก โปรตีโอมิกส์ น้ำลาย ซีรั่ม สุนัข ตัวบ่งชี้ทางชีวภาพ

Abstract

Project Code : RSA5980053

Project Title : Salivary and serum proteomic analyses to monitor treatment response and early detect recurrence and/or metastasis in canine oral melanoma

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Project Period : 3 years 5 months (16 June 2016 – 30 November 2019)

Oral melanoma is one of the most common and aggressive oral malignancies in dogs and often found at the late-clinical stages. The present study aimed to use Matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF MS), Liquid chromatography-tandem MS (LC-MS/MS) and In-gel digestion coupled with mass spectrometry (GeLC-MS/MS) to search for particular peptide mass fingerprints (PMFs) and conceivable biomarkers in serum and saliva of dogs with early- and late-stage oral melanoma (EOM and LOM, respectively), OM treated with surgery and chemotherapy, oral squamous cell carcinoma (OSCC), benign oral tumors (BN), and periodontitis and healthy controls (CP). Specific peptide fragments from peptide mass fingerprints (PMFs) were sequenced by LC-MS/MS and BLAST-searched with mammalian protein databases. Western blot analysis was used to confirm protein expression of the diseases. Expressed protein sequences from western blots were verified by LC-MS/MS. The results of salivary proteomics using MALDI-TOF MS showed unique peptide fragments appeared in the tumor groups (EOM, LOM, OSCC and BN), in the LOM and OSCC groups and in the CP controls. Western blot analysis exhibited increased expression of sentrin-specific protease 7 (SENP7) in OSCC and Toll-like receptor 4 (TLR4) in LOM and OSCC, compared with the CP group. The expression of nuclear factor kappa B (NF-**K**B), a TLR4 partner, was notably increased in OSCC compared with CP, BN and EOM and in LOM compared with EOM. For the GeLC-MS/MS-based salivary proteomic profiling, increased expression of protein tyrosine phosphatase non-receptor type 5 (PTPN5) was shown in all tumor groups compared with the CP group. Marked expression of PTPN5 was also observed in LOM and OSCC compared with that in BN and EOM. In addition, tumor protein p53 (p53), which appeared in

the PTPN5–drug interactions, was exhibited to be expressed in all tumor groups compared with that in the CP group. For the salivary proteomics of LOM treated with surgery and chemotherapy drugs, carboplatin, for 1-7 times. In dogs with short-term survival (less than 12 months after surgery), a significantly increased ratio of free ubiquitin D (fUBD) to conjugated ubiquitin D (cUBD) was shown in the pre-surgery stage (PreS) compared with that after being treated with surgery and carboplatin for 2, 4 and 5 times (AT2, AT4 and AT5). In addition, the ratio was also shown to be significantly augmented in PreS group with short-term survival compared with that in PreS group with long-term survival (more than 12 months after surgery). In addition, the expression of fUBD was enhanced in PreS compared with that of AT2 in STS group. In conclusion, discrete clusters of EOM, LOM, OSCC, BN and CP groups and potential protein candidates associated with the diseases were demonstrated by salivary and serum proteomics. Western blot analysis verified SENP7, TLR4, NF-**K**B, PTPN5 and p53 as potential salivary biomarkers of canine oral tumors. A ratio of fUBD to cUBD in PreS was plausibly shown to be a potential prognostic biomarker for survival in dogs with LOM.

Keywords: Oral Melanoma, Oral tumor, Proteomics, Saliva, Serum, Dog, Biomarker

Executive Summary

Canine oral melanoma is one of the most common and aggressive oral malignancies in dogs and makes up to 44% of all canine oral cancer diagnoses. As the oral cavity is not routinely examined by owners or veterinarians, oral cancers are usually detected at a late clinical stage (stages III and IV), based on the World Health Organization (WHO) clinical staging system for tumors of the oral cavity. Dogs with a late clinical stage have a high mortality rate. Hence, early diagnosis and screening are important for successful treatment. The gold standard for oral tumor diagnosis is a tissue biopsy for histopathological examination; however, it is an invasive technique and impractical for oral cancer screening. Serum and salivary collections are more easily accessible and less invasive. In order to discover novel biomarkers for canine oral tumors, a proteomic approach has been performed. MALDI-TOF MS has been used to show unique PMFs and clusters of samples, whereas LC-MS/MS and GeLC-MS/MS have been used to identify peptide sequences, leading to potential disease-associated peptides and proteins. The present study aimed to explore novel PMFs and conceivable biomarkers in serum and saliva of dogs with EOM, LOM, OM treated with surgery and chemotherapy, OSCC, BN, P and C, using MALDI-TOF MS, LC-MS/MS and GeLC-MS/MS. The associations of disease-perturbed proteins with chemotherapy drugs, cisplatin, cyclophosphamide, piroxicam and doxorubicin, were exhibited. MS results were validated by western blot analysis and expressed protein sequences from western blots were verified by LC-MS/MS.

For the results of salivary proteomics of canine oral tumors using MALDI-TOF MS and LC-MS/MS, unique PMFs and homogeneous clusters of each EOM, LOM, SCC and CP were obtained. Using MALDI-TOF MS, seven peptide fragments appeared in the tumor groups (EOM, LOM, OSCC and BN) at 1096, 1208, 1322, 1794, 1864, 2354 and 2483 Da, two peptide fragments appeared in the LOM and OSCC groups at 2450 and 3492 Da, and in the CP controls at 2544 and 3026 Da. Using western blot analysis, the expression of SENP7, a peptide fragment at 1096 Da, in OSCC was significantly increased, as was the expression of TLR4, a peptide fragment at 3492 Da, in LOM and OSCC, compared with the CP group. The expression of NF-**K**B, a TLR4

partner, was notably increased in OSCC compared with CP, BN and EOM. The expression was also enhanced in LOM compared with EOM.

For the GeLC-MS/MS-based salivary proteomic profiling of canine oral tumors, increased expression of PTPN5 was shown in all tumor groups compared with the CP group. Marked expression of PTPN5 was also observed in LOM and OSCC compared with that in BN and EOM. In addition, p53, which appeared in the PTPN5–drug interactions, was exhibited to be expressed in all tumor groups compared with that in the CP group.

For the salivary proteomics of LOM treated with surgery and chemotherapy drugs, carboplatin, for 1-7 times. In dogs with short-term survival (less than 12 months after surgery), a significantly increased ratio of free ubiquitin D (fUBD) to conjugated ubiquitin D (cUBD) was shown in the pre-surgery stage (PreS) compared with that after being treated with surgery and carboplatin for 2, 4 and 5 times (AT2, AT4 and AT5). In addition, the ratio was also shown to be significantly augmented in PreS group with short-term survival compared with that in PreS group with long-term survival (more than 12 months after surgery). In addition, the expression of fUBD was enhanced in PreS compared with that of AT2 in STS group.

In conclusion, discrete clusters of EOM, LOM, OSCC, BN and CP groups and potential protein candidates associated with the diseases were demonstrated by salivary and serum proteomics. Western blot analysis verified SENP7, TLR4, NF-**K**B, PTPN5 and p53 as potential salivary biomarkers of canine oral tumors. A ratio of fUBD to cUBD in PreS was plausibly shown to be a potential prognostic biomarker for survival in dogs with LOM. Further studies of the role of these proteins and related proteins should be performed in larger populations to confirm the results. In addition, the possibility to use fUBD and a ratio of fUBD to cUBD as prognostic markers of survival. Suitable drugs or treatment might be reconsidered for treating the STS group with high ratios of fUBD to cUBD, regarding to the concept of precision medicine for canine oral cancer.

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1. Introduction

Oral neoplasms represent approximately 7% of all types of tumors in dogs (Bronden et al., 2009). Among these, oral melanoma (OM) is the most aggressive, with high prevalence, accounting for 20% of all oral tumors (Bergman, 2007; Liptak and Withrow, 2013). According to the World Health Organization (WHO) clinical staging scheme of OM, the prognosis is based on tumor size, lymph node involvement and distant metastasis or TNM system. Stage I is a tumor $<$2 cm in diameter; stage II is a 2 to <4 cm diameter tumor; stage III is a tumor $\geq$4 cm in diameter with or without lymph node metastasis, and stage IV is a tumor with distant metastasis (Bergman, 2007). As the oral cavity is not routinely examined by owners or veterinarians, oral cancers are usually detected at a late clinical stage (stages III and IV) with poor prognosis (Smith et al., 2002; Prein et al., 2012; Brockley et al., 2013). Surgery resection is the primary option for canine oral tumors. The combination of surgery and chemotherapy drugs is considered for late-stage cancer. The common anti-cancer drugs used are carboplatin, a derivative of the anti-cancer drug cisplatin, doxorubicin (also called adriamycin), cyclophosphamide and piroxicam. The last two drugs are widely used in metronomic chemotherapy (Ogilvie et al., 1989; Elmslie et al., 2008; McWhinney et al., 2009). Dogs with a late clinical stage have a high mortality rate (Liptak and Withrow, 2013). Hence, early diagnosis and screening are important for successful treatment. The gold standard for oral tumor diagnosis is a tissue biopsy for histopathological examination; however, it is an invasive technique and impractical for oral cancer screening. Serum collection is a more easily accessible and less invasive technique (Tuck et al., 2009). In addition, serum is commonly used for protein marker identification especially in metastatic cancers (Chai et al., 2016). The gene expression of several cytokines and their receptors in serum (e.g., IL-1, IL-2, IL-4, IL-6, IL-8 and IL-10) is metastatic-, proliferation- and/or angiogenesis-associated at either the protein or mRNA levels. They could potentially be used to monitor melanoma progression and the efficacy of immunomodulatory therapy (Porter et al., 2001; Ottaiano et al., 2006; Yurkovetsky et al., 2007). Since biomolecules can be transferred from blood to saliva and saliva is directly contacted to the oral tumor lesion, saliva collection becomes an attractive method for biomarker mining (Zhang et al., 2010a; Zhang et al., 2010b; Lee et al., 2012; Jancsik et al., 2014). It is safe, non-invasive, painless and cost effective (Zhang et al., 2013-RSA Proposal). Salivary biomarkers have been recently used in human oral cancer detection such as IL-1B, IL-8, Mac-2 binding protein (M2BP),

MMP-9, and cancer antigen (CA125) but not yet in dog (Shpitzer et al., 2009; Brinkmann et al., 2011; Balan et al., 2012; Cheng et al., 2014).

Recent, several potential saliva and serum protein biomarkers of several cancers in minute details have been discovered by mass spectrometric technologies. Mass spectrometry (MS) is the high-throughput technology for protein profiling. A matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), composed of a MALDI source and a TOF mass analyzer, is used for searching peptide mass fingerprints (PMFs). MS spectra are obtained and compared to get fingerprints of ions that are characteristic of the cell/tissue/organism. In addition, three-dimensional principal component analysis (3D PCA) scatterplot has been used to reveal the uniformity and homogeneity of the sample group [21-Nan-PlosOne]. MALDI-TOF MS was demonstrated as a rapid screening method to differentiate oral cancer, oral lichen planus, and chronic periodontitis in human saliva (Chaiyarit et al., 2015). In dog, MALDI-TOF was used to study different protein expression in tears from dogs with cancers (transmissible venereal tumor, mammary gland adenocarcinoma, squamous cells carcinoma, fibrosarcoma, etc.) and normal dogs in order to develop tear film analysis for cancer diagnosis and management in dogs (de Freitas Campos et al., 2008). Specific mass spectra peaks on the PMF map can be further analyzed using MALDI-TOF/TOF MS, which was used to identify protein biomarkers in canine lymphoma, mammary tumor, prostate tumor and mast cell tumor (LeRoy et al., 2007; McCaw et al., 2007; Wilson et al., 2008; Klopfleisch et al., 2010; Klose et al., 2011; Atherton et al., 2013; Schlieben et al., 2013; Zamani-Ahmadmahmudi et al., 2014). Another tandem MS, liquid chromatography-tandem mass spectrometry (LC-MS/MS), is used for routine identification of proteins. LC-MS/MS use electrospray ionization (ESI) whereas MALDI-TOF uses MALDI as an ionization source with different operation and performance characteristics (Jiang et al., 2015). In-gel digestion coupled with mass spectrometric analysis (GeLC-MS/MS) is a one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by in-gel digestion and LC-MS/MS. GeLC-MS/MS is suitable for qualitative and quantitative complex protein identification (Dzieciatkowska et al., 2014). In dogs, LC-MS/MS was used to measure plasma free metanephrine and free normetanephrine in dogs with pheochromocytoma for disease diagnosis and in lymphoma (Wilson et al., 2008; Gostelow et al., 2013). In addition, MALDI-TOF MS coupled with LC-MS/MS and GeLC-MS/MS were used to analyze proteome of canine oral tumor tissues (Pisamai et al., 2018).

However, the study of salivary and serum proteomics of dogs with oral diseases has not been demonstrated. There remain knowledge gaps in proteome profiles of COM therapeutic response. Our study demonstrated candidate salivary biomarkers of canine oral tumors that might help diagnosis and treatment plan of the diseases.

2. Objectives

To study and compare serum and salivary biomarker and peptide mass fingerprint profiles of canine oral melanoma during treatments and follow-up for monitoring treatment response and for early diagnosis of disease recurrence and/or metastasis by mass spectrometry-based proteomic analyses.

3. Materials and Methods

Animals

Saliva samples were collected from patients with oral tumors scheduled for surgical excision at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University and private animal hospitals, including 5 EOM, 24 LOM, 10 OSCC and 11 BN, respectively (age range 7—14 years). For the saliva samples for monitoring treatment response, short-term survivors (STS) and long-term survivors (LTS) were defined as patients with late-clinical stage OM and living shorter than 1 y or longer than 1 y after surgery resection, respectively. Animal histories and treatment histories of LOM patients treated with surgery and chemotherapy drugs were shown in Tables 1—2, respectively. Serum samples were collected from patients with oral tumors scheduled for surgical excision at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University and private animal hospitals, including 5 EOM, 24 LOM, 9 OSCC and 10 BN, respectively (age range 7—14 years). Inclusion criteria included the presence of benign oral tumors, OM and OSCC, diagnosed without previous treatment either chemotherapy or radiotherapy. The staging of OM and OSCC were determined according to the WHO (Owen and Organization, 1980). Dogs were examined for an oral, regional lymph node, and physical condition; moreover, the regional lymph nodes were required for cytological examination to rule out metastasis. Skull-to-abdomen radiography was evaluated by a Brivo DR-F digital X-ray system (GE

Table 1. Patient history

	ID	Age at initial treatment (m)	Breed	Sex	Clinical stage	Survival time (m)
1	10	9 y 4 m	German shepherd	F	III	8 m 23 d
2	11	10 y 4 m	Poodle	F	III	6 m 9 d
3	16	11 y 5 m	Poodle	M	III	3 m 19 d
4	44	10 y 9 m	Mixed	Fs	III	1 m 10 d
5	46	7 y 7 m	Golden retriever	M	III	3 m 22 d
6	31	12 y 8 m	Shih tzu	F	III	24 m 24 d
7	71	12 y 3 m	Terrier	M	III	14 m 12 d
8	72	10 y	Mixed	Fs	III	13 m 12 d
9	86	13 y 1 m	Poodle	M	III	15 m 21 d

*M = Male, Mc = Male castration, F = Female, Fs = Female spray

Table 2. Patient treatment history

No.	PreS	PostS	AT1	AT2	AT3	AT4	AT5	AT6	AT7	R	M1	M2	C1	C2	C3	C4	Remarks
1	•	•	•	•	•	•	•				•	•					Metastasis
2	•				•	•	•	•					•	•			Recurrence
3	•	•	•		•	•											Recurrence
4		•	•	•													Metastasis
5		•	•	•	•												Recurrence
6	•	•		•	•	•		•					•	•	•	•	Seizures
7	•	•	•	•	•	•											Metastasis
8		•	•	•	•	•											Metastasis
9	•	•	•	•	•	•											Recurrence

Healthcare, Chicago, IL) or Optima CT660 CT-scanner (GE Healthcare). OM and OSCC metastasis to abdominal organs was checked by ultrasonography. Seven saliva and 6 serum samples were gathered from dogs with normal oral health with normal blood profiles and no history or clinical signs of oral cavity or cancerous problems (age range 7—8 years). For a chronic periodontitis group, 5 dogs demonstrated gingivitis, dental tartar and/or periodontal attachment loss (age range 7—13 years). The samples were obtained with the consent of owners and sample collection protocol was approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand (Approval number 1631042).

Sample preparation

To collect saliva, dogs were fasted for at least 1 h before saliva collection and mouths were cleaned with 0.9% sterile normal saline solution (Jou et al., 2010). Saliva was collected on the day of surgery without mechanical and chemical stimulation (PreS group). For the saliva samples for monitoring treatment response, samples were also obtained at 14 days after operation (PostS group). Adjuvant chemotherapeutic protocol, carboplatin, was given at a dosage of 250 mg/m2 of a 3-week interval for 6-7 treatments. Saliva was collected post chemotherapy treatments for 1—7 times (AT1—AT7) and during following-up for 1 or 2 month-intervals for 1—4 times after treatment ends (C1—C4). Whole saliva (0.5—1.0 mL) was collected for 5—10 min using a sterile cotton swab. Samples were centrifuged at 2600 x*g* for 15 min at 4°C (Henson and Wong, 2010). Approximate 200 µL of supernatant was mixed with Halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA) and kept at -20°C until analysis. Total protein concentrations from salivary supernatants were evaluated by Lowry's assay at 690 nm, using bovine serum albumin as a standard (Lowry et al., 1951).

Serum samples (from the matched dogs used to collect saliva if possible) were obtained at the initial visit for surgical excision. Sera were immediately kept on ice and centrifuged at 3,000x*g* for 15 min at 4°C and subsequently at 15,000 x *g* for 15 min at 4°C to remove platelets and particulate matter. Serum will be aliquoted and kept at -80°C until use (Tolek et al., 2012). Total protein concentrations from salivary supernatants were evaluated by Lowry's assay at 690 nm, using bovine serum albumin as a standard (Lowry et al., 1951).

Preparation of saliva samples for LC-MS analysis

Total protein of samples was measured by Lowry's assay (Lowry et al., 1951). Each sample was prepared to 1.5 µg/µL in 10 mM ammonium bicarbonate. Disulfide bonds were reduced by 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate for 1 h at room temperature and alkylated in 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate for 1 h at room temperature in the dark. After that, the protein in each sample was digested with the sequencing grade modified trypsin (Promega, Madison, WI) of 50% acetonitrile (ACN) in 10 mM ammonium bicarbonate for overnight. Then, the solvent was removed. Finally, each sample was dissolved with 20 µl of 0.1% formic acid and centrifuged 10,000 rpm for 5 min before LC-MS/MS analysis. Spike BSA as internal standard was prepared by using 1.5 µg/µL in 10 mM ammonium bicarbonate.

Analysis of peptides by MALDI-TOF MS

The protein sample of each dog was prepared with 0.1% trifluoroacetic acid (TFA) to the final concentration of 0.2 and 0.1 µg/µL for salivary and serum samples, respectively. Samples were mixed with MALDI matrix solution, consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 100% acetonitrile (ACN) and 5% trifluoroacetic acid, at the ratio of 1:1, and directly applied onto the MTP384 target plate (Bruker Daltonics, Billercia, MA) and air dried. Eight replicates were performed to prevent sample preparation bias. Mass spectra were obtained with an Ultraflex III TOF/TOF (Bruker Daltonics) in a linear positive mode with a mass range of 1000—20000 Da and 1000—5000 Da for salivary and serum samples, respectively. External calibrations were performed using a ProteoMass Peptide & Protein MALDI-MS Calibration Kit (Sigma Aldrich, St. Louis, MO) that consists of human angiotensin II (m/z 1046), P14R (m/z 1533), human adrenocorticotropic hormone fragment 18—39 (m/z 2465), bovine insulin oxidized B chain (m/z 3465), bovine insulin (m/z 5731), and cytochrome *c* (m/z 12362). Saliva peptide mass spectra were determined by flexAnalysis 3.3 software (Bruker Daltonics). Peptide mass spectral peaks were analyzed using Quick Classifier (QC)/ Different Average, Supervised Neural Network (SNN), Anderson-Darling (AD), t-test/ANOVA (TTA), Wilcoxon/Kruskal-Wallis (W/KW) and the Genetic Algorithm (GA) statistical algorithms incorporated in the ClinProTools v. 3.0 software (Bruker Daltonics) to reveal the uniformity and homogeneity of the sample group as PMF, pseudo-gel view and principal

component analysis (PCA) (Shao et al., 2012; Chaiyarit et al., 2015; Rungruengphol et al., 2015). A dendrogram of each dog was constructed, using ClinProTools v. 3.0.

According to dendrograms and PCA plots, four from eight replicates were selected and pooled. Thirty-two replicates of pooled samples were applied twice to MALDI-TOF MS as mentioned above. The recognition capability and cross-validation values of more than 90% exhibited the reliability of the peak selection (Niyompanich et al., 2015). ClinProTools v. 3.0 was used to analyze intensity values. Results with $p < 0.05$ were considered significant and peaks were then selected to be analyzed by LC MS/MS.

Peptide and protein identification by LC-MS/MS

With the limitation of the LC MS/MS, peptide samples at 1000–4000 Da, respectively, were selected by ClinProTools v. 3.0 and purified using C18 ZipTip (MilliporeSigma, Burlington, MA). Each peptide was diluted in ACN for 51 different dilution ratios equally spaced in the range 0—100%. Amino acid sequences of gradient-eluted peptides were identified by reversed-phase high performance liquid chromatography and a PTM Discovery System (Bruker Daltonics) coupled to an UltiMate 3000 LC System (Thermo Fisher Scientific, Waltham, MA). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 µm diameter × 50 mm length). The nanoLC system was connected to an electrospray ionization in the positive ion mode and quadrapole ion-trap MS (Bruker Daltonics). Eluent A and eluent B solutions were prepared from a 0.1% formic acid dilution and from 50% ACN in water containing 0.1% formic acid, respectively. Peptide separation was achieved with a 4—70% linear gradient of eluent B at a flow rate of 1000 nL/min for 7.5 min. A regeneration step and an equilibration step were performed with 90% and 4% eluent B, respectively, for 20 min per run. Peptide fragment mass spectra were acquired in the data-dependent AutoMS mode with a scan range of 400—1500 m/z, 3 averages, and up to 5 precursor ions, selected from the MS scan at 200—2800 m/z.

The results of LC MS/MS were converted into an mzXML file by CompassXport software (Bruker Daltonics). DeCyder MS Differential Analysis software (Amersham Biosciences, Little Chalfont, UK) was used for protein quantification (Johansson et al., 2006; Thorsell et al., 2007). The PepDetect module was used in MS mode for automated peptide detection, charge state assignments, and peptide ion signal intensities. The proteins were identified from MS/MS peptide

mass values using Mascot software (Matrix Science, London, UK) (Perkins et al., 1999). The data were searched against the NCBI mammal database for protein identification. Proteins were identified from one or more peptides with an individual MASCOT score corresponding to $p<0.05$. The information about particular proteins and detailed analysis of the protein sequences were used in the annotation of UniProtKB/Swiss-Prot entries [26]. The relationship of candidate proteins and chemotherapy drugs were performed by the Stitch program, version 5.0 (Szklarczyk et al., 2016).

Analysis of peptides by GeLC-MS/MS

Peptides were analysed by GeLC-MS/MS as previously described with some modifications (Pisamai et al., 2018). Briefly, 50 μg of pooled samples in each group (CP, BN, EOM, LOM and OSCC) were mixed with loading buffer [0.5M dithiothreitol (DTT), 10% w/v SDS, 0.4 M Tris-HCl pH 6.8, 50% v/v glycerol, 0.1 mg/ml Bromophenol Blue] and boiled at 90°C for 5 min prior to separating on 12.5% SDS-PAGE (Atto, Tokyo, Japan). Gels were fixed using 50% methanol, acetic acid and 37% formaldehyde and stained with silver nitrate solution, before being scanned using a GS-710 scanner (Bio-Rad Laboratories, Benicia, CA, USA) and stored in 0.1% acetic acid. After that in-gel tryptic digestion was performed where protein bands in each lane were divided into 17 segments and chopped into 1 mm^3 pieces. Gel pieces were dehydrated using 100% acetonitrile (ACN) and dried. Cysteines were reduced and alkylated by 10 mM DTT in 10 mM ammonium bicarbonate and 100 mM iodoacetamide in 10 mM ammonium bicarbonate, respectively, prior to dehydrating twice in 100% ACN. Trypsin digestion was performed in 50 mM NH4HCO3 (pH 7.8) overnight at 37°C. The tryptic peptides were extracted from the gels using 50% ACN in 0.1% formic acid (FA). Pooled samples were submitted to a reversed-phase high performance liquid chromatography (HPLC). The gradient-eluted peptides were analysed using an Ultimate 3000 LC System coupled to an HCTUltra PTM Discovery System (Bruker Daltonics, Bremen, Germany). Peptides were separated on a PepSwift monolithic column (100 μm internal diameter × 50 mm) (Thermo Fisher Scientific). Peptide separation was achieved with a linear gradient at a flow rate of 1000 nL/min from 4% ACN, 0.1% FA to 70% ACN, 0.1% FA for 7.5 min with a regeneration step at 90% ACN, 0.1% FA and an equilibration step at 4% ACN, 0.1% FA. The entire process took 20 min. Peptide fragment mass spectra were acquired in a data-dependent Auto MS mode with a

scan range 400—1500 m/z. However, in the case of having more than 5 precursor fragments, peptides would be selected from the MS scan at 200—2800 m/z. CompassXport software (Bruker Daltonics) was used to convert data from LC-MS/MS into the mzXML format. Protein quantitation was performed using DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare) [16, 17-Sek GeLC]. The peptide sequences were searched against the NCBI mammal database for protein identification using MASCOT software, version 2.2 (Matrix Science, London, UK) [18-Sek GeLC]. Database interrogation included taxonomy (mammals), enzyme (trypsin), variable modifications (oxidation of methionine residues), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (1.2 Da), fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and maximum number of missed cleavages. Proteins were identified from one or more peptides with an individual MASCOT score corresponding to $p<0.05$. Proteins were annotated by UniProtKB/Swiss-Prot entries (http://www.uniprot.org/) and classified according to their molecular function, biological process and cellular component using the PANTHER classification system, version 8.1 (www.pantherdb.org/) [19-Sek GeLC]. Protein list comparison among different sample groups was displayed using jvenn diagram (http://bioinfo.genotoul.fr/jvenn/example. html) (Bardou et al., 2014). The interaction network of candidate proteins and chemotherapy drugs was explored using the Stitch program, version 5.0 (http://stitch.embl.de/) (Szklarczyk et al., 2016).

Validation of MS results by western blot analysis

Pooled saliva samples of 5 µg for detecting SENP7 and TLR4, 12 µg for detecting NF-**K**B, 10 µg for detecting PTPN5 and p53, individual saliva samples of 15 µg for detecting UBD and pooled serum samples of 5 µg for detecting IGSF10 and TRAF3IP1 were mixed with loading dye [0.5 M dithiothreitol, 10% (w/v) SDS, 0.4 M Tris-HCl, pH 6.8, and 50% (v/v) glycerol]. Samples were heated at 85°C for 10 min prior to loading on pre-cast NuPAGE 4–12% (w/v) Bis-Tris 1.0-mm minigel (Thermo Fisher Scientific), using RunBlue MES Run Buffer (Expedeon, Heidelberg, Germany) in an XCell SureLock Mini-Cell electrophoresis system (Thermo Fisher Scientific) at 200 V for 90 min. PageRuler prestained protein ladder (molecular weight range 10–180 kDa) (Thermo Fisher Scientific) was used. Subsequently, proteins were transferred to Trans-Blot Turbo mini-sized nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) at 25 V for 14 min using Trans-Blot

Turbo 5× transfer buffer (Bio-Rad Laboratories). A Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes (Thermo Fisher Scientific) was used to detect total proteins in each well according to the manufacturer's instructions. Nonspecific binding was blocked with 5% bovine serum albumin (GoldBio, St Louis, MO) in Tris-buffered saline containing 0.1% Tween 20 (TBST) overnight. After washing with TBST, primary antibodies diluted at 1:1000 were incubated with a membrane at 4°C overnight (Table 3). A membrane was washed and subsequently incubated with 1:10000 horseradish peroxidase-conjugated rabbit anti-mouse antibody (ab6728, Abcam, Cambridge, UK) or 1:10000 horseradish peroxidase-conjugated goat anti-rabbit antibody (ab205718, Abcam) at 25°C for 1 h. The proteins of interest were detected using ECL western blotting detection reagents (GE Healthcare). Western blots were imaged with a ChemiDoc Touch Imaging System (Bio-Rad Laboratories). Protein band intensities were analyzed by Image Lab 6.0.1 software (Bio-Rad Laboratories). For western blot normalization, total protein normalization, modified from Aldridge et al. (2008) was used (Aldridge et al., 2008). The ratios of target band intensities to the total proteins in each lane in the first or second half of a membrane were calculated according to the sizes of target proteins. The western blotting was performed in triplicate.

Verification of expressed protein sequences by LC-MS/MS

To confirm SENP7, TLR4, NF-**K**B, PTPN5, p53, Ub, IGSF10 and TRAF3IP1 protein identities, antibodies were removed from nitrocellulose membranes by incubating with Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific) at room temperature for 15 min. After washing 4 times with TBST, protein bands were cut and incubated with 10 mM DTT in 10 mM ammonium bicarbonate (Ambic) at room temperature overnight. Samples were then incubated with 10 ng trypsin in 10 mM Ambic at 37°C for 3 h and concentrated by the speed-vac (Thermo Fisher Scientific). Fifteen µL of 0.1% formic acid was used to dissolve proteins prior to applying to the LC-MS/MS as mentioned above.

Table 3. Primary antibodies used for western blot in the present study

Antibody for	Catalog No.	Company
Salivary samples for MALDI-TOF coupled with LC-MS/MS:		
Mouse monoclonal anti-human sentrin-specific protease 7 (SENP7) (E-8)	sc-373821	Santa Cruz Biotechnology, Dallas, TX
Mouse monoclonal anti-mouse toll-like receptor 4 (TLR4) (25)	sc-293072	Santa Cruz Biotechnology, Dallas, TX
Mouse monoclonal anti-human nuclear factor kappa B (NF-**K**B) p65	sc-8008	Santa Cruz Biotechnology, Dallas, TX
Salivary samples for GeLC-MS/MS:		
Mouse monoclonal anti-human protein tyrosine phosphatase non-receptor type 5 (PTPN5)	sc-514678	Santa Cruz Biotechnology, Dallas, TX
Novocastra liquid mouse monoclonal anti-human tumor protein 53 (p53) (DO-1)	-	Leica Biosystems, Newcastle upon Tyne, UK
Salivary samples from chemotherapy:		
Mouse monoclonal anti-human ubiquitin (Ub) (A-5)	sc-166553	Santa Cruz Biotechnology, Dallas, TX
Serum samples for MALDI-TOF coupled with LC-MS/MS:		
Mouse polyclonal anti-human immunoglobulin superfamily member 10 (IGSF10) (A-1)	H00285313-A01	Abnova, Taipei City, Taiwan
Serum samples for GeLC-MS/MS:		
Rabbit polyclonal anti-human tumor necrosis factor receptor associated factor 3 interacting protein 1 (TRAF3IP1)	ab153860	Abcam, Cambridge, UK

Statistical analysis

ClinProTools v. 3.0 and MASCOT softwares were used to analyze peak intensities of peptides in MALDI-TOF MS spectra and MASCOT LC-MS/MS scores, respectively. For the GeLC-peak intensities and MASCOT LC-MS/MS scores, respectively. Western blot band intensity ratios MS/MS results, ANOVA statistical analysis, incorporated into the DeCyder MS differential analysis software, and MASCOT software, version 2.2 were used to analyse significantly different peptide were tested for normality and statistical differences were analyzed by ordinary one-way ANOVA with Tukey's multiple comparisons for SENP7 (pooled samples), ordinary one-way ANOVA with Bonferroni's multiple comparisons for TLR4 and NF-**K**B (pooled samples), Kruskall Wallis with Dunn's multiple comparisons for SENP7, TLR4 and NF-**K**B (individual samples), ordinary one-way ANOVA with Tukey's multiple comparisons for PTPN5 and p53, Friedman test with Dunn's multiple comparisons for Ub, and Kruskall Wallis for IGSF10 and TRAF13. Statistical analyses of protein expression data were conducted using GraphPad Prism, version 8.2.1 (GraphPad Software, La Jolla, CA). Significance was accepted at the $p<0.05$ level.

4. Results

4.1. Salivary proteomics of canine oral tumors using MALDI-TOFMS coupled with LC-MS/MS

All 32 replicates in each pooled sample group demonstrated the homogeneity within the group. A 3-dimensional view of the PCA plot showed distinct clusters among the EOM, LOM, OSCC and BN groups, whereas periodontitis and healthy controls were shown to be in the same cluster and classified as a control (CP) group (Fig. 1). Divergent PMFs of CP, EOM, LOM, OSCC and BN groups were observed, and peptide masses at 1000—5000 Da were selected by ClinProTools software and specific peptide sequences were analyzed by LC MS/MS. Seven peptide fragments appeared in the tumor groups (EOM, LOM, OSCC and BN) at 1096, 1208, 1322, 1794, 1864, 2354 and 2483 Da (SENP7 or KAT2B, PPRC1 or RMND1, DTX3L, ZNF699, MAP3K15 or ATP6V1E2, PLCL2 and COL12A1, respectively), two peptide fragments appeared in the LOM and OSCC groups at 2450 and 3492 Da (TNRC18 and TLR4, respectively), two peptide fragments appeared only in the CP controls at 2544 and 3026 Da (ZNF451 and CASPL4A2, respectively) (Fig. 2). Candidate protein biomarkers were evaluated for biological processes and location in the cell by UniProtKB/Swiss-

Prot (Table 4) (UniProt Consortium, 2018). Networks of protein-protein and protein-chemotherapy drug interactions were performed by the Stitch program, version 5.0 and pathways with high edge confidence scores (>0.700) represented the strength of the protein-protein interactions at the functional level (Figs 3-5) (Szklarczyk et al., 2016). Several candidate proteins presented in this study showed a strong relationship with chemotherapy drugs, including KAT2B, PPRC1, DTX3L, ZNF699 and MAP3K15. Also, p53 was noticeable in all of these pathways as well as the pathways of SENP7-doxorubicin except SENP7-cyclophosphamide/piroxicam and SLC30A10-cyclophosphamide/piroxicam pathways which involved the cytochrome P450 family 2 (CYP2) family. We did not find an association of TLR4 with chemotherapy drugs. However, western blot analysis revealed protein expression of SENP7 in EOM, LOM, OSCC and BN, and TLR4 and NF-**K**B in LOM and OSCC (Figs 6-8). The protein bands of SENP7, TLR4 and NF-**K**B on the membranes were verified by LC-MS/MS. From the Mascot search results, MS/MS fragmentations of KFRKTLPR, NLRYLDISYTR and MLLAVQR were found to be matched with SENP7, TLR4, and NF-**K**B, respectively (Fig. 9). Western blots were then performed in individual samples. The results were shown in Figs. 10-12. The increased expression of SENP7, TLR4 and NF-**K**B was observed in LOM and OSCC compared with CP and BN.

4.2. Salivary proteomics of canine oral tumors using GeLC-MS/MS

A total of 3726 proteins were identified. The distribution of the individual and overlapped proteins in EOM, LOM, OSCC, BN and CP groups was illustrated by a Venn diagram (Fig. 13). In addition, the molecular function, biological process, cellular component and the relative expression levels of the proteins uniquely expressed in each group and commonly expressed in all cancerous groups was analysed using the PANTHER software tools (Tables 5 and 6). For the networks of protein–protein and protein–chemotherapy drug interactions, analysed by the Stitch program, version 5.0, edge confidence scores demonstrated the strength of the interactions at the functional level. Pathways with high edge confidence scores (>0.700) were presented as thick lines. The associations of protein tyrosine phosphatase non-receptor type 5 (PTPN5) and tumor protein p53 (p53) with cisplatin and doxorubicin drugs were shown.

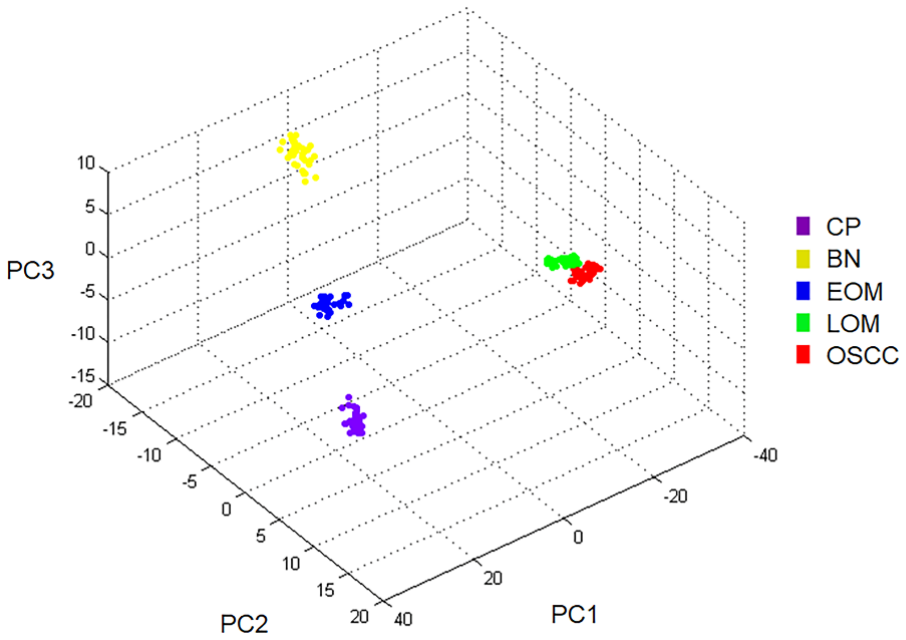


Fig. 1. Three-dimensional principal component analysis scatterplot of normal and periodontitis gingiva (CP), benign tumors (BN), early stage oral melanoma (EOM), late-stage OM (LOM) and oral squamous cell carcinoma (OSCC). Thirty two dots represent replicate in each pooled sample group.

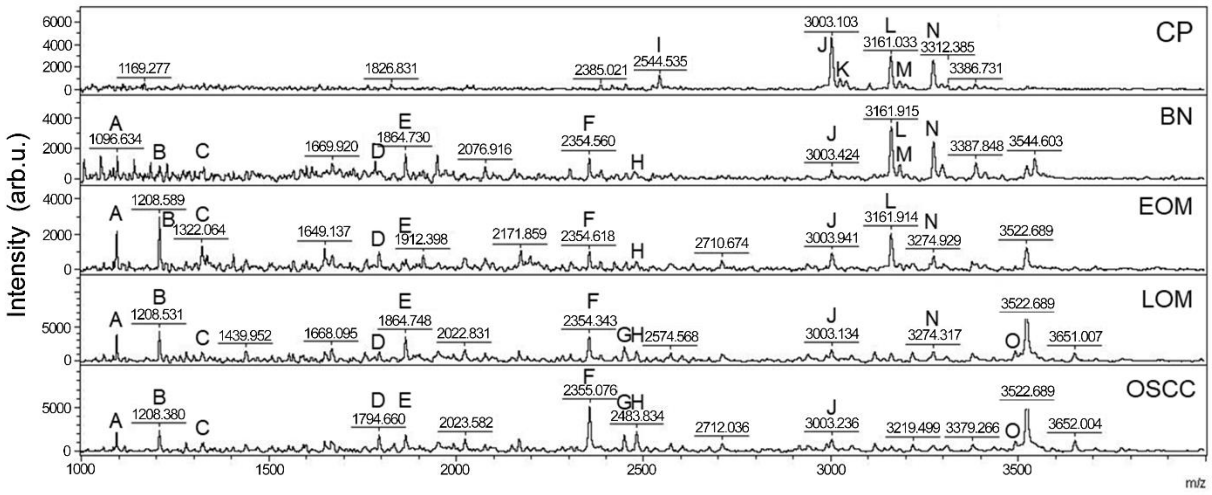


Fig. 2. Peptide mass fingerprint (PMF) of normal and periodontitis gingiva (CP), benign tumors (BN), early-stage oral melanoma (EOM), late-stage OM (LOM) and oral squamous cell carcinoma (OSCC) in the range 1000–5000 Da with identified proteins of each mass spectrum. A: SENP7 or KAT2B (1096 Da); B: PPRC1 or RMND1 (1208 Da); C: DTX3L (1322 Da); D: ZNF699) (1794 Da); E: MAP3K15 or ATP6V1E2 (1864 Da); F: PLCL2 (2354 Da); G: TNRC18 (2450 Da); H: COL12A1 (2483 Da); I: ZNF451 (2544 Da); J: protocadherin FAT1 (3003 Da); K: CASPL4A2 (3026 Da); L: centrosomal protein 192 (3161 Da); M: glypican 5 (3184 Da); N: cell-cycle checkpoint protein RAD17 (3274 Da); O: TLR4 (3492 Da). arb. u., arbitrary unit.

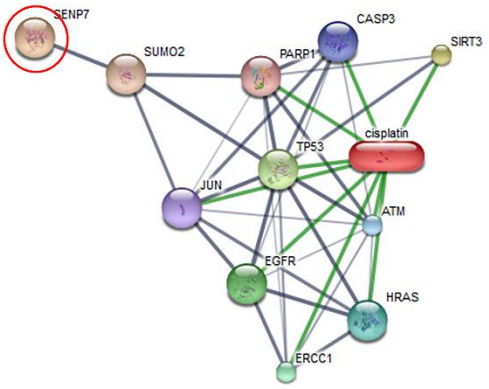
Table 4. Nominated proteins based on biological process involvement and protein score, using MALDI-TOF MS and LC-MS/MS data

Database	Protein name	Protein score	Peptide sequence	Biological process	Subcellular distribution
XP_010329708.1	Sentrin-specific protease 7 isoform X1 (SENP7)	29	LNLSERIPR	Adenylate cyclase-modulating G-protein-coupled receptor signaling pathway	Nucleus and plasma membrane
XP_021512678.1	Histone acetyltransferase KAT2B (KAT2B)	25	VYPGLLCFK	Cell cycle arrest, chromatin remodeling	Nucleus and cytoskeleton
EHB18528.1	Peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (PPRC1)	17	AHDHYQRQR	Positive regulation of DNA-binding transcription factor activity	Nucleus
ERE86066.1	Required for meiotic nuclear division protein 1-like protein (RMND1)	9	TLALSTYFHR	Positive regulation of mitochondrial translation	Mitochondrion
A0A286Y4B2	E3 ubiquitin-protein ligase DTX3L (DTX3L)	15	TLYGIQTGNQPK	Histone ubiquitination	Cytosol, endosome, lysosome and nucleus
XP_012863361.1	Zinc finger protein 699 (ZNF699)	14	EYGEACSSPSSIGPPVR	Regulation of transcription	Nucleus

XP_004612329.1	Mitogen-activated protein kinase kinase kinase 15 (MAP3K15)	20	TDSMEILTSDIIDGLLK	Activation of MAPKK activity	Nucleus and cytoplasm
XP_021074063.1	V-type proton ATPase subunit E 2 (ATP6V1E2)	16	VCNTLESRLNLAAMQK	ATP hydrolysis coupled proton transport	Membrane
XP_010964328.1	Inactive phospholipase C-like protein 2 (PLCL2)	17	VMVMTSPNVEESYLPSPDVLK	Intracellular signal transduction	Cytoplasm
ELK17433.1	Trinucleotide repeat-containing protein 18 protein (TNRC18)	16	NSSGKLSGKPLLTSDAYELGAGMR	Chromatin silencing	Cytosol, mitochondrion, nucleus and other locations
XP_007951446.1	Collagen type XII alpha-1 chain (COL12A1)	23	DYKPQVGVIVDPSTKTLSFFNK	Cell adhesion	Extracellular matrix
XP_004267830.1	Zinc finger protein 451 isoform X2 (ZNF451)	18	DTSPFQPNPPAGGPIVEALEHSKR	Nucleic acid binding	Nucleus
XP_015104668.1	Protocadherin Fat 1 isoform X3 (FAT1)	16	GNPPMSEITSVHIFVTIADNASPKFTSK	Homophilic cell adhesion via plasma membrane adhesion molecules	Plasma membrane and integral component of membrane
XP_016818046.1	CASP-like protein 4A2 (CASPL4A2)	22	SAASPGPAPAAGDPGGSARPRPAAPLGSALALAF	Iron—sulfur cluster binding	Plasma membrane

XP_003924923.1	Centrosomal protein of 192 kDa isoform X1 (CEP192)	21	SGNLLETHEVDLTSNSEELD PIRLALLGK	Centrosome-templated microtubule nucleation	Cytoskeleton
ERE87034.1	Glypican-5 (GPC5)	13	GMCKDLTKPMQHHVTVIA ASTECVVTLK	Regulation of signal transduction	Plasma membrane and extracellular space
XP_010635607.1	Cell-cycle checkpoint protein RAD17 isoform X2 (RAD17)	13	LLFPKEIQEECSILNISFNPVA PTIMMK	Cell cycle	Nucleus
ABU41662.1	Toll-like receptor 4 variant 1 (TLR4)	3	MMSASRLAGTLIPAMAFLS CVRPESWEPCVE	Activation of MAPK activity	Early endosome and cell membrane

A.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CASP3
SIRT3
TP53
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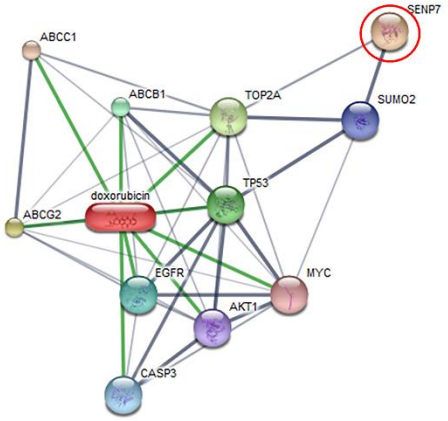
JUN
ATM
EGFR
HRAS
ERCC1

B.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ABCB1
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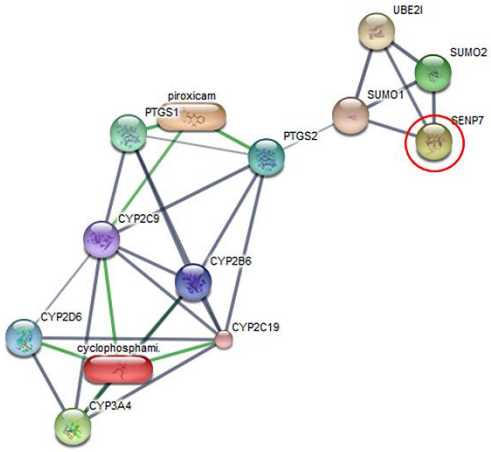
SUMO2
TP53
doxorubicin
ABCG2
EGFR
MYC
AKT1
CASP3

C.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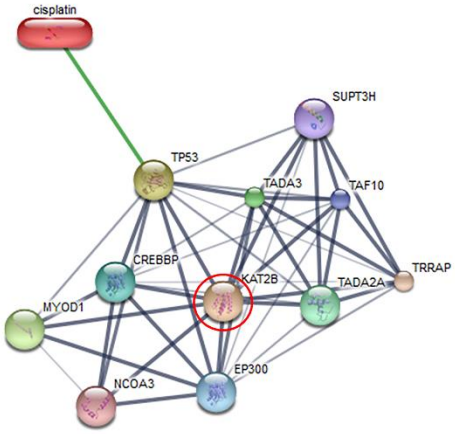
SUMO2
SUMO1
SENP7
piroxicam
PTGS1
PTGS2
CYP2C9
CYP2B6
CYP2D6
CYP2C19
cyclophosphami.
CYP3A4

D.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TP53
TADA3
TAF10
CREBBP
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TADA2A
TRRAP
MYOD1
EP300
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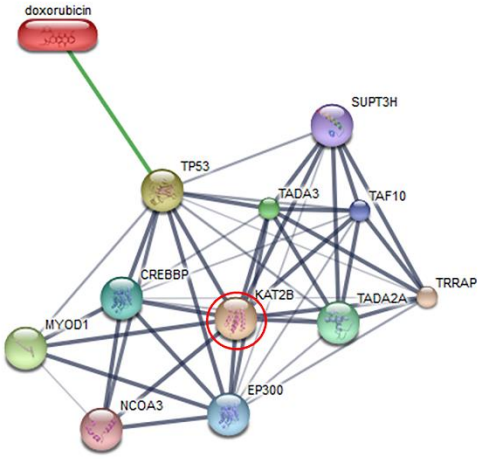
E.


doxorubicin
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TP53
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TAF10
CREBBP
KAT2B
TADA2A
TRRAP
MYOD1
EP300
NCOA3

F.

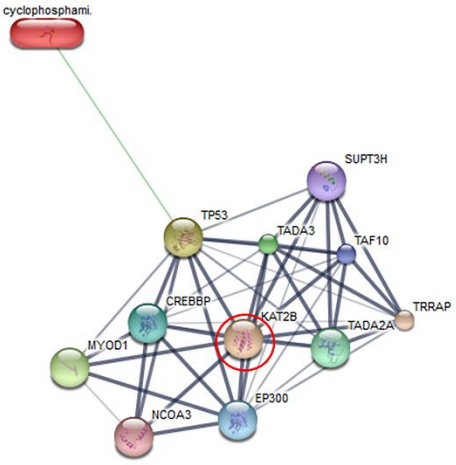
cyclophosphami.
SUPT3H
TP53
TADA3
TAF10
CREBBP
KAT2B
TADA2A
TRRAP
MYOD1
EP300
NCOA3

Fig. 3. Involvement of sentrin-specific protease 7 (SENP7) and K(lysine) acetyltransferase 2B (KAT2B) in networks of protein–chemotherapy drug interactions, cisplatin, doxorubicin, cyclophosphamide and piroxicam. Interactions of SENP7 with cisplatin (A), SENP7 with doxorubicin (B), SENP7 with cyclophosphamide and piroxicam (C), KAT2B with cisplatin (D), KAT2B with doxorubicin (E), KAT2B with cyclophosphamide and piroxicam (F) were exhibited. Red circles: SENP7 and KAT2B. Abbreviations: ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), ataxia telangiectasia mutated (ATM), ATP-binding cassette, sub-family C (CFTR/MRP), member 1 (ABCC1), ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2), v-akt murine thymoma viral oncogene homolog 1 (AKT1), caspase 3 (CASP3), c-jun (JUN), CREB binding protein (CREBBP), cytochrome P450 family 2 subfamily B member 6 (CYP2B6), cytochrome P450 family 2 subfamily C member 9 (CYP2C9), cytochrome P450 family 2 subfamily C member 19 (CYP2C19), cytochrome P450 family 2 subfamily D member 6 (CYP2D6), cytochrome P450 family 3 subfamily A member 4 (CYP3A4), E1A binding protein p300 (EP300), epidermal growth factor receptor (EGFR), v-myc myelocytomatosis viral oncogene homolog (MYC), DNA excision repair protein ERCC-1, endonuclease non-catalytic subunit (ERCC1), Harvey rat sarcoma viral oncogene homolog (HRAS), myogenic differentiation 1 (MYOD1), nuclear receptor coactivator 3 (NCOA3), poly [ADP-ribose] polymerase 1 (PARP-1), prostaglandinendoperoxide synthase 1 (PTGS1), prostaglandin-endoperoxide synthase 2 (PTGS2), sirtuin 3 (SIRT3), solute carrier family 30 (zinc transporter), member 6 (SLC30A6), suppressor of Ty 3 homolog (S. cerevisiae) (SUPT3H), SMT3 suppressor of mif two 3 homolog 1 (SUMO1), SMT3 suppressor of mif two 3 homolog 2 (SUMO2), transcriptional adaptor 2A (TADA2A), transcriptional adaptor 3 (TADA3), TAF10 RNA polymerase II (TAF10), topoisomerase (DNA) II alpha (TOP2A), transformation/transcription domain-associated protein (TRRAP), tumor protein p53 (TP53), ubiquitinconjugating enzyme E2I (UBE2I).

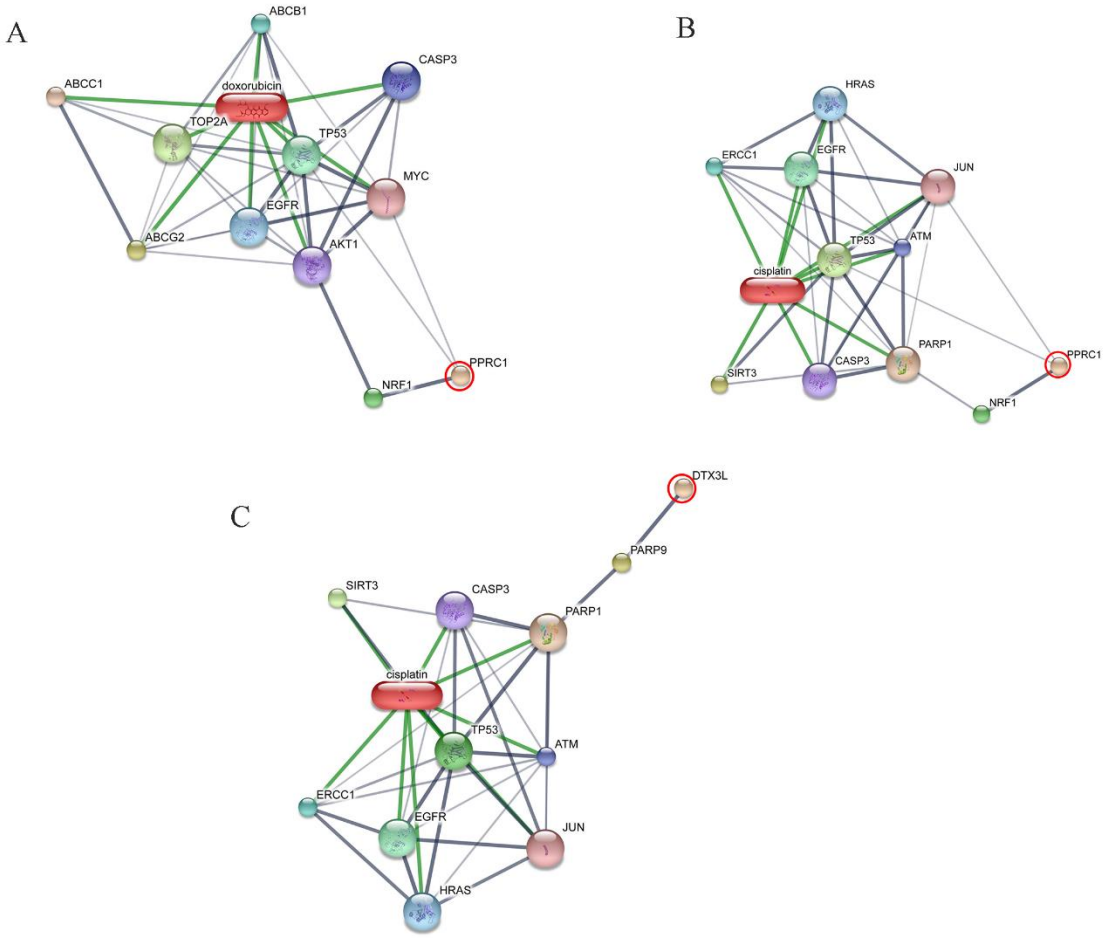

Fig. 4. Involvement of peroxisome proliferator-activated receptor gamma, coactivator-related 1 (PPRC1) and deltex 3-like (DTX3L) in networks of protein–chemotherapy drug interactions, cisplatin and doxorubicin. Interactions of PPRC1 with doxorubicin (A), PPRC1 with cisplatin (B) and DTX3L with cisplatin (C) are shown. Red circles: PPRC1 and DTX3L. Abbreviations: ABCB1, ATP-binding cassette, sub-family B, member 1; ABCC1, ATP-binding cassette, sub-family C, member 1; ABCG2, ATP-binding cassette, sub-family G, member 2; AKT1, v-akt murine thymoma viral oncogene homolog 1; ATM, ataxia telangiectasia mutated; CASP3, caspase 3; EGFR, epidermal growth factor receptor; ERCC1, excision repair crosscomplementing rodent repair deficiency, complementation group 1; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; JUN, jun protooncogene; MYC, v-myc myelocytomatosis viral oncogene homolog; nuclear respiratory factor 1 (NRF1), PARP1, poly (ADP-ribose) polymerase 1; PARP9, poly (ADP-ribose) polymerase family, member 9; SIRT3, sirtuin 3; TOP2A, topoisomerase II alpha; TP53, tumor protein p53.

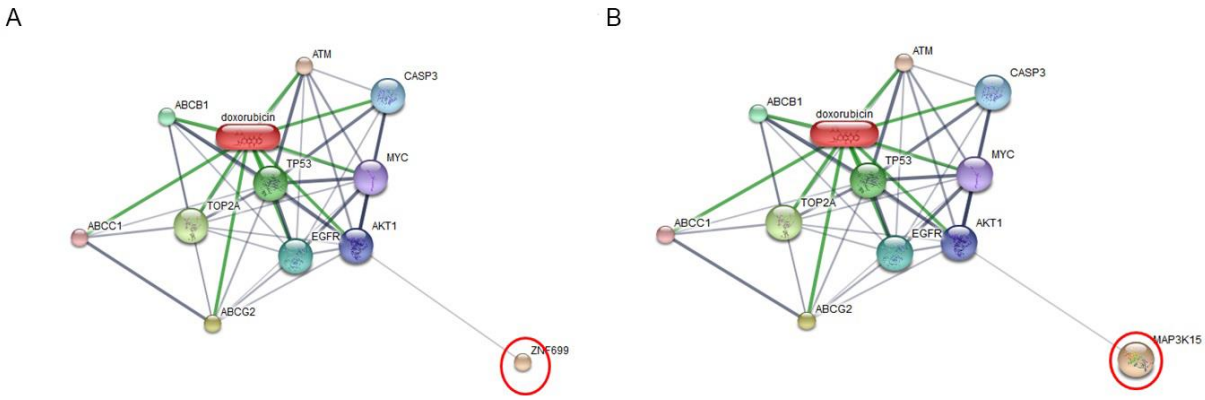

Fig. 5. Involvement of zinc finger protein 699 (ZNF699) (A) and mitogen-activated protein kinase kinase kinase 15 (MAP3K15) (B) in networks of protein–chemotherapy drug interactions, doxorubicin. Red circles: ZNF699 and MAP3K15. Abbreviations: ABCB1, ATP-binding cassette, subfamily B, member 1; ABCC1, ATP-binding cassette, sub-family C, member 1; ABCG2, ATP-binding cassette, sub-family G, member 2; AKT1, v-akt murine thymoma viral oncogene homolog 1; ATM, ataxia telangiectasia mutated; CASP3, caspase 3; EGFR, epidermal growth factor receptor; MYC, vmyc myelocytomatosis viral oncogene homolog; TOP2A, topoisomerase II alpha; TP53, tumor protein p53.

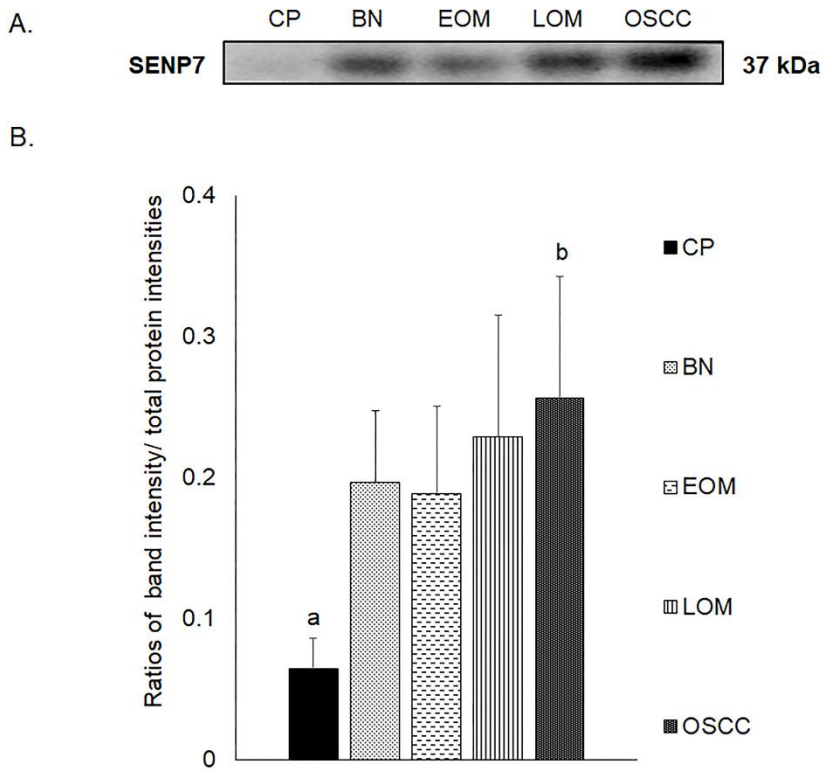

Fig. 6. Western blot analysis of salivary sentrin-specific protease 7 (SENP7) of pooled saliva samples from dogs with periodontitis and normal controls (CP), benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively) and oral squamous cell carcinoma (OSCC). Representative western blot for SENP7 at 37 kDa (A) and bar graph of ratios of SENP7 protein intensity to total blotted protein intensities in each lane in the second half of a membrane (B). a–b denote a significant difference at $p<0.05$.

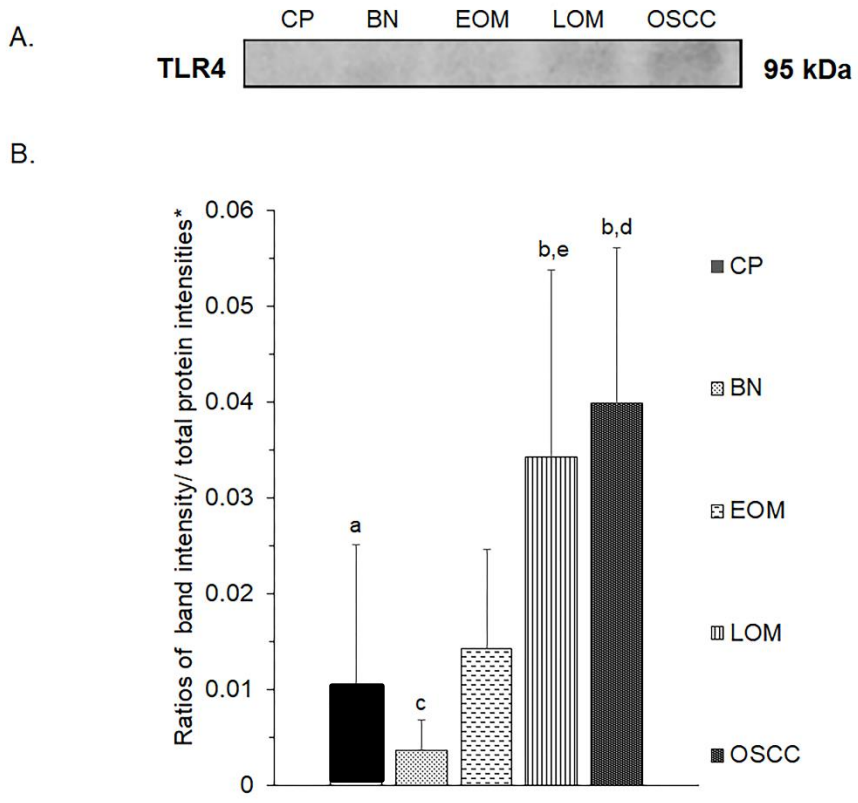

Fig. 7. Western blot analysis of salivary toll like receptor 4 (TLR4) of pooled saliva samples from dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). Representative western blot for TLR4 at 95 kDa (A) and bar graph of ratios of TLR4 protein intensity to total blotted proteins in each lane in the first half of a membrane (B). a–b denote a significant difference at $p<0.05$. c–d denote a significant difference at $p<0.01$.

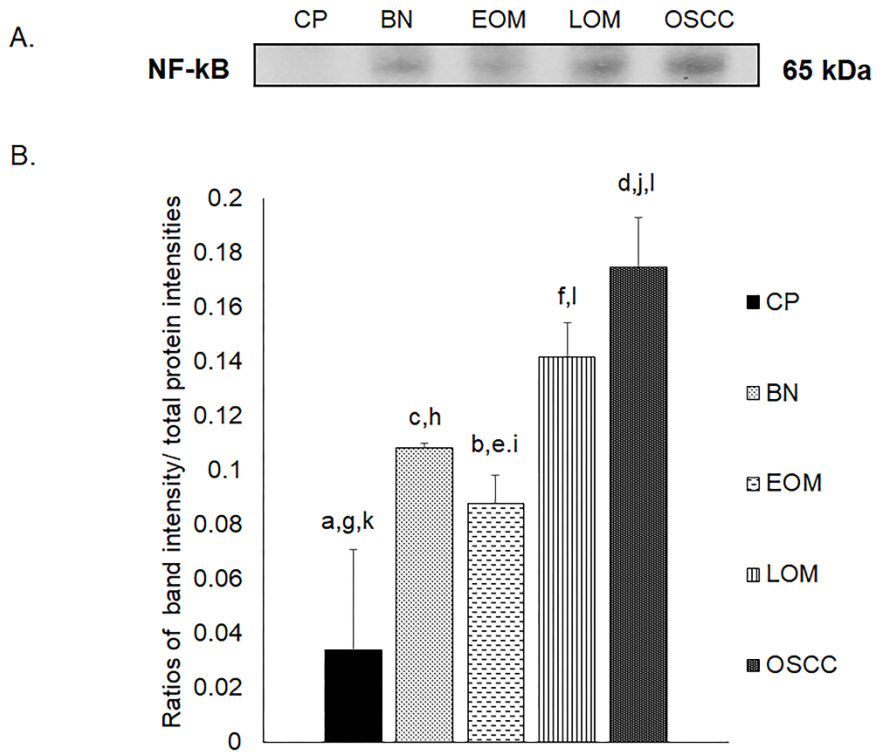

Fig. 8. Western blot analysis of nuclear factor kappa B (NF-**K**B) of pooled saliva samples from dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). Representative western blot for NF-**K**B at 65 kDa (A) and bar graph of ratios of NF-**K**B protein intensity to total blotted proteins in each lane in the first half of a membrane (B). a–b, c–d and e–f denote a significant difference at $p<0.05$. g–h and i–j denote a significant difference at $p<0.01$. k–l denote a significant difference at $p<0.001$.

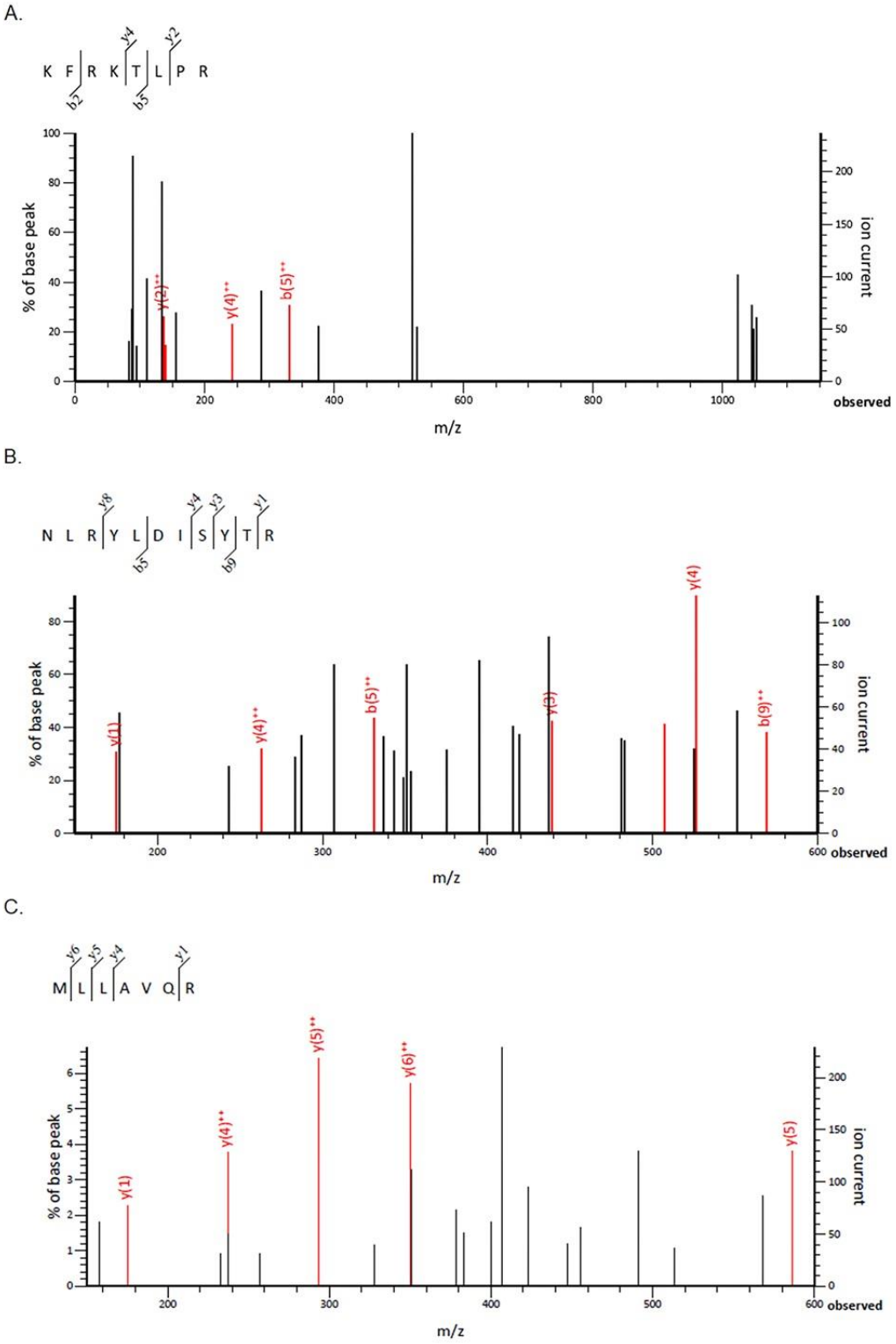

Fig. 9. Verification of expressed protein sequences by LC-MS/MS. MS/MS fragmentations of KFRKTLPR found in SENP7 (A), NLRYLDISYTR found in TLR4 (B), and MLLAVQR found in NF-**K**B **(C)** were shown.

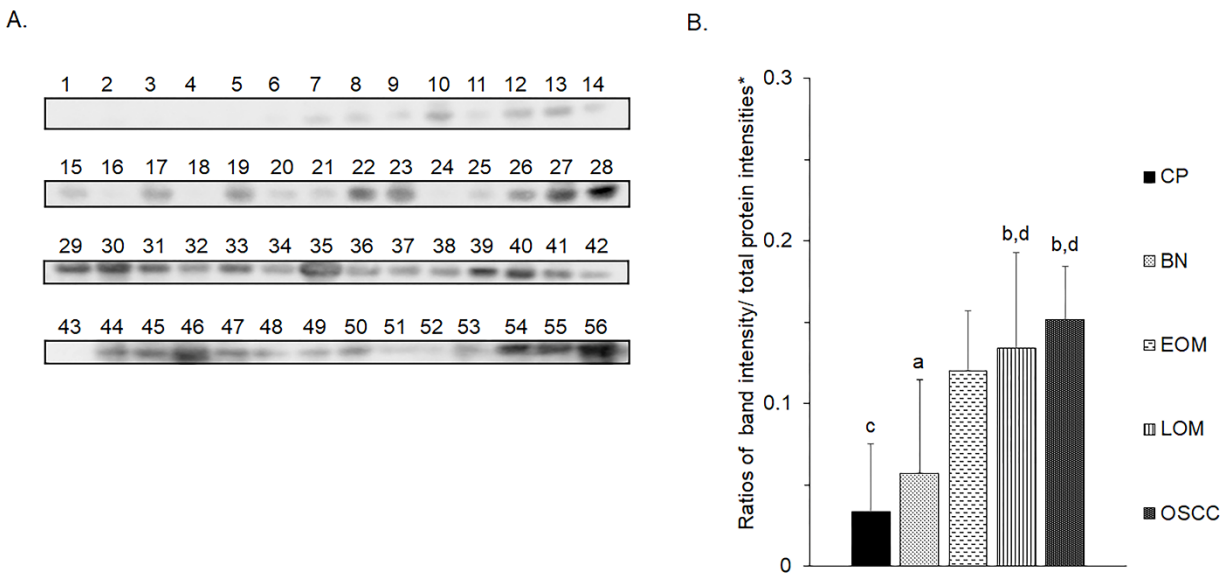

Fig. 10. Western blot analysis of salivary sentrin-specific protease 7 (SENP7) of individual saliva from dogs with benign oral tumors (BN, lanes 15-25), early- and late-stage oral melanoma (EOM, lanes 10-14, and LOM, lanes 35-56, respectively), oral squamous cell carcinoma (OSCC, lanes 26-34) and periodontitis (lanes 7-9) and normal controls (lanes 1-6) (CP). Representative western blot for SENP7 at 37 kDa (A) and bar graph of ratios of SENP7 protein intensity to total blotted protein intensities in each lane in the second half of a membrane (B). a–b and c-d denote a significant difference at $p<0.05$ and $p<0.01$, respectively.

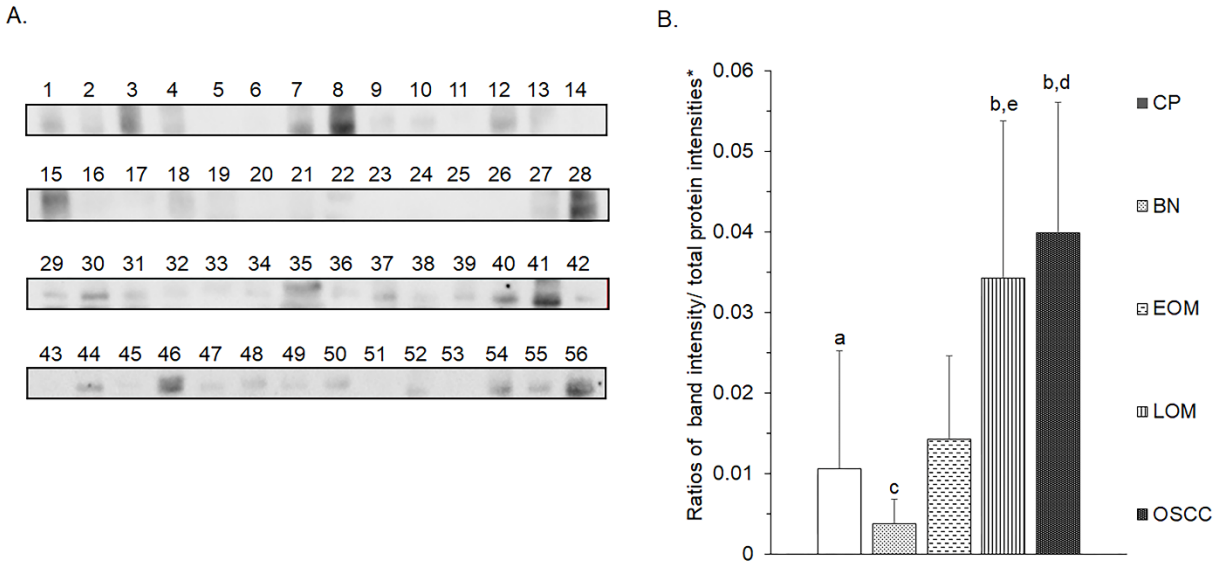

Fig. 11. Western blot analysis of salivary toll like receptor 4 (TLR4) of individual saliva samples from dogs with benign oral tumors (BN, lanes 15-25), early- and late-stage oral melanoma (EOM, lanes 10-14, and LOM, lanes 35-56, respectively), oral squamous cell carcinoma (OSCC, lanes 26-34) and periodontitis (lanes 7-9) and normal controls (lanes 1-6) (CP). Representative western blot for TLR4 at 95 kDa (A) and bar graph of ratios of TLR4 protein intensity to total blotted proteins in each lane in the first half of a membrane (B). a–b, c-d and c-e denote a significant difference at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

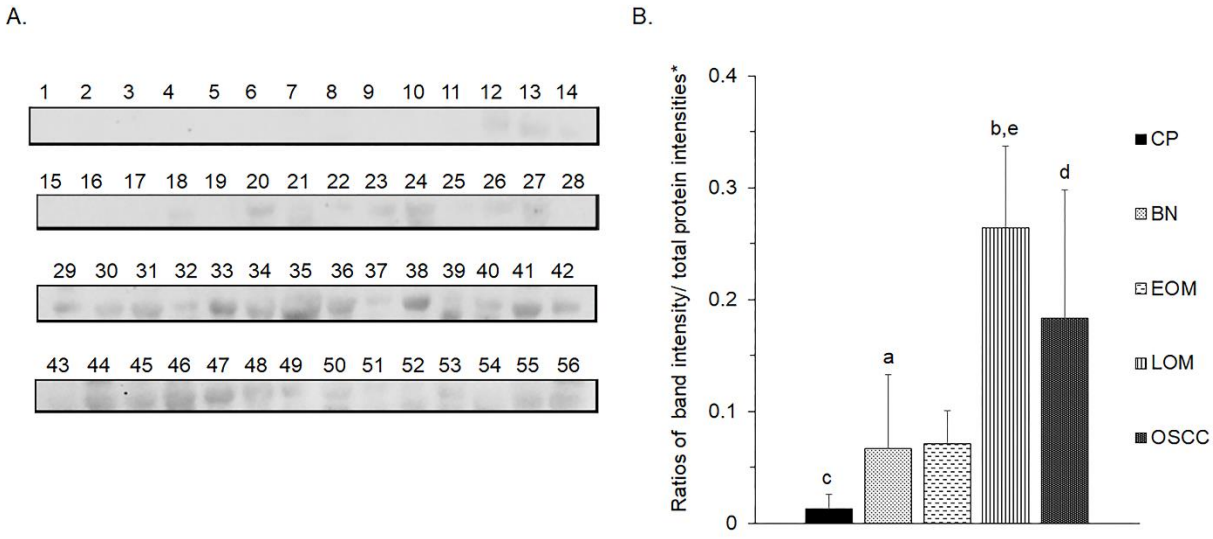

Fig. 12. Western blot analysis of nuclear factor kappa B (NF-**K**B) of individual saliva samples from dogs with benign oral tumors (BN, lanes 15-25), early- and late-stage oral melanoma (EOM, lanes 10-14, and LOM, lanes 35-56, respectively), oral squamous cell carcinoma (OSCC, lanes 26-34) and periodontitis (lanes 7-9) and normal controls (lanes 1-6) (CP). Representative western blot for NF-**K**B at 65 kDa (A) and bar graph of ratios of NF-**K**B protein intensity to total blotted proteins in each lane in the first half of a membrane (B). a–b, c-d and c-e denote a significant difference at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

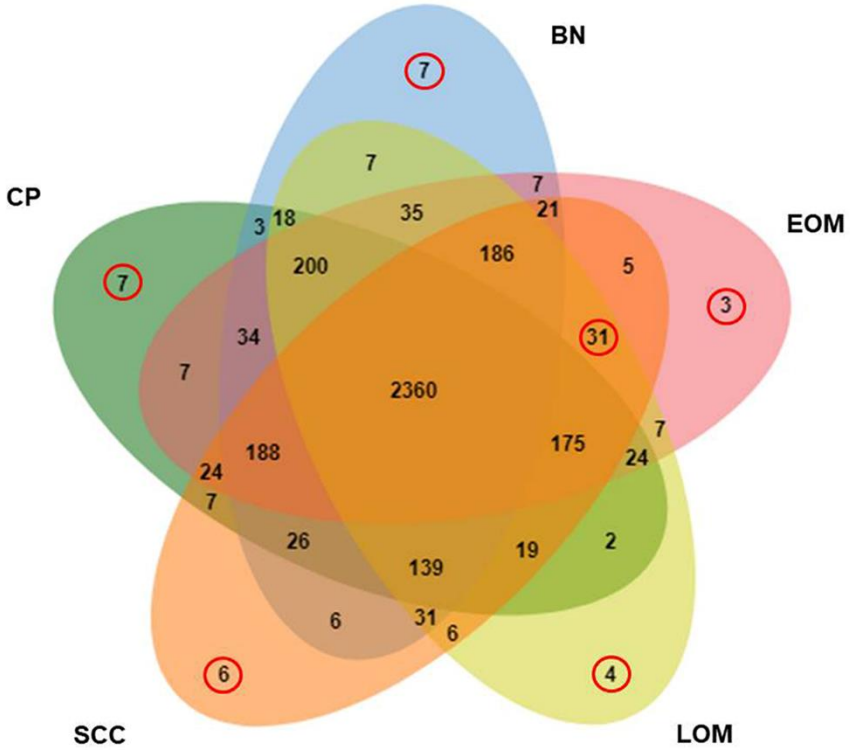

Fig. 13. Venn diagram of proteins differentially expressed in early-stage OM (EOM), late-stage OM (LOM), oral squamous cell carcinoma (OSCC), benign oral tumors (BN) and normal and periodontitis (CP). Circles indicate overexpressed proteins uniquely found in each group and commonly found in all cancerous groups.

Table 5. Overexpressed proteins uniquely found in normal controls and periodontitis, benign oral tumors, early-stage oral melanoma, late-stage oral melanoma and oral squamous cell carcinoma based on biological process involvement and protein score

Database	Protein name	Protein ID score	Peptides	Biological process	Subcellular distribution
Normal controls and periodontitis:					
XP_016007048.1	Semaphorin-4B isoform X1	13.9	QLVASYCPK	1. Negative chemotaxis 2. Semaphorin–plexin signalling pathway	1. Extracellular space 2. Integral component of plasma membrane
XP_011988340.1	Visual system homoeobox 1 isoform X2	16.98	FPGRPLPSAARQK	1. Multicellular organism development 2. Regulation of transcription	1. Nucleus 2. Cytoskeleton
XP_013973434.1	CDK5 regulatory subunit-associated protein 2 isoform X1	12.52	FTNQGKR	Microtubule organizing center	
XP_002689199.3	Olfactory receptor 2M5	26.19	MCWQVAAMSWAGGAR	Olfaction	Plasma membrane
XP_008048855.1	Potassium voltage-gated channel subfamily Q member 1	34.67	LNIEDFR	1. Potassium ion export across plasma membrane 2. Cellular response to cAMP	1. Endoplasmic reticulum 2. Endosome 3. Plasma membrane
XP_007125871.1	GLIPR1-like protein 1	14.03	AHNEAR	Single fertilization	Plasma membrane

EHB15707.1	Transient receptor potential cation channel subfamily M member 5	26.14	TVAPKSLLFR	Ion transmembrane transport	Plasma membrane
Benign oral tumors:					
KFO21119.1	Germ cell-less protein-like 1	7.86	KAVAAR	Cell differentiation	Nucleus
XP_004629194.1	Poly [ADP-ribose] polymerase 12	21.09	KLGMSSELVHR	Protein auto-ADP-ribosylation	Nucleus
XP_015289690.1	Lamin tail domain-containing protein 2	8.98	GLLPPMSSGK	Cell population proliferation	1. Cytoskeleton 2. Nucleus
XP_012868232.1	Telomeric repeat-binding factor 2-interacting protein 1	16.48	AEPDPEAAESVEPQTK	1. Negative regulation of DNA recombination at telomere 2. Positive regulation of NF-**K**B transcription factor activity	Nucleus
XP_012373519.1	Myb-related protein B	16.69	MLPGRYVPGGGVGAR	1. Mitotic cell cycle 2. Regulation of cell cycle	Nucleus
XP_012865682.1	Erythrocyte membrane protein band 4.2	12.59	QWSAVVEDR	1. Cell morphogenesis 2. Hemoglobin metabolic process	Cytoskeleton 1. Cytoplasm 2. Membrane
XP_005371197.1	Long-chain-fatty-acid–CoA ligase ACSBG2	5.91	APGTGFLTEMLR	cell differentiation	

Early-stage oral melanoma:					
XP_011760132.1	Putative protein SSX6	12.53	GGNMPGPTGCVR	Regulation of transcription, DNA-templated	Nucleus
XP_004326275.1	Bromodomain testis-specific protein-like	14.28	DNAKPMNYDEKR	Chromatin remodelling	Nucleus
XP_006868797.1	Zinc finger protein GLI2-like	16.61	GGSLENSSIPDLSR	Nucleic acid binding	Nucleus
Late-stage oral melanoma:					
EPQ15807.1	Transformation/transcription domain-associated protein	9.28	AMAILTPAVPAR	1. DNA repair 2. Histone deubiquitination	1. Golgi apparatus 2. Nucleus
XP_009240233.1	Glutathione S-transferase-like	20.93	ARISHILTINK	Glutathione transferase activity	Cytoplasm
XP_011282224.1	Protein FAM186A	32.14	SVEQSFLELLIEEDR	No data	1. Nucleus 2. Cytoplasm
XP_004412391.1	Deleted in lung and oesophageal cancer protein 1	7.49	AGPPKNK	Negative regulation of cell population proliferation	Cytoplasm
Oral squamous cell carcinoma:					
XP_007944568.1	Ankyrin repeat domain-containing protein 26-like	6.56	ADIKENMVIDMQANCMILXK	Protein interaction	Cytoplasm

XP_012392091.1	Cytohesin-4 isoform X2	9.84	YPGELSSGEAEELQR	Regulation of ARF protein signal transduction	Nucleus
XP_007532207.2	Probable C-mannosyltransferase DPY19L4	17.69	KPKSSGNK	Protein C-linked glycosylation via 2'-alpha-mannosyl-L-tryptophan	Membrane
EHB17858.1	Dynein heavy chain 11, axonemal	3.80	ATSEMR	Determination of left/right symmetry	Cytoskeleton
XP_004275614.1	Fanconi anaemia-associated protein of 100 kDa	7.99	XGMDDR	Interstrand cross-link repair	Nucleus
OBS77059.1	Protein A6R68_16468	7.01	DQVSDDVSVQSSGPNCQR	Regulation of transcription by RNA polymerase II	Nucleus

Table 6. Overexpressed proteins commonly found in early-stage oral melanoma, late-stage oral melanoma and oral squamous cell carcinoma based on biological process involvement and protein score

Database	Protein name	Protein ID score	Peptides	Biological process	Subcellular distribution
XP_005376885.1	ATP synthase subunit s, mitochondrial isoform X1	4.77	HQTMLFGK	ATP biosynthetic process	Mitochondria
XP_004411845.1	Carbonic anhydrase 12 isoform X1	33.40	SLHAAAVLLLLCFK	Carbonate dehydratase activity	Integral component of membrane
XP_015354861.1	Cell division cycle-associated protein 2	17.63	RSFCAPTLSSK	Cell cycle cell division	Nucleus
XP_004625867.1	dihydroorotate dehydrogenase (quinone), mitochondrial	17.17	IPIIGVGGVSSGQDAMDK	'de novo' UMP biosynthetic process	Mitochondrion inner membrane
XP_014948096.1	Hermansky–Pudlak syndrome 3 protein isoform X1	9.93	ACPPISMDVCALR	Organelle organization, pigmentation	Cytosol
XP_004644982.1	KN motif and ankyrin repeat domain-containing protein 3	14.22	FALNQNLPDLGGSR	Negative regulation of actin filament polymerization	Cytoplasm
XP_008158631.1	Leucocyte immunoglobulin-like receptor subfamily A member 6	3.43	EPAEVEELK	Adaptive immune response	Membrane
XP_003787787.1	Negative elongation factor C/D	7.47	SNFIMMN	Transcription by RNA polymerase II	Nucleus
XP_011285357.1	Neurexin-2-β	13.66	VVVVLGGQGSSG	Neuron cell–cell adhesion signal transduction	Membrane
XP_005629058.1	Origin recognition complex subunit 1 isoform X1	6.66	SRPTPSHPATPRAK	DNA replication, mitotic cell cycle	Nucleus

XP_006896914.1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP] isoform X1	18.32	ARVSQM	Gluconeogenesis	Cytosol
XP_004620060.1	Phospholipase B1, membrane-associated-like	11.55	RMENNSGINFNEDWK	Phospholipase activity	Integral component of membrane
XP_012626009.1	Progesterone receptor isoform X2	17.75	VLLLLNTTR	DNA-binding transcription factor activity	Nucleus
XP_008151988.1	Secernin-2	13.13	QGGITAEAMMDILRDK	Exocytosis	Extracellular exosome
XP_007489730.1	Sodium/iodide cotransporter	6.99	DSKEYPQEVK	Cellular response to cAMP	Membrane
XP_016811442.1	T-box transcription factor TBX18 isoform X2	12.54	MYSGELGPI	DNA-binding transcription factor activity	Nucleus
XP_004045865.1	Uncharacterized protein LOC101132572	12.64	RFTLSLDAPAPTQGVCK	Unknown	Unknown
XP_006190947.1	Zinc finger protein ZIC 3	8.6	THTGKGEGGR	Cell differentiation	Nucleus
XP_011744397.1	28S ribosomal protein S14, mitochondrial	16.97	KNTXLPK	Mitochondrial translational elongation and translation	Mitochondria
XP_007505382.1	3-hydroxyisobutyrate dehydrogenase, mitochondrial isoform X1	8.97	SMASKTPVGFVGLGNM GNPMAK	3-hydroxyisobutyrate dehydrogenase activity	Mitochondria
XP_004448347.1	α-ketoglutarate-dependent dioxygenase alkB homolog 4 isoform X1	7.08	LVSLNLLSSTVLSMSR	Demethylation	Mitochondria
XP_005065718.1	Ankyrin repeat domain-containing protein 34B	20.75	QKALMTTNGPK	Unknown	Nucleus

NP_036833.1	β1 adrenergic receptor	13.02	QGFSSESK	Adenylate cyclase-activating adrenergic receptor signalling pathway	Endosome, plasma membrane
ELK12127.1	Cytochrome b-c1 complex subunit 2, mitochondrial	11.51	DNMAYTGEGLR	Aerobic respiration	Mitochondria
XP_006883886.1	E3 SUMO-protein ligase RanBP2	11.07	LSQSGHMLINLSRGK	centrosome localization	Nucleus
BAD96349.1	Heme oxygenase (decyclizing) 2 variant	11.2	KSSGALEK	Heme oxygenase (decyclizing) 2 variant	Endoplasmic reticulum
OBS70980.1	Pyrroline-5-carboxylate reductase	9.86	LTAFXPAPK	L-proline biosynthetic process	Mitochondria
XP_015976454.1	Laminin subunit α1	15.83	YXNGTWYK	Cell adhesion	Extracellular region or secreted
KFO28259.1	Mitochondrial import receptor subunit TOM20 like protein	10.02	LFSVQMPLAKLPTTGQR	Protein import into mitochondrial matrix	Mitochondria
EAW72809.1	Signal sequence receptor, delta (translocon-associated protein delta), isoform CRA_c	3.09	APTQAPMR	Regulate the retention of ER resident proteins	Endoplasmic reticulum
XP_006865897.1	Tyrosine-protein phosphatase non-receptor type 5	21.9	AEGLRGSHR	Cellular response to cytokine stimulus	Endoplasmic reticulum

Additionally, the correlation of PTPN5 and cyclophosphamide was demonstrated (Fig. 14). Western blot analysis unveiled an enhanced expression of PTPN5 and p53 in tumor groups compared with that in the CP group (Figs. 15 and 16). In addition, the expression of PTPN5 in LOM and OSCC was augmented compared with that in BN and EOM (Fig. 15). Peptide sequences of PTPN5 and p53 western blot analysis were verified by LC-MS/MS (Fig. 17).

4.3. Salivary proteomics in monitoring therapeutic response of COM using LC-MS/MS

The STS and LTS had median survivals of 3 and 14.5 m, respectively. The two survival curves were illustrated with a p value of 0.0046 (Fig. 18). A total of 132, 29 and 74 proteins were commonly found in individuals of PreS, PostS, and R and M, respectively. Predicted ubiquitin D (*Rousettus aegyptiacus*) and predicted transient receptor potential cation channel subfamily M (melastatin) member 8 (TRPM8) channel-associated factor 2 (*Monodelphis domestica*) appeared in all samples in every group of chemotherapy treatment, excluding the PreS, PostS, R and M groups. Western blot analysis showed the expression of fUBD and cUBD in STS and LTS samples was illustrated in Figs. 19—20. In dogs with STS, a significantly increased ratio of fUBD to cUBD was shown in PreS compared with that of AT2, AT4 and AT5 (Fig. 21). The ratio was also shown to be significantly augmented in PreS group with STS compared with that with LTS ($p<0.01$). In addition, the expression of fUBD was enhanced in PreS compared with that of AT2 (Fig. 22). UBD sequence was confirmed by LC-MS/MS (Fig. 23).

4.4. Serum proteomics of canine oral tumors using MALDI-TOF MS coupled with LC-MS/MS

All 32 replicates in each pooled sample group demonstrated the homogeneity within the group. A 3-dimensional view of the PCA plot showed distinct clusters of each group (Fig. 24). PMFs of CP, EOM, LOM, OSCC and BN groups were observed, and peptide masses at 1000—5000 Da were selected by ClinProTools software. A peptide fragment of IgSF10 appeared in the EOM and LOM at 2690 Da (Fig. 25). However, western blot analysis showed no statistically significant difference was observed among groups (Fig. 26). IgSF10 sequence was confirmed by LC-MS/MS (Fig. 27).

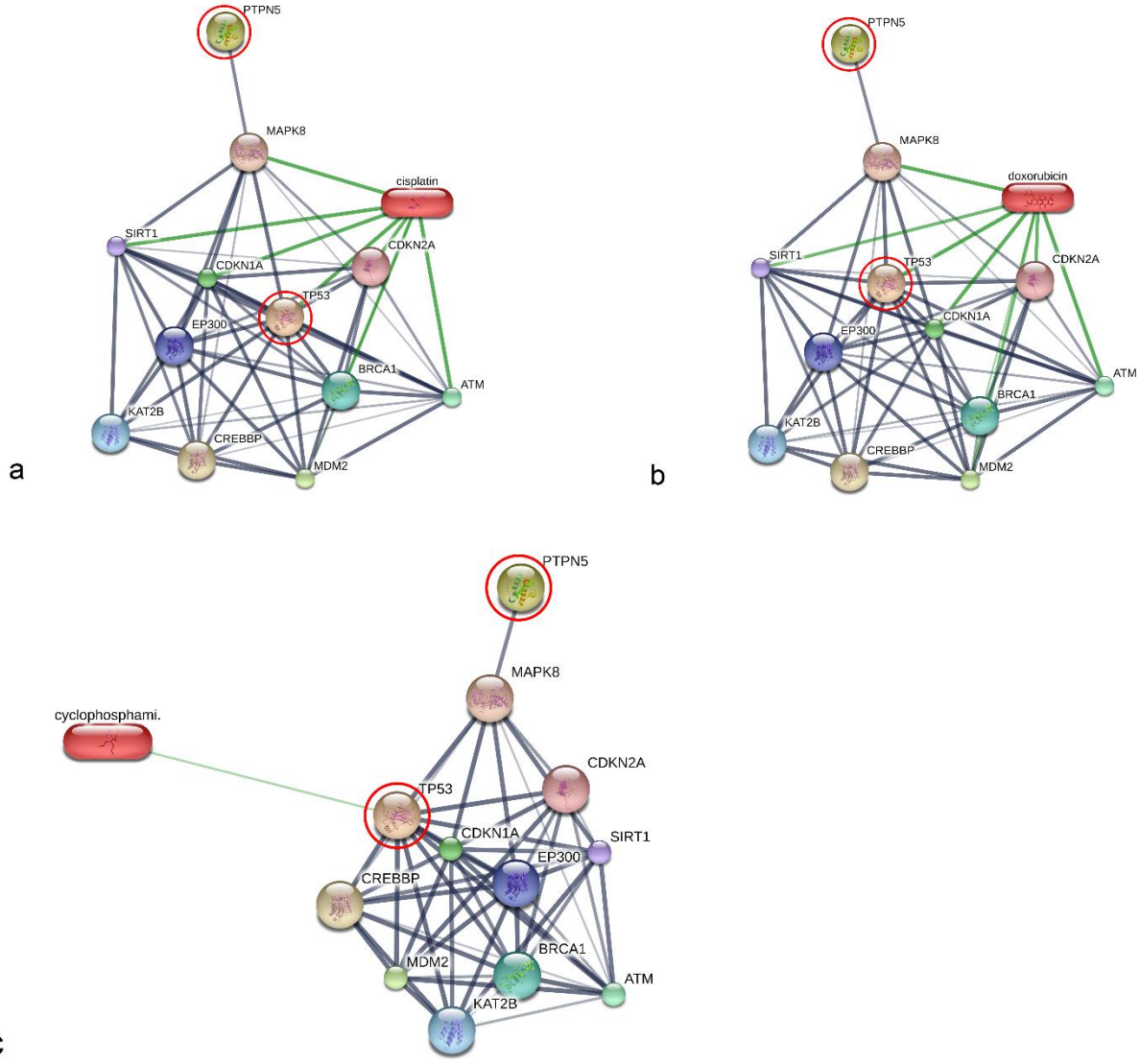

Fig. 14. Involvement of tyrosine-protein phosphatase non-receptor type 5 (PTPN5) and tumor protein p53 (TP53) in networks of protein chemotherapy drug interactions, cisplatin and doxorubicin, analysed by Stitch, version 5.0. Interactions of PTPN5 and TP53 with cisplatin (A). Interactions of PTPN5 and TP53 with doxorubicin (B), Interactions of PTPN5 and TP53 with cyclophosphamide (C). Red circles: PTPN5 and TP53. Abbreviations: ataxia telangiectasia mutated (ATM), breast cancer 4721, early onset (BRCA1), cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), cyclin-34473 dependent kinase inhibitor 2A (CDKN2A), CREB binding protein (CREBBP), E1A binding 474 protein p300 (EP300), K(lysine) acetyltransferase 2B (KAT2B), mitogen-activated protein kinase 4758 (MAPK8), Mdm2 (MDM2) and sirtuin 1 (SIRT1).

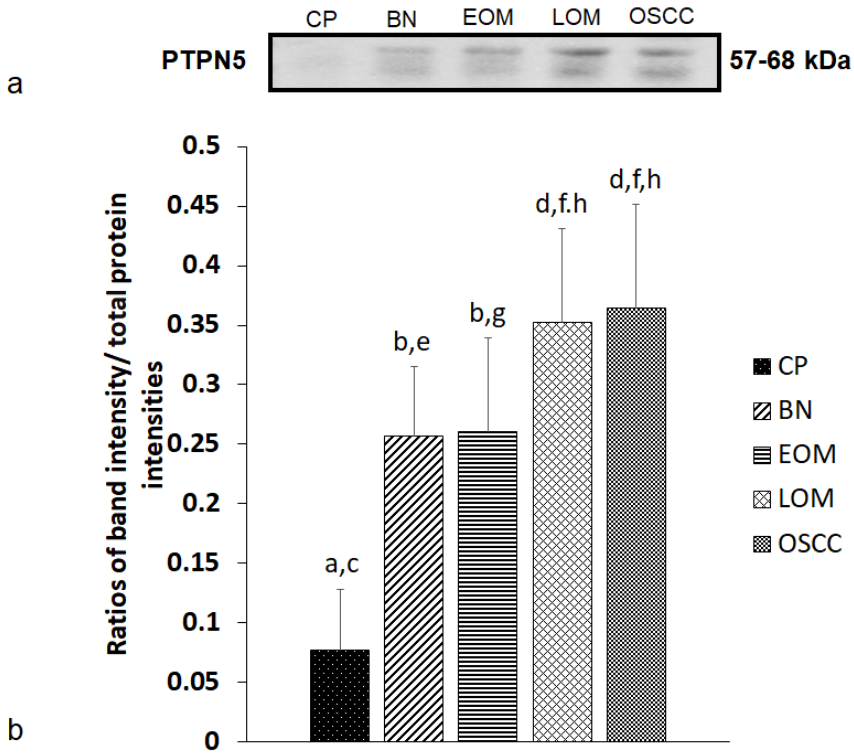

Fig. 15. Western blot analysis of salivary tyrosine-protein phosphatase non-receptor type 5 (PTPN5) of dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). Representative western blot for PTPN5 at 57–68 kDa (A). Bar graph of ratios of PTPN5 protein intensity to total blotted proteins in each lane in a membrane (B). a-b denote a significant difference at $p<0.05$; c-d denote a significant difference at $p<0.001$.

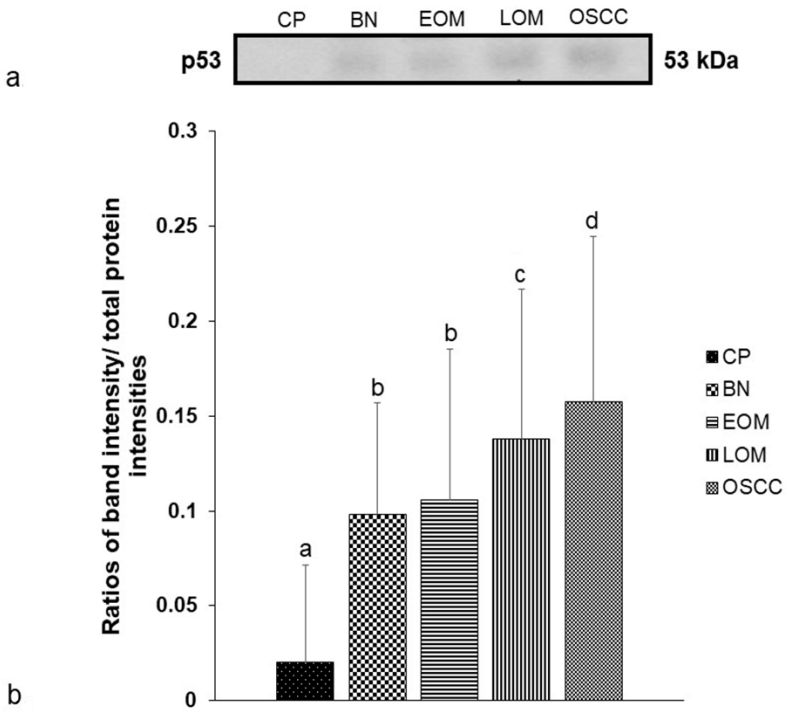

Fig. 16. Western blot analysis of salivary tumor protein p53 (p53) of dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). A representative western blot for P53 at 53 kDa (A). Bar graph of ratios of P53 protein intensity to total blotted proteins in each lane in a membrane (B). a-b denote a significant difference at $p<0.05$; a-c denote a significant difference at $p<0.01$; a-d denote a significant difference at $p<0.001$.

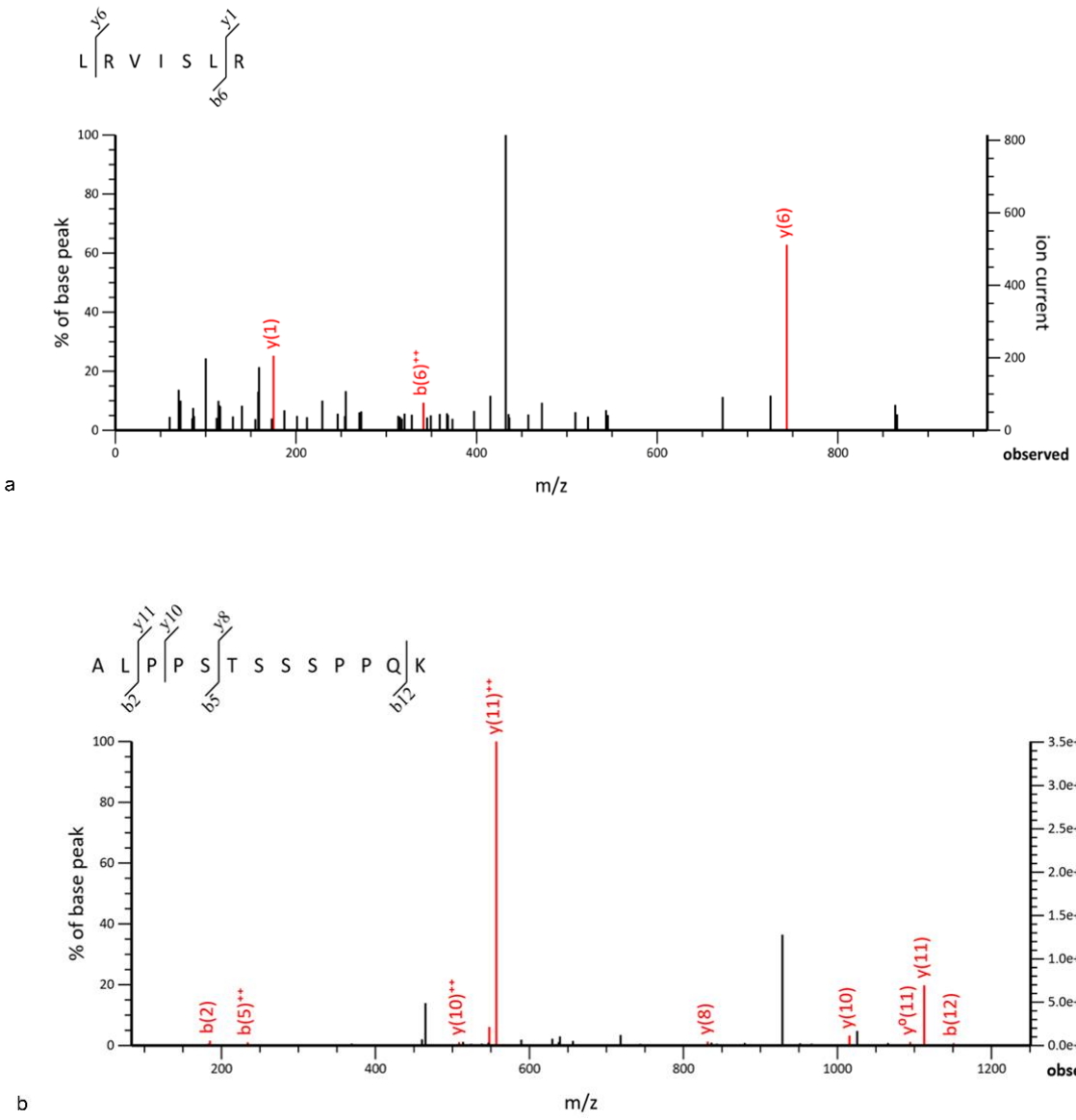

Fig. 17. Verification of expressed protein sequences by LC-MS/MS. MS/MS fragmentations of LRVISLR found in salivary tyrosine-protein phosphatase non-receptor type 5 (PTPN5) (a). ALPPSTSSSPPQK found in salivary tumor protein p53 (p53) (b).

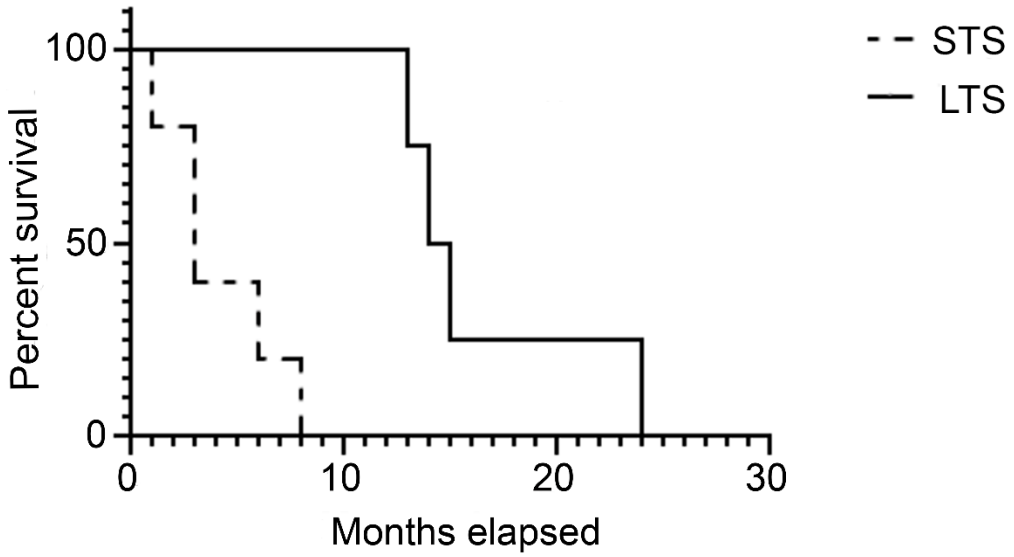

Fig. 18. Probability of overall survival of dogs with oral melanoma treated with surgery and chemotherapy with the short-term (<12 months after surgery, in dot line) and long-term survival periods (>12 months after surgery, in full line).

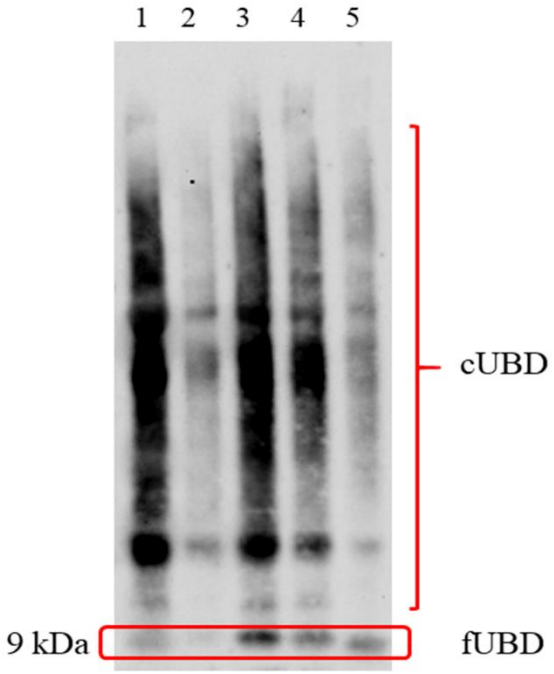

Fig. 19. Representative western blot of patients with short-term survival for free ubiquitin D (fUBD) at 9 kDa and conjugated ubiquitin D (cUBD) in saliva. Lane 1: Pre-surgery (PreS); Lane 2: Post-surgery (PostS); Lane 3: After treating with chemotherapy drug for 1 time; Lane 4: After treating with chemotherapy drug for 2 times; Lane 5: After treating with chemotherapy drug for 3 times.

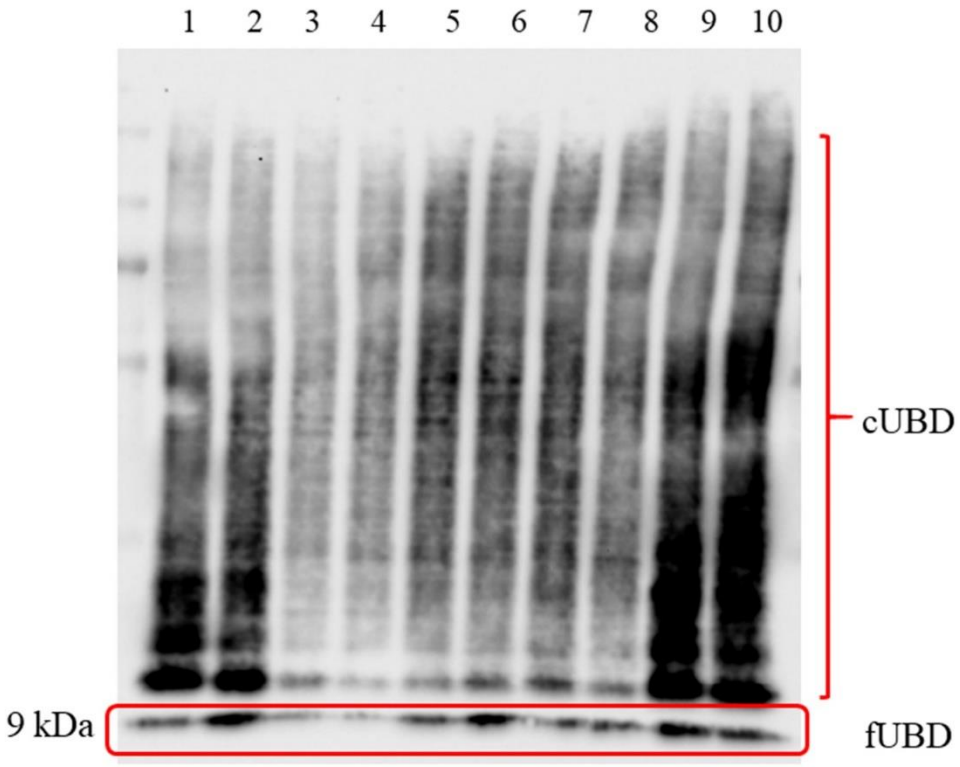

Fig. 20. Representative western blot of patients with long-term survival for free ubiquitin D (fUBD) at 9 kDa and conjugated ubiquitin D (cUBD) in saliva. Lane 1: Pre-surgery (PreS); Lane 2: Post-surgery (PostS); Lane 3: After treating with chemotherapy drug for 2 times; Lane 4: After treating with chemotherapy drug for 3 times; Lane 5: After treating with chemotherapy drug for 4 times; Lane 6: After treating with chemotherapy drug for 6 times; Lane 7: 1st Checking up after treating with chemotherapy drug; Lane 8: 2nd Checking up after treating with chemotherapy drug; Lane 9: 3rd Checking up after treating with chemotherapy drug; Lane 10: 4th Checking up after treating with chemotherapy drug.

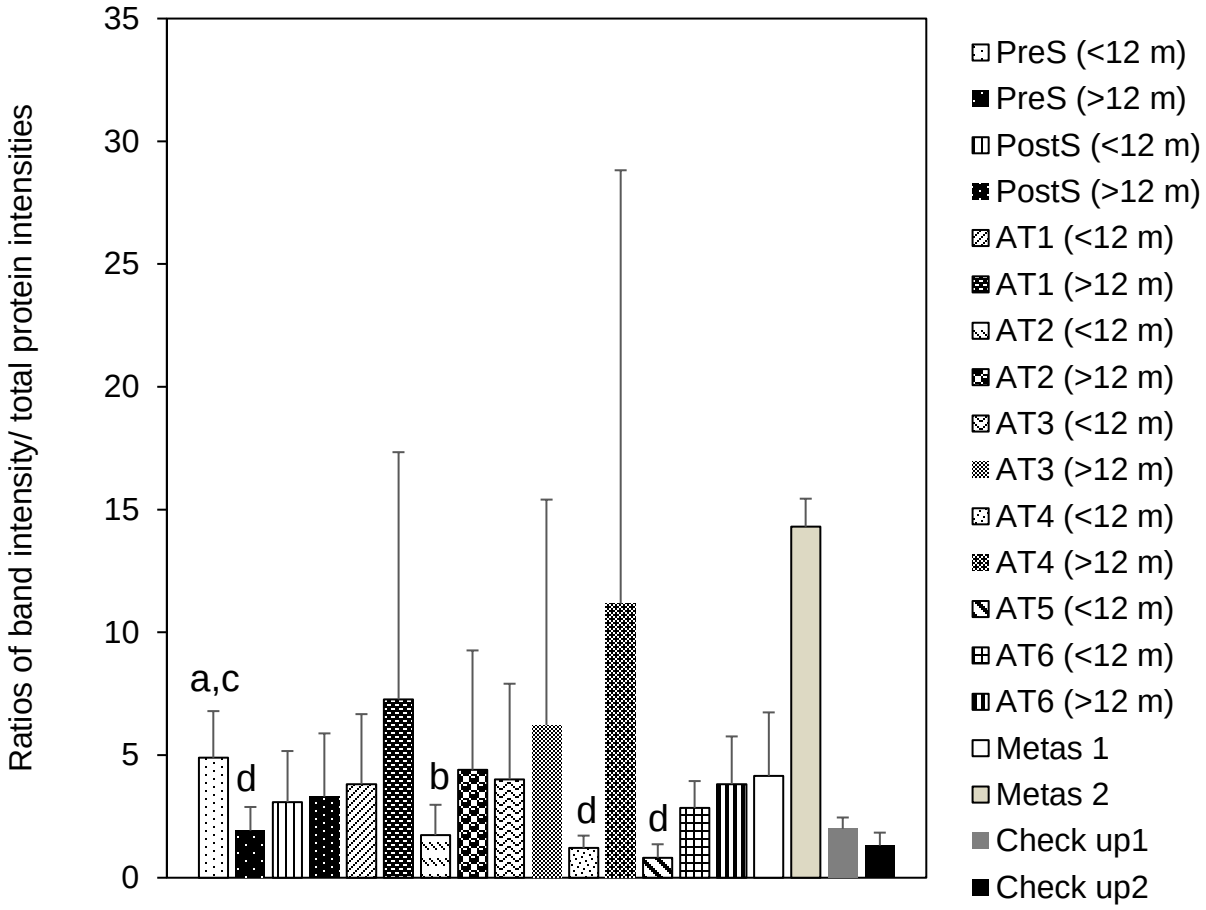

Fig. 21. Western blot analysis of ratios of free ubiquitin D (fUBD) to conjugated ubiquitin D (cUBD) in short-term survival (<12 m) and long-term survival groups (>12 m). PreS: Pre-surgery; PostS: Post-surgery; AT1-6: After treating with chemotherapy drug for 1-6 times; Metas: Metastasis after treatment; Check up: Check up after treatment.

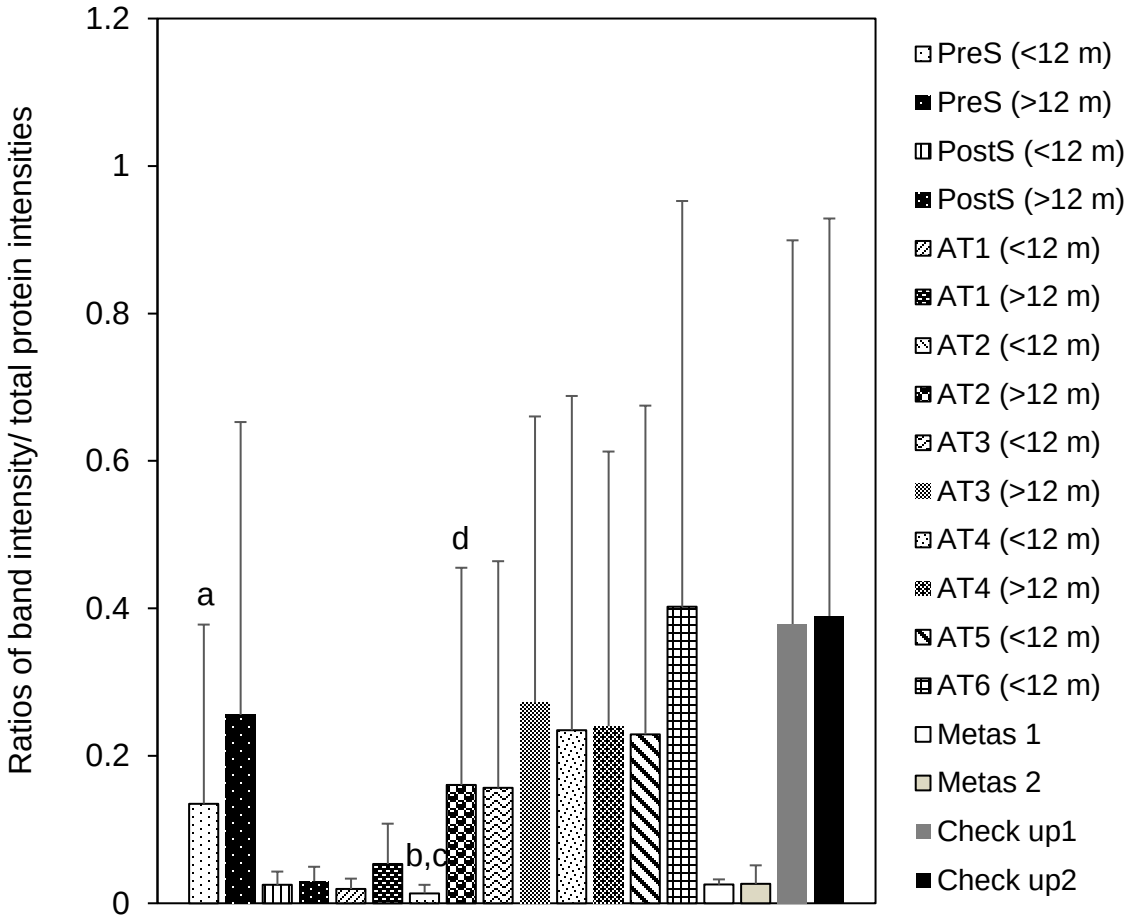

Fig. 22. Western blot analysis of free ubiquitin D (fUBD) in short-term survival and long-term survival groups. PreS: Pre-surgery; PostS: Post-surgery; AT1-6: After treating with chemotherapy drug for 1-6 times; Metas: Metastasis after treatment; Check up: Check up after treatment.

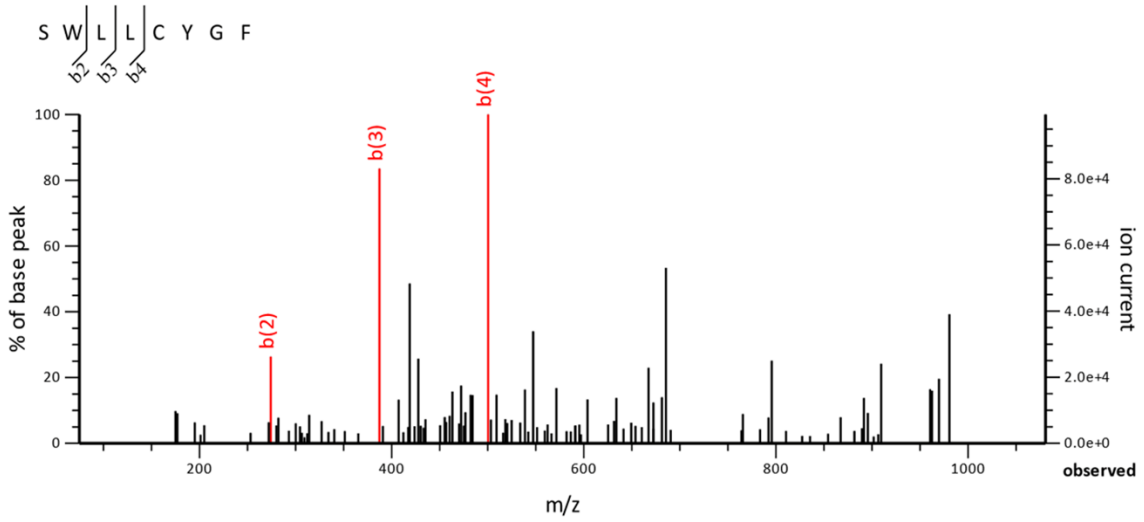

Fig. 23. Verification of UBD sequence by LC-MS/MS. MS/MS fragmentation of SWLLCYGF found in free ubiquitin D (fUBD) was shown

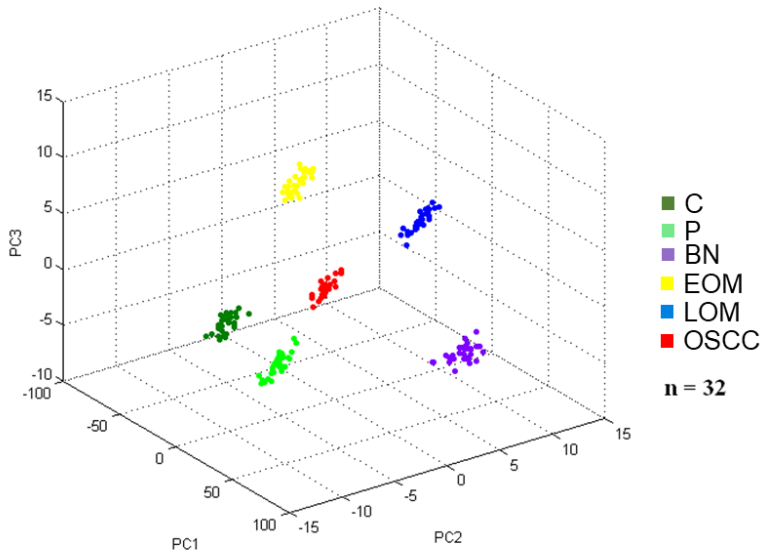

Fig. 24. Three-dimensional principal component analysis scatterplot of normal (C), periodontitis gingiva (P), benign tumors (BN), early stage oral melanoma (EOM), late-stage OM (LOM) and oral squamous cell carcinoma (OSCC). Thirty two dots represent replicate in each pooled sample group.

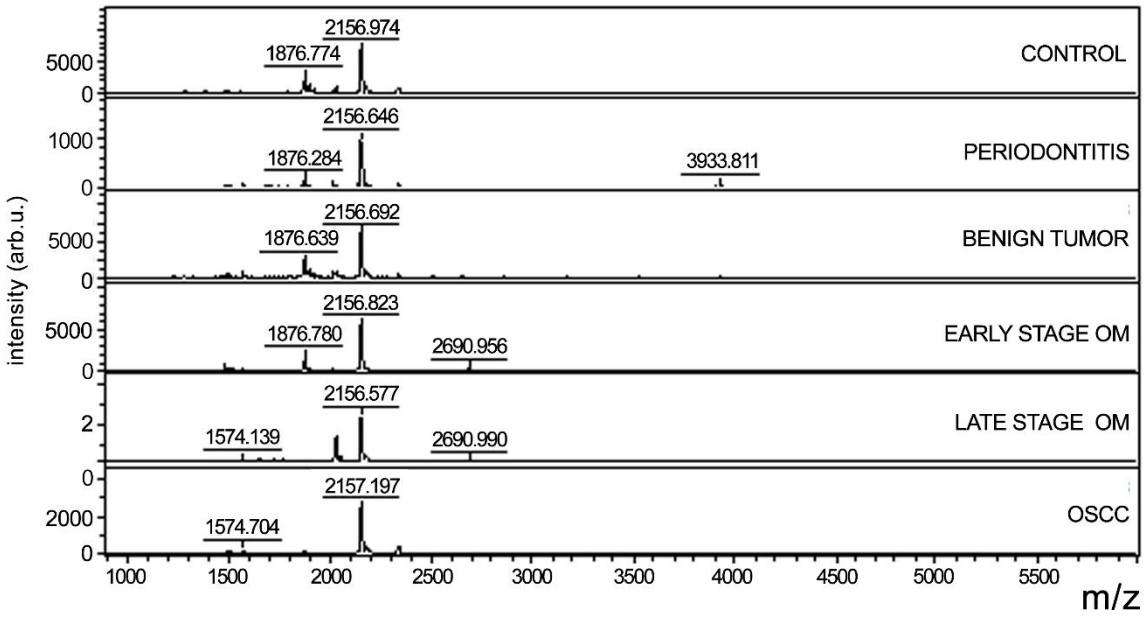

Fig. 25. Serum peptide mass fingerprint (PMF) of normal controls, periodontitis, benign tumors, early-stage oral melanoma (OM), late-stage OM and oral squamous cell carcinoma (OSCC) in the range 1000–5000 Da.

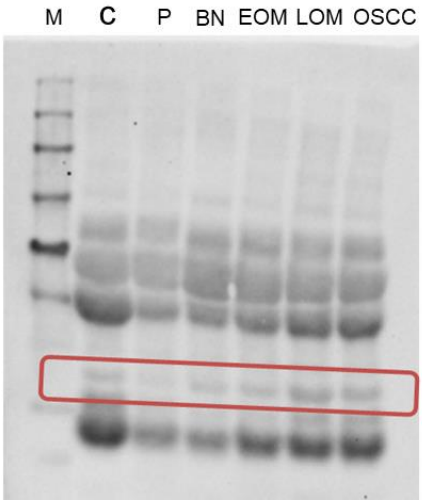

Fig. 26. Western blot analysis of immunoglobulin superfamily member 10 (IgSF10) of pooled serum samples from dogs with periodontitis (P), normal controls (C), benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively) and oral squamous cell carcinoma (OSCC). Representative western blot for IgSF10 at 37 kDa.

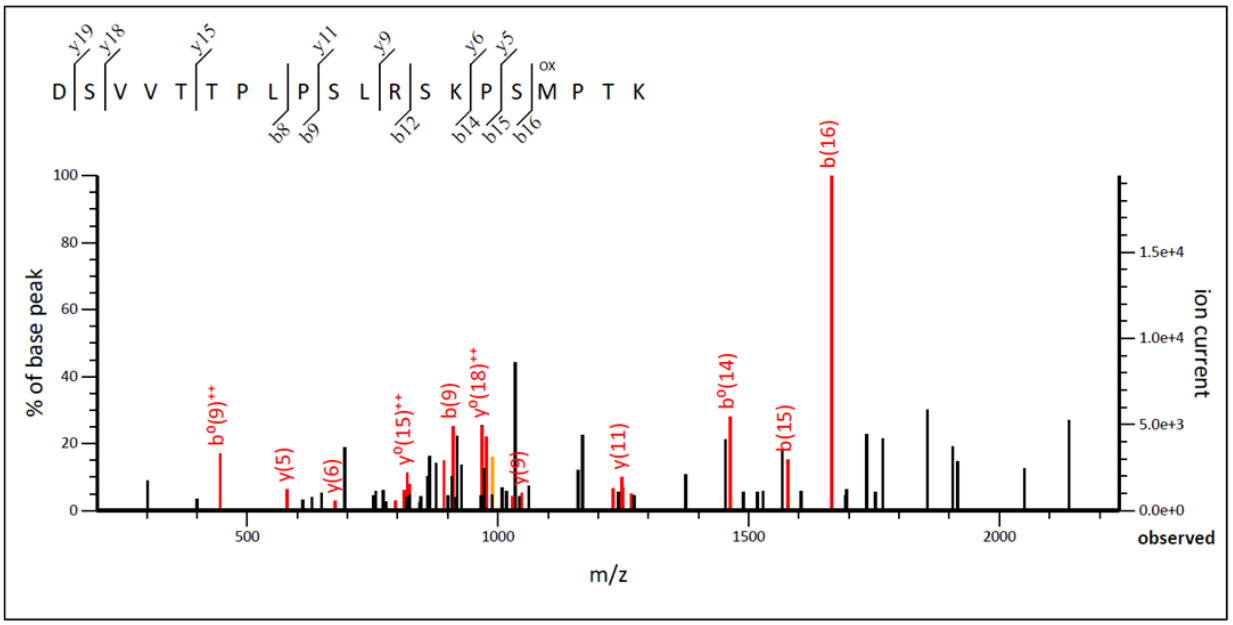

Fig. 27. Verification of expressed protein sequences by LC-MS/MS. MS/MS fragmentations of DSVVTTPLPSLRSKPSMPTK found in IgSF10 was shown.

4.5. Serum proteomics of canine oral tumors using GeLC-MS/MS

A total of 3907 proteins were identified. The distribution of the individual and overlapped proteins in EOM, LOM, OSCC, BN, P and C groups was illustrated by a Venn diagram (Fig. 28). TRAF3IP1 appeared in the EOM, LOM, and OSCC groups. However, western blot analysis showed no statistically significant difference was observed among groups (Fig. 29). TRAF3IP1 sequence was confirmed by LC-MS/MS (Fig. 30).

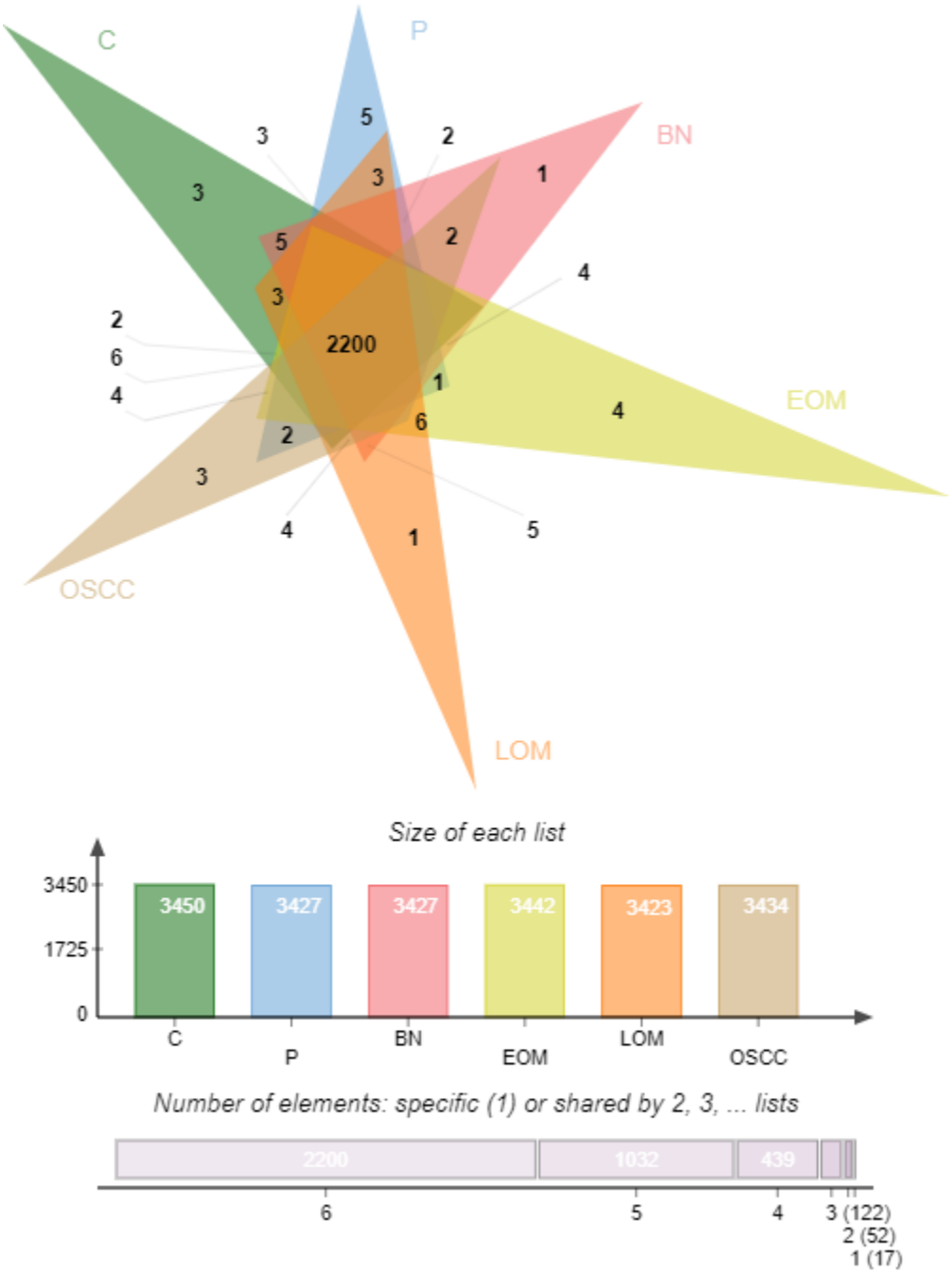

Fig. 28. Venn diagram of proteins differentially expressed in early-stage OM (EOM), late-stage OM (LOM), oral squamous cell carcinoma (OSCC), benign oral tumors (BN), periodontitis (P) and normal (C).

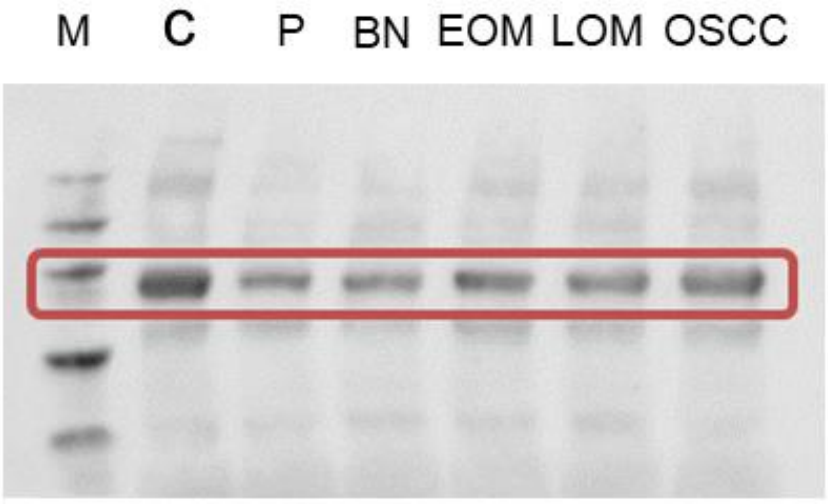

Fig. 29. Western blot analysis of TNF receptor-associated factor 3 interacting protein 1 (TRAF3IP1) of pooled serum samples from dogs with periodontitis (P), normal controls (C), benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively) and oral squamous cell carcinoma (OSCC). Representative western blot for TRAF3IP1 at 95 kDa.

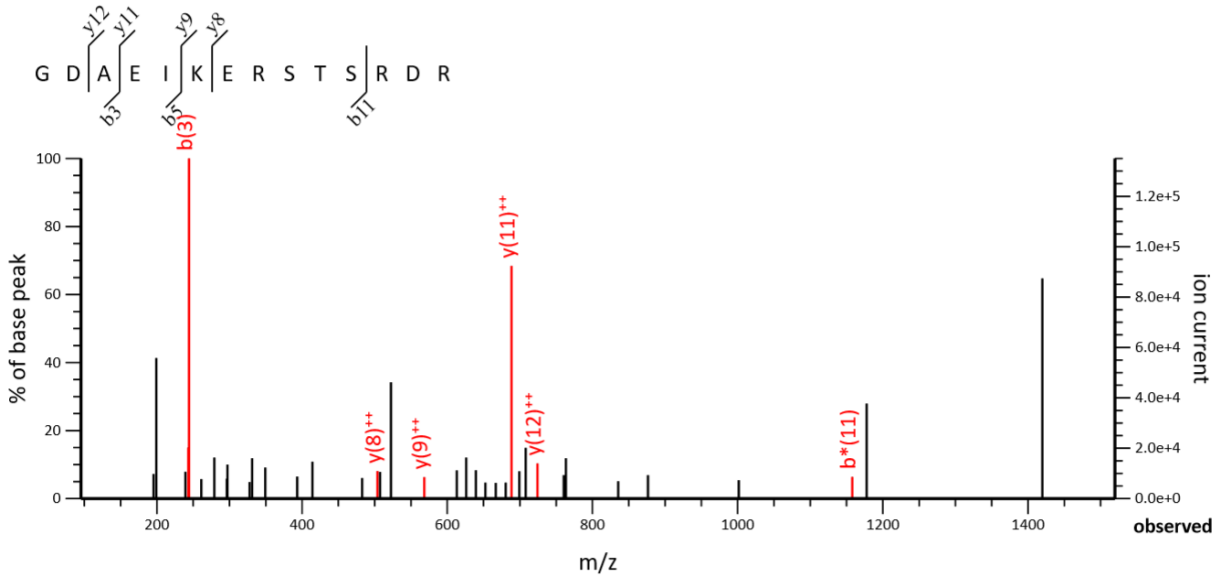

Fig. 30. Verification of expressed protein sequences by LC-MS/MS. MS/MS fragmentations of GDAEIKERSTSRDR found in TRAF3IP1 was shown.

5. Discussion

In the first study of salivary proteomics of canine oral tumors using MALDI-TOFMS coupled with LC-MS/MS, discrete sample groups from the PCA showed that MALDI-TOF MS could possibly be used as a rapid and reliable method for detection of canine oral tumors. MALDI-TOF MS has been reported to be a potential tool to characterize human head and neck squamous cell carcinoma from oral brush biopsy, human OSCC from oral fluid and canine oral tumors from tissues (Hu et al., 2007; Remmerbach et al., 2011; Maurer et al., 2013). Specific mass signals were found from saliva of oral lichen planus (OLP) patients as well as the discovery of a relationship between OLP and oral cancer (Hu et al., 2008). Moreover, salivary PMFs showed a number of proteins that were differently expressed in the early-stage OSCC compared with healthy patient, using MALDI-TOF MS combined with magnetic beads (Jiang et al., 2015). The fast, high accuracy and high sensitivity of MALDI-TOF MS made it suitable for screening oral cancers from biological fluids, especially saliva, which was easy to collect. This could help reduce the recurrence of the disease in the future.

Mass spectral peaks were analyzed for peptide and nominated protein identification. Differential protein expression has been revealed in canine oral tumors by MALDI-TOF MS coupled with LC-MS/MS and by in-gel digestion coupled with mass spectrometry (GeLC MS/MS) from tissues of oral tumors (Pisamai et al., 2018). We did not find similar proteins to those from tumor tissues which was probably due to the different source of samples, as proteins in saliva could be either secretary proteins from zygomatic, parotid, mandibular and sublingual salivary glands or proteins from oral tumors. Compared with the previously reported normal canine salivary proteomics, a number of different proteins in the CP group were observed (Lucena et al., 2018; Pasha et al., 2018; Sanguansermsri et al., 2018; Torres et al., 2018) This was possibly owing to different groups of dogs in the study as we combined periodontitis and healthy dogs as a CP group. In addition, different dog breeds and environments as well as different proteomic approaches could affect the results (Lucena et al., 2018; Pasha et al., 2018; Torres et al., 2018).

Our study showed for the first time elevated SENP7 in canine oral tumors. SENPs are small ubiquitin-like modifier (SUMO)-specific protease. SENP7 functioned to deconjugate SUMO from cellular substrates (Bawa-Khalfe and Yeh, 2010). The long SENP7 transcript has been reported to promote epithelial–mesenchymal transition and decrease the cell adhesion molecule E-cadherin

(CDH1) in breast cancer cell line, which could lead to the metastasis of the disease (Bawa-Khalfe et al., 2012). SENP7 also showed a strong relationship with the chemotherapy drugs. The networks of SENP7 as well as other targets, KAT2B and DTX3L, with chemotherapy drugs showed the strong relationship with p53 protein, a biomarker of oral cancers (Carlos de Vicente et al., 2004; Pandya et al., 2018). The association of target proteins with p53 and chemotherapy drugs, especially carboplatin and doxorubicin, should be further investigated.

TLR4 expression has been reported in several cancers, including human head and neck cancer (Mai et al., 2013), human laryngeal and oral cancer cell line and melanoma (Molteni et al., 2006; Szczepanski et al., 2007; Goto et al., 2008). In fact, TLR4 has been reported to play divergent roles, either as a pro- or an anti-tumor agent (Awasthi, 2014). Our study demonstrated that TLR4 and NF-**K**B expressions were elevated in LOM and OSCC, which displayed intimate clusters in a PCA plot; hence, TLR4 promoted cancer progression and possibly served as a prognostic factor. In addition, TLR4 has been reported to regulate the inflammatory response by activating NF-**K**B either via the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway or the Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway (Mai et al., 2013; Kawasaki and Kawai, 2014). The TLR4—NF-**K**B pathway has been intensively studied in several cancers such as laryngeal carcinoma and ovarian carcinoma as prognostic markers and inhibiting of the pathway might serve as a potential treatment of the cancers. Suppressor of cytokine signaling 1 (SOCS1), a regulator of cytokine-mediated innate and adaptive immunity, has been reported to inhibit the TLR4—NF-**K**B pathway in laryngeal carcinoma. In addition, decreased TLR4 expression has been observed in ovarian carcinoma after treating with NF-**K**B inhibitor (Starska et al., 2009).

In the second study, GeLC-MS/MS was used to identify novel salivary biomarker candidates in canine oral tumors. PTPN5 and p53 were plausibly shown to be candidates in LOM and OSCC. PTP is a group of protein tyrosine phosphatases that have divergent functions, either promoting or suppressing cancer. Several oncogenic PTPs have been reported to be highly expressed in human breast cancer (Thorsell et al., 2007). PTPN5 is in the same non-receptor Cys-based classical PTPs as PTPN1 and PTPN11, which promoted tumorigenesis in ovarian cancer, gastric cancer, prostate cancer, breast cancer, leukaemia, colorectal cancer and uveal melanoma (Wiener et al.,

1994; Xu et al., 2005; Zhou et al., 2008; Wang et al., 2012; Hoekstra et al., 2016; Maacha et al., 2016; Bollu et al., 2017). To the best of our knowledge, this study presented for the first time the association of PTPN5 expression and canine oral cancers, particularly LOM and OSCC. Likewise, PTPN1 has been reported to be increased in canine oral cancer tissues by MALDI-TOF MS plus LC-MS/MS (Pisamai et al., 2018), indicating that expression of PTPNs might play a role in canine oral cancer. PTPN1 functioned via Src/Ras/Erk and PI3K/Akt pathways, whereas PTPN11 functioned via EGFR/Ras/MAPK pathways (Julien et al., 2007; Wang et al., 2012; Liu et al., 2015; den Hollander et al., 2016; Yu and Zhang, 2018). We also exhibited the enhanced expression of p53, in tumor groups, particularly in LOM and OSCC. Likewise, p53 was found in the interaction networks of PTPN5 and the chemotherapy drugs cisplatin and doxorubicin. p53 is a tumor suppressor protein; however, mutant p53 protein has been shown to be a biomarker in several cancers, such as human breast cancer, colorectal cancer, ovarian cancer, oesophageal squamous cell carcinoma, non-small cell lung cancer, and a prognostic marker in breast cancer, oesophageal squamous cell carcinoma, colon cancer, non-small cell lung cancer and B cell lymphoma (Samowitz et al., 2002; Balogh et al., 2006; Patil et al., 2011; Huang et al., 2014; Molina-Vila et al., 2014; Soragni et al., 2016; Solomon et al., 2018; Zaky et al., 2019). In human head and neck squamous cell carcinoma, p53 mutation played an important role in tumorigenesis and progression. It has been used not only as a risk and prognostic biomarker, but also as a predictive biomarker in the clinical response to chemotherapy treatments (Poeta et al., 2007; Peltonen et al., 2011; Sano et al., 2011; Gross et al., 2014; Osman et al., 2015). Several studies, aiming to treat cancer in humans, have investigated the promoting function of wild-type p53 and degradation of mutant p53 (Alexandrova et al., 2015; Parrales and Iwakuma, 2015; Soragni et al., 2016).

In the third study, LC-MS/MS and western blot were used to identify novel salivary biomarker candidates of COM during pre-operation, post-operation, after treatments with carboplatin for 1—7 times, metastasis and recurrence, and checking up. GeLC-MS/MS and LC-MS/MS have been used to compare salivary proteomes of healthy dogs with different breeds and between healthy dogs and human (Lucena et al., 2018; Pasha et al., 2018; Sanguansermsri et al., 2018; Torres et al., 2018). One publication has reported the salivary proteome in dogs infected with *Leishmania infantum*, using LC-MS/MS (Franco-Martinez et al., 2019). For canine oral tumor proteomics, MALDI-TOF MS has been used to analyze PMF, PCA scatterplots and potential protein candidates

in saliva and tissues of dogs with oral tumors (Pisamai et al., 2018; Ploypetch et al., 2019). To the best of our knowledge, this study has shown for the first time of salivary proteomics of OM in dogs for monitoring surgery and chemotherapy responses. From LC-MS/MS results, the expression of UBD appeared in all samples with chemotherapy treatment. The expression of UBD in different forms was further confirmed by western blot analysis. Ratios of fUBD to cUBD in PreS of LOM with STS were significantly higher than those in AT in contrast to the trends of those of LOM with LTS, possibly showing the potential prognostic biomarker for survival of LOM. In patients with stage IIB-IIC colon cancer, the expression of UBD has been identified as a recurrent risk and associated with STS after surgery (Yan et al., 2010; Zhao et al., 2015) UBD was overexpressed in cervical squamous cell carcinoma tissues and associated with tumor size and lymphatic metastasis (Peng et al., 2015). Silenced expression of UBD, regulated by miR-24-1-5p could enhance autophagy and apoptosis of human skin melanoma cells (Xiao et al., 2017). In fact, ubiquitination (UBQ) or the conjugation of ubiquitin to target proteins leads to the protein degradation by 26S proteasome (Fu et al., 1998). In our study, according to the higher ratios of fUBD to cUBD in dogs with STS treated with chemotherapy, the increased cUBD after therapy in individuals with STS and the increased fUBD in individuals with LTS in PreS group, it might be implied that the lower fUBD expression (or the higher UBQ) during treatment associated with the STS. Since several formula of chemotherapy drugs and treatment have been currently used in treating canine oral cancers, other suitable drugs or treatment might be considered for treating the STS group with high ratios of fUBD to cUBD, regarding to the concept of precision medicine for canine oral cancer (Ogilvie et al., 1989; Elmslie et al., 2008; Pang and Argyle, 2016). However, with the limit samples in this study, the study should be further investigated in larger populations. In addition, misregulated expression of several ubiquitin-conjugating enzymes used in UBQ, contributes to eccentric expression of NF**K**B and TGFβ and their signaling, leading to angiogenesis, increased invasiveness, chemotherapy resistance and metastasis of several cancers (Gallo et al., 2017). In fact, expression of NF**K**B has been reported in saliva of canine LOM and OSCC and the expression of SENP7, small ubiquitin-like modifier (SUMO)-specific protease 7, has been reported in saliva of dogs with oral squamous cell carcinoma (Ploypetch et al., 2019). The link of NF**K**B with Ub should be further investigated. For the serum proteomics, protein expression could not be verified by western blot. This might possibly due to the antibodies used not indeed for dogs as they are anti-human IgSF10 and anti-

human TRAF3IP1 antibodies. In addition, different epitopes were used to prepare antibodies from the peptide sequences from proteomics.

6. Conclusion and Suggestion for the future work

Taken together, these studies revealed the discrete clusters of EOM, LOM, OSCC, BN and CP groups, using salivary MALDI-TOF MS. With the combination of MALDI-TOF MS and LC MS/MS, potential protein candidates associated with the diseases were identified. Western blot analysis could verify SENP7, TLR4 and NF-**K**B as potential salivary biomarkers of canine oral tumors. In addition, the potential salivary biomarkers of canine oral tumors, PTPN5 and p53 were revealed using GeLC-MS/MS and western blot. A ratio of fUBD to cUBD in PreS as a candidate prognostic biomarker for survival in dogs with LOM was also shown.

For the future works, the role of TLR4 and NF-**K**B as prognostic markers of progressive tumors and of the signal transduction of SENP7 and TLR4—NF-**K**B pathways and the mechanisms to inhibit the pathways should be performed. Since most families of PTPs served as biomarker targets of several anticancer drugs, potential inhibitors of PTPN5 as candidate anticancer drugs for oral tumors should be investigated. Further investigation of p53 in canine oral tumors for potential prognostic and therapeutic biomarkers should be performed. In addition, the study of expression of ubiquitin-conjugating enzymes used in UBQ and proteins involving in autophagy and apoptosis should be performed in larger populations. Suitable drugs or treatment might be reconsidered for treating the STS group with high ratios of fUBD to cUBD.

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8. Research Output

8.1. Ploypetch S, Roytrakul S, Jaresitthikunchai J, Phaonakrop N, Krobthong S, **Suriyaphol G*** 2019. Salivary proteomics of canine oral tumors using MALDI-TOF mass spectrometry and LC-tandem mass spectrometry. PLoS One. 14: e0219390.

8.2. Ploypetch S, Roytrakul S, Phaonakrop N, Kalpravidh C, Rungsipipat A, **Suriyaphol G***. In-gel digestion coupled with mass spectrometry (GeLC-MS/MS)-based salivary proteomic profiling of canine oral tumors (Submitted to BMC Veterinary Research - revision in process).

8.3. Ploypetch S, Roytrakul S, Jaresitthikunchai J, Phaonakrop N, Krobthong S, Teewasutrakul P, **Suriyaphol G***. Salivary proteomics in monitoring therapeutic response of canine oral melanoma (Expected to be submitted to Scientific Reports).

8.4. Ploypetch S*, Roytrakul S, Jaresitthikunchai J, Phaonakrop N, **Suriyaphol G**. Serum proteomic analysis by MALDI-TOF MS for rapid screening of canine oral melanoma at different clinical stages. The 34th World Veterinary Association Congress. 5-8 May 2018. Barcellona, Spain (Oral presentation).

8.5. Ploypetch S, Roytrakul S, Jaresitthikunchai J, **Suriyaphol G***. Saliva peptide finger print analysis of canine oral squamous cell carcinoma by MALDI-TOF mass spectrometry. The 17th Chulalongkorn University Veterinary Conference (CUVC 2018). 25-27 April 2018. Nonthaburi, Thailand (Poster presentation).

8.6. Ploypetch S, Roytrakul S, Jaresitthikunchai J, Rungsipipat A, Kalpravidh C, **Suriyaphol G*.** Salivary proteomic analysis of canine oral melanoma at different clinical stages by MALDI-TOF mass spectrometry. The 19th International Symposium of World Association of Veterinary Laboratory Diagnosticians (ISWAVLD). 19-22 June 2019. Chiang Mai, Thailand (Poster presentation).

8.7. Ploypetch S. Analysis of salivary proteome for identification of novel candidate biomarkers/ in canine oral tumors. The 18th Chulalongkorn University Veterinary Science Conference 2019 (CUVC 2019). 24-26 April 2019. Nonthaburi, Thailand (3-minute pitching).

9. Appendix

PLOS ONE

RESEARCH ARTICLE

Salivary proteomics of canine oral tumors using MALDI-TOF mass spectrometry and LC-tandem mass spectrometry

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Abstract

Canine oral tumors are relatively common neoplasms in dogs. For disease monitoring and early diagnosis, salivary biomarkers are appropriate because saliva collection is non-invasive and requires no professional skills. In the era of omics, matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF MS) coupled with liquid chromatography-tandem MS (LC-MS/MS) are suitable to identify potential disease-associated peptides and proteins. The present study aimed to use MALDI-TOF MS and LC-MS/MS to search for particular peptide mass fingerprints (PMFs) and conceivable biomarkers in saliva of dogs with early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC), benign oral tumors (BN), and periodontitis and healthy controls (CP). Pooled saliva samples in each group were used to be representative of population change. Unique PMFs were obtained and specific peptide fragments were sequenced by LC-MS/MS and BLAST-searched with mammalian protein databases. Seven peptide fragments appeared in the tumor groups (EOM, LOM, OSCC and BN) at 1096, 1208, 1322, 1794, 1864, 2354 and 2483 Da, two peptide fragments appeared in the LOM and OSCC groups at 2450 and 3492 Da, and in the CP controls at 2544 and 3026 Da. Also, protein–chemotherapy drug interaction networks were exhibited. Using western blot analysis, the expression of sentrin-specific protease 7 (SENP7), a peptide fragment at 1096 Da, in OSCC was significantly increased, as was the expression of TLR4, a peptide fragment at 3492 Da, in LOM and OSCC, compared with the CP group. The expression of nuclear factor kappa B (NF-κB), a TLR4 partner, was notably increased in OSCC compared with CP, BN and EOM. The expression was also enhanced in LOM compared with EOM. Expressed protein sequences from western blots were verified by LC-MS/MS. Western blots were then performed with individual samples in each group. The results showed the elevated expression of TLR4 in LOM and OSCC, compared with that in CP and BN, the increased expression of NF-κB in LOM and OSCC, compared with CP and in LOM compared with BN, and the enhanced expression of SENP7 in LOM and OSCC, compared with that in CP and BN. In

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conclusion, discrete clusters of EOM, LOM, OSCC, BN and CP groups and potential protein candidates associated with the diseases were demonstrated by salivary proteomics. Western blot analysis verified SENP7, TLR4 and NF-κB as potential salivary biomarkers of canine oral tumors.

Introduction

Tumors in the oral cavity and gastrointestinal system account for 18% of tumors in dogs and tumors in the oral cavity account for 46% of canine head and neck cancers [1, 2]. As the oral cavity is not routinely examined by owners or veterinarians, oral cancers are usually detected at a late clinical stage (stages III and IV), based on the World Health Organization (WHO) clinical staging system for tumors of the oral cavity [3]. As defined by their primary sizes and metastatic profile, the tumor, node and metastasis (TNM) stages comprise stage I (a $<$2 cm diameter tumor), stage II (a 2 to $<$4 cm diameter tumor), stage III (a $\geq$4 cm tumor and/or lymph node metastasis) and stage IV (a tumor with distant metastasis). Surgery resection is the primary option for canine oral tumors. The combination of surgery and chemotherapy drugs is considered for late-stage cancer. The common anti-cancer drugs used are carboplatin, a derivative of the anti-cancer drug cisplatin, doxorubicin (also called adriamycin), cyclophosphamide and piroxicam. The last two drugs are widely used in metronomic chemotherapy [4–6]. Dogs with a late clinical stage have a high mortality rate [7]. Hence, early diagnosis and screening are important for successful treatment. The gold standard for oral tumor diagnosis is a tissue biopsy for histopathological examination; however, it is an invasive technique and impractical for oral cancer screening. Salivary biomarkers are suitable for early detection or monitoring of oral tumors because saliva contacts directly with an oral mass, and saliva collection is non-invasive and easy to perform. In order to discover novel salivary proteins for human oral tumors, a proteomic approach has been performed [8–11]. In healthy dogs, salivary proteomic analysis as well as the comparison of canine salivary proteomics with that of healthy humans have recently been reported [12–15]. However, the study of salivary proteomics of dogs with oral diseases has not been demonstrated. The present study aimed to explore novel peptide mass fingerprints (PMFs), clusters, and conceivable biomarkers in saliva of dogs with early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC), benign oral tumors (BN), periodontitis and healthy controls, using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI--TOF MS), coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The associations of disease-perturbed proteins with chemotherapy drugs, cisplatin, cyclophosphamide, piroxicam and doxorubicin, were exhibited. The candidate protein expressions were verified by western blot analysis. Our study demonstrated candidate salivary biomarkers of canine oral tumors that might help diagnosis and treatment plan of the diseases.

Materials and methods

Animals

Saliva samples were collected from patients with oral tumors scheduled for surgical excision at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University and private animal hospitals, including 5 EOM, 24 LOM, 10 OSCC and 11 BN, respectively (age range 7–14 years). OM was classified as early- or late-clinical stages, according to the

TNM staging system of the WHO [16]. Inclusion criteria included the presence of benign oral tumors, OM and OSCC, diagnosed without previous treatment either chemotherapy or radiotherapy. The staging of OM and OSCC were determined according to the WHO [17]. Dogs were examined for an oral, regional lymph node, and physical condition; moreover, the regional lymph nodes were required for cytological examination to rule out metastasis. Skull-to-abdomen radiography was evaluated by a Brivo DR-F digital X-ray system (GE Healthcare, Chicago, IL) or Optima CT660 CT-scanner (GE Healthcare). OM and OSCC metastasis to abdominal organs was checked by ultrasonography. Seven samples were gathered from dogs with normal oral health with normal blood profiles and no history or clinical signs of oral cavity or cancerous problems (age range 7–8 years). For a chronic periodontitis group, 5 dogs demonstrated gingivitis, dental tartar and/or periodontal attachment loss (age range 7–13 years). The samples were obtained with the consent of owners and sample collection protocol was approved by the Chulalongkorn University Animal Care and Use Committee (CU-A-CUC), Thailand (Approval number 1631042).

Sample preparation

To collect saliva, dogs were fasted for at least 1 h before saliva collection and mouths were cleaned with 0.9% sterile normal saline solution [10]. Saliva was collected on the day of surgery without mechanical and chemical stimulation. Whole saliva (0.5–1.0 mL) was collected for 5–10 min using a sterile cotton swab. Samples were centrifuged at 2600 x*g* for 15 min at 4 ˚C [18]. Approximate 200 μL of supernatant was mixed with Halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA) and kept at -20 ˚C until analysis. Total protein concentrations from salivary supernatants were evaluated by Lowry's assay at 690 nm, using bovine serum albumin as a standard [19].

Analysis of salivary peptides by MALDI-TOF MS

The salivary protein sample of each dog was prepared with 0.1% trifluoroacetic acid (TFA) to the final concentration of 0.2 μg/μL. Samples were mixed with MALDI matrix solution, consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 100% acetonitrile (ACN) and 5% trifluoroacetic acid, at the ratio of 1:1, and directly applied onto the MTP384 target plate (Bruker Daltonics, Billercia, MA) and air dried. Eight replicates were performed to prevent sample preparation bias. Mass spectra were obtained with an Ultraflex III TOF/TOF (Bruker Daltonics) in a linear positive mode with a mass range of 1000–20000 Da. External calibrations were performed using a ProteoMass Peptide & Protein MALDI-MS Calibration Kit (Sigma Aldrich, St. Louis, MO) that consists of human angiotensin II (m/z 1046), P14R (m/z 1533), human adrenocorticotropic hormone fragment 18–39 (m/z 2465), bovine insulin oxidized B chain (m/z 3465), bovine insulin (m/z 5731), and cytochrome *c* (m/z 12362). Saliva peptide mass spectra were determined by flexAnalysis 3.3 software (Bruker Daltonics). Peptide mass spectral peaks were analyzed using Quick Classifier (QC)/ Different Average, Supervised Neural Network (SNN), Anderson-Darling (AD), t-test/ANOVA (TTA), Wilcoxon/Kruskal-Wallis (W/KW) and the Genetic Algorithm (GA) statistical algorithms incorporated in the ClinProTools v. 3.0 software (Bruker Daltonics) to reveal the uniformity and homogeneity of the sample group as PMF, pseudo-gel view and principal component analysis (PCA) [11, 20, 21]. A dendrogram of each dog was constructed, using ClinProTools v. 3.0.

According to dendrograms and PCA plots, four from eight replicates were selected and pooled. Thirty-two replicates of pooled samples were applied twice to MALDI-TOF MS as mentioned above. The recognition capability and cross-validation values of more than 90% exhibited the reliability of the peak selection [22]. ClinProTools v. 3.0 was used to analyze

intensity values. Results with $p<0.05$ were considered significant and peaks were then selected to be analyzed by LC MS/MS.

Peptide and protein identification by LC-MS/MS

With the limitation of the LC MS/MS, salivary peptide samples at 1000–4000 Da were selected by ClinProTools v. 3.0 and purified using C18 ZipTip (MilliporeSigma, Burlington, MA). Each peptide was diluted in ACN for 51 different dilution ratios equally spaced in the range 0–100%.

Amino acid sequences of gradient-eluted peptides were identified by reversed-phase high performance liquid chromatography and a PTM Discovery System (Bruker Daltonics) coupled to an UltiMate 3000 LC System (Thermo Fisher Scientific, Waltham, MA). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 μm diameter × 50 mm length). The nanoLC system was connected to an electrospray ionization in the positive ion mode and quadrapole ion-trap MS (Bruker Daltonics). Eluent A and eluent B solutions were prepared from a 0.1% formic acid dilution and from 50% ACN in water containing 0.1% formic acid, respectively. Peptide separation was achieved with a 4–70% linear gradient of eluent B at a flow rate of 1000 nL/min for 7.5 min. A regeneration step and an equilibration step were

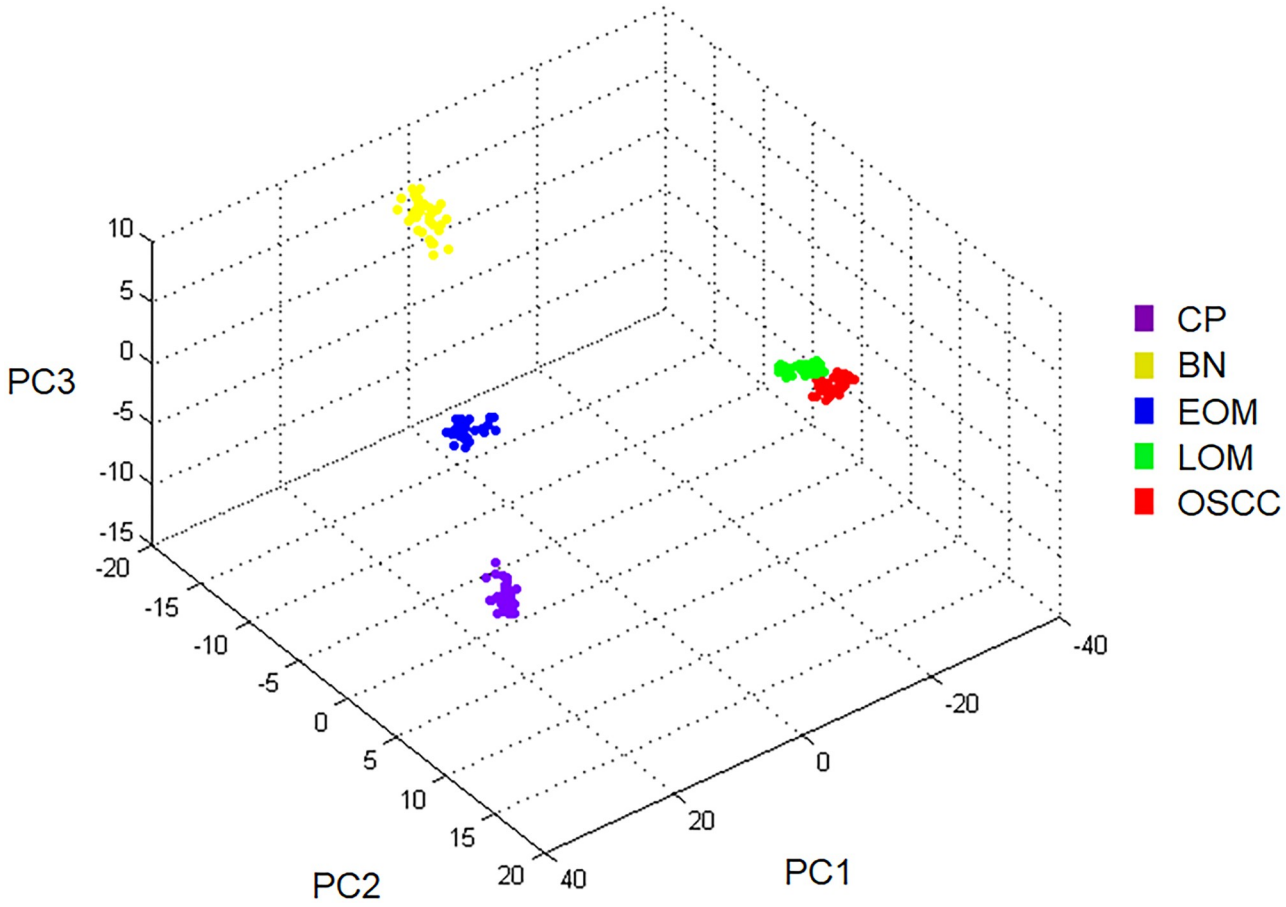

Fig 1. Three-dimensional principal component analysis scatterplot of normal and periodontitis gingiva tissues (CP), benign tumors (BN), early-stage oral melanoma (EOM), late-stage OM (LOM) and oral squamous cell carcinoma (OSCC). Thirty two dots represent replicate in each pooled sample group.

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performed with 90% and 4% eluent B, respectively, for 20 min per run. Peptide fragment mass spectra were acquired in the data-dependent AutoMS mode with a scan range of 400–1500 m/z, 3 averages, and up to 5 precursor ions, selected from the MS scan at 200–2800 m/z.

The results of LC MS/MS were converted into an mzXML file by CompassXport software (Bruker Daltonics). DeCyder MS Differential Analysis software (Amersham Biosciences, Little Chalfont, UK) was used for protein quantification [23, 24] The PepDetect module was used in MS mode for automated peptide detection, charge state assignments, and peptide ion signal intensities. The proteins were identified from MS/MS peptide mass values using Mascot software (Matrix Science, London, UK) [25]. The data were searched against the NCBI mammal database for protein identification. Proteins were identified from one or more peptides with an individual MASCOT score corresponding to $p<0.05$. The information about particular proteins and detailed analysis of the protein sequences were used in the annotation of UniProtKB/Swiss-Prot entries [26]. The relationship of candidate proteins and chemotherapy drugs were performed by the Stitch program, version 5.0 [27].

Validation of MS results by western blot analysis

Pooled saliva samples of 5 μg for SENP7 and TLR4 and of 12 μg for NF-κB were mixed with loading dye [0.5 M dithiothreitol, 10% (w/v) SDS, 0.4 M Tris-HCl, pH 6.8, and 50% (v/v) glycerol]. Samples were heated at 85 ˚C for 10 min prior to loading on pre-cast NuPAGE 4–12% (w/v) Bis-Tris 1.0-mm minigel (Thermo Fisher Scientific), using RunBlue MES Run Buffer (Expedeon, Heidelberg, Germany) in an XCell SureLock Mini-Cell electrophoresis system (Thermo Fisher Scientific) at 200 V for 90 min. PageRuler prestained protein ladder (molecular weight range 10–180 kDa) (Thermo Fisher Scientific) was used. Subsequently, proteins were transferred to Trans-Blot Turbo mini-sized nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) at 25 V for 14 min using Trans-Blot Turbo 5× transfer buffer (Bio-Rad Laboratories). A Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes (Thermo

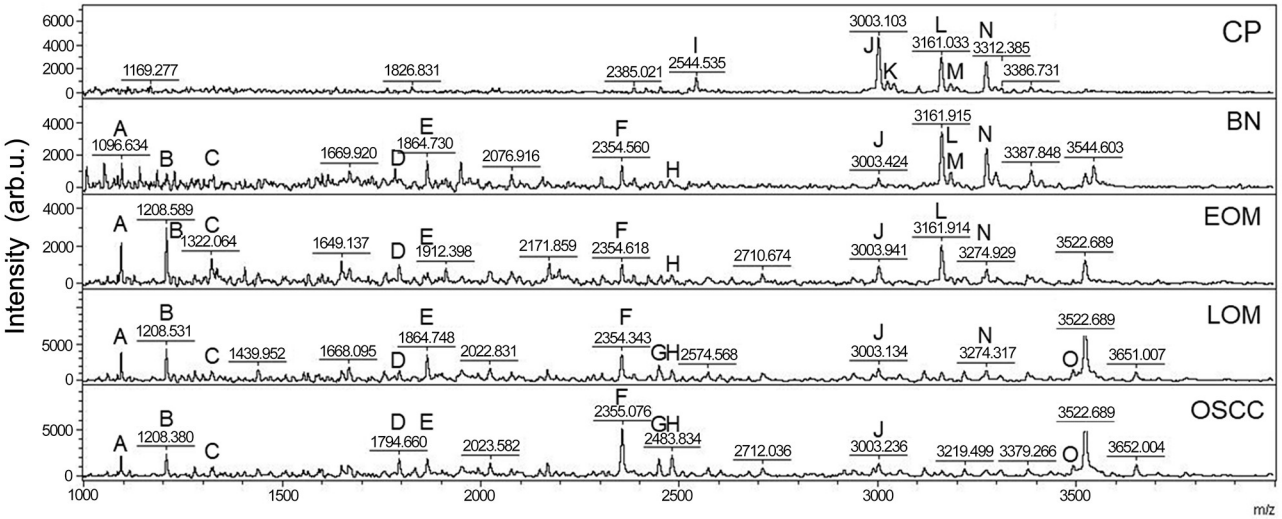

Fig 2. Peptide mass fingerprint (PMF) of normal and periodontitis gingiva tissues (CP), benign tumors (BN), early-stage oral melanoma (EOM), late-stage OM (LOM) and oral squamous cell carcinoma (OSCC) in the range 1000–5000 Da with identified proteins of each mass spectrum. A: SENP7 or KAT2B (1096 Da); B: PPRC1 or RMND1 (1208 Da); C: DTX3L (1322 Da); D: ZNF699) (1794 Da); E: MAP3K15 or ATP6V1E2 (1864 Da); F: PLCL2 (2354 Da); G: TNRC18 (2450 Da); H: COL12A1 (2483 Da); I: ZNF451 (2544 Da); J: protocadherin FAT1 (3003 Da); K: CASPL4A2 (3026 Da); L: centrosomal protein 192 (3161 Da); M: glypican 5 (3184 Da); N: cell-cycle checkpoint protein RAD17 (3274 Da); O: TLR4 (3492 Da). arb. u., arbitrary unit.

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Fisher Scientific) was used to detect total proteins in each well according to the manufacturer's instructions. Nonspecific binding was blocked with 5% bovine serum albumin (GoldBio, St Louis MO) in Tris-buffered saline containing 0.1% Tween 20 (TBST) overnight. After washing with TBST, primary antibodies, 1:1000 mouse monoclonal anti-mouse TLR4 (25) (sc-293072, Santa Cruz Biotechnology, Dallas, TX), 1:1000 mouse monoclonal anti-human SENP7 (E-8) (sc- 373821, Santa Cruz Biotechnology) or 1:1000 mouse monoclonal anti-human NF-κB p65 (sc- 8008, Santa Cruz Biotechnology) were incubated with a membrane at 4 ˚C overnight. A membrane was washed and subsequently incubated with 1:10,000 horseradish peroxidase-conjugated rabbit anti-mouse antibody (ab6728, Abcam, Cambridge, UK) at 25 ˚C for 1 h. The proteins of interest were detected using ECL western blotting detection reagents (GE Healthcare). Western blots were imaged with a ChemiDoc Touch Imaging System (Bio-Rad Laboratories). Protein band intensities were analyzed by Image Lab 6.0.1 software (Bio-Rad Laboratories). For western blot normalization, total protein normalization, modified from Aldridge et al. (2008) was used [28]. The ratios of target band intensities to the total proteins in each lane in the first or second half of a membrane were calculated according to the sizes of

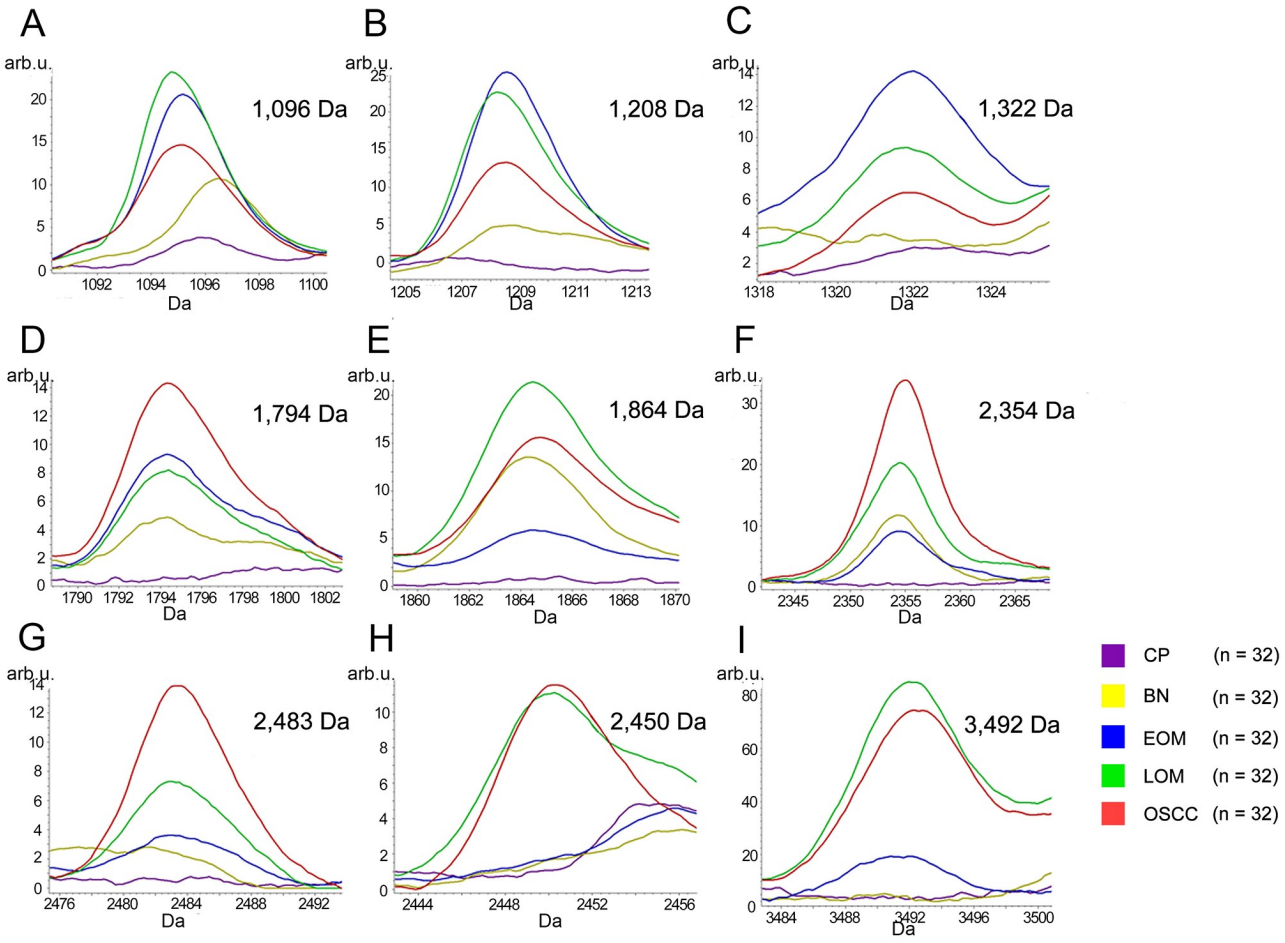

Fig 3. MALDI-TOF intensity profiles of salivary proteins from benign tumors (BN), early-stage oral melanoma (EOM), late-stage OM (LOM) and oral squamous cell carcinoma (OSCC). Percentages of interpretable mass signals are shown: SENP7 or KAT2B at 1096 Da (A); PPRC1 or RMND1 at 1208 Da (B); DTX3L at 1322 Da (C); ZNF699 at 1794 Da (D); MAP3K15 or ATP6V1E2 at 1864 Da (E); PLCL2 at 2354 Da (F); COL12A1 at 2483 Da (G); TNRC18 at 2450 Da (H); TLR4 at 3492 Da (I). arb. u., arbitrary unit.

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target proteins. The western blotting was performed in triplicate. Statistical analyses of protein expression data were conducted using GraphPad Prism v. 8.0.1 (GraphPad Software, La Jolla, CA). Western blots were also performed with individual samples in each group after the target protein sequences were confirmed by LC-MS/MS.

Verification of expressed protein sequences by LC-MS/MS

To confirm SENP7, TLR4 and NF-κB protein identities, Antibodies were removed from nitrocellulose membranes by incubating with Restore Plus Western Blot Stripping Buffer (Thermo

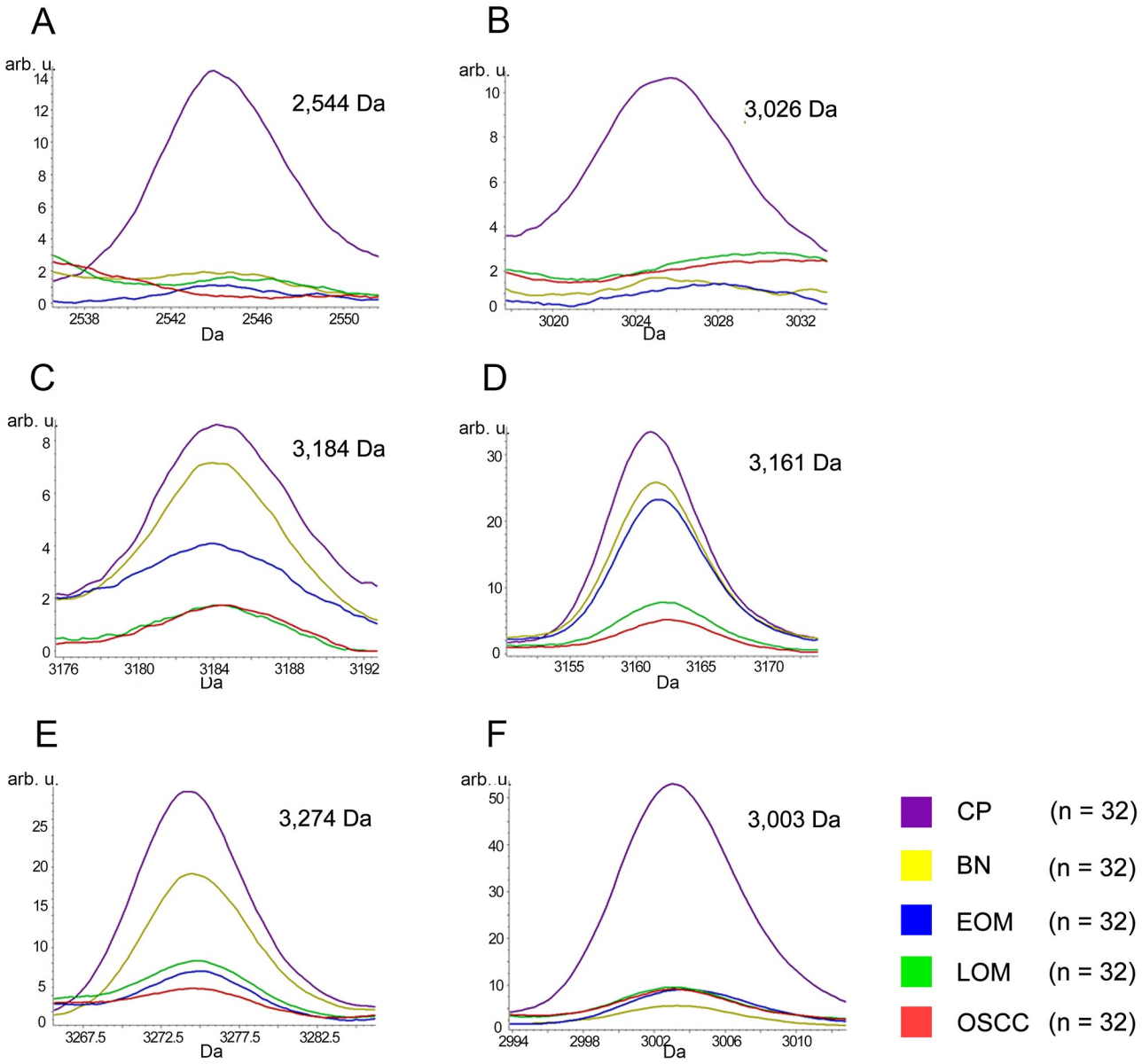

Fig 4. MALDI-TOF intensity profiles of salivary proteins from normal and periodontitis gingiva tissues (CP), benign tumors (BN), early-stage oral melanoma (EOM), late-stage OM (LOM) and oral squamous cell carcinoma (OSCC). Percentages of interpretable mass signals are shown: ZNF451 at 2544 Da (A); CASPL4A2 at 3026 Da (B); glypican 5 at 3184 Da (C); CEP192 at 3161 Da (D); cell-cycle checkpoint protein RAD17 at 3274 Da (E); protocadherin FAT1 at 3003 Da (F). arb. u., arbitrary unit.

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Fisher Scientific) at room temperature for 15 min. After washing 4 times with TBST, protein bands were cut and incubated with 10 mM DTT in 10 mM ammonium bicarbonate (Ambic) at room temperature overnight. Samples were then incubated with 10 ng trypsin in 10 mM Ambic at 37 ˚C for 3 hr and concentrated by the speed-vac (Thermo Fisher Scientific). Fifteen μL of 0.1% formic acid was used to dissolve proteins prior to applying to the LC-MS/MS as mentioned above.

Table 1. Nominated proteins based on biological process involvement and protein score, using MALDI-TOF MS and LC-MS/MS data.

Database	Protein name	Protein score	Peptide sequence	Biological process	Subcellular distribution
XP_010329708.1	Sentrin-specific protease 7 isoform X1 (SENP7)	29	LNLSERIPR	Adenylate cyclase-modulating G-protein-coupled receptor signaling pathway	Nucleus and plasma membrane
XP_021512678.1	Histone acetyltransferase KAT2B (KAT2B)	25	VYPGLLCFK	Cell cycle arrest, chromatin remodeling	Nucleus and cytoskeleton
EHB18528.1	Peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (PPRC1)	17	AHDHYQRQR	Positive regulation of DNA-binding transcription factor activity	Nucleus
ERE86066.1	Required for meiotic nuclear division protein 1-like protein (RMND1)	9	TLALSTYFHR	Positive regulation of mitochondrial translation	Mitochondrion
A0A286Y4B2	E3 ubiquitin-protein ligase DTX3L (DTX3L)	15	TLYGIQTGNQPK	Histone ubiquitination	Cytosol, endosome, lysosome and nucleus
XP_012863361.1	Zinc finger protein 699 (ZNF699)	14	EYGEACSSPSSIGPPVR	Regulation of transcription	Nucleus
XP_004612329.1	Mitogen-activated protein kinase kinase kinase 15 (MAP3K15)	20	TDSMEILTSDIIDGLLK	Activation of MAPKK activity	Nucleus and cytoplasm
XP_021074063.1	V-type proton ATPase subunit E 2 (ATP6V1E2)	16	VCNTLESRLNLAAMQK	ATP hydrolysis coupled proton transport	Membrane
XP_010964328.1	Inactive phospholipase C-like protein 2 (PLCL2)	17	VMVMTSPNVEESYLPSPDVLK	Intracellular signal transduction	Cytoplasm
ELK17433.1	Trinucleotide repeat-containing protein 18 protein (TNRC18)	16	NSSGKLSGKPLLTSDAYELGAGMR	Chromatin silencing	Cytosol, mitochondrion, nucleus and other locations
XP_007951446.1	Collagen type XII alpha-1 chain (COL12A1)	23	DYKPQVGVIVDPSTKTLSFFNK	Cell adhesion	Extracellular matrix
XP_004267830.1	Zinc finger protein 451 isoform X2 (ZNF451)	18	DTSPFQPNPPAGGPIVEALEHSKR	Nucleic acid binding	Nucleus
XP_015104668.1	Protocadherin Fat 1 isoform X3 (FAT1)	16	GNPPMSEITSVHIFVTIADNASPKFTSK	Homophilic cell adhesion via plasma membrane adhesion molecules	Plasma membrane and integral component of membrane
XP_016818046.1	CASP-like protein 4A2 (CASPL4A2)	22	SAASPGPAPAAGDPGGSARPRPAAPLGSALALAF	Iron–sulfur cluster binding	Plasma membrane
XP_003924923.1	Centrosomal protein of 192 kDa isoform X1 (CEP192)	21	SGNLLETHEVDLTSNSEELDPIRLALLGK	Centrosome-templated microtubule nucleation	Cytoskeleton
ERE87034.1	Glypican-5 (GPC5)	13	GMCKDLTKPMQHHVTVIAASTECVVTLK	Regulation of signal transduction	Plasma membrane and extracellular space
XP_010635607.1	Cell-cycle checkpoint protein RAD17 isoform X2 (RAD17)	13	LLFPKEIQEECSILNISFNPVAPTIMMK	Cell cycle	Nucleus
ABU41662.1	Toll-like receptor 4 variant 1 (TLR4)	3	MMSASRLAGTLIPAMAFLSCVRPESWEPCVE	Activation of MAPK activity	Early endosome and cell membrane

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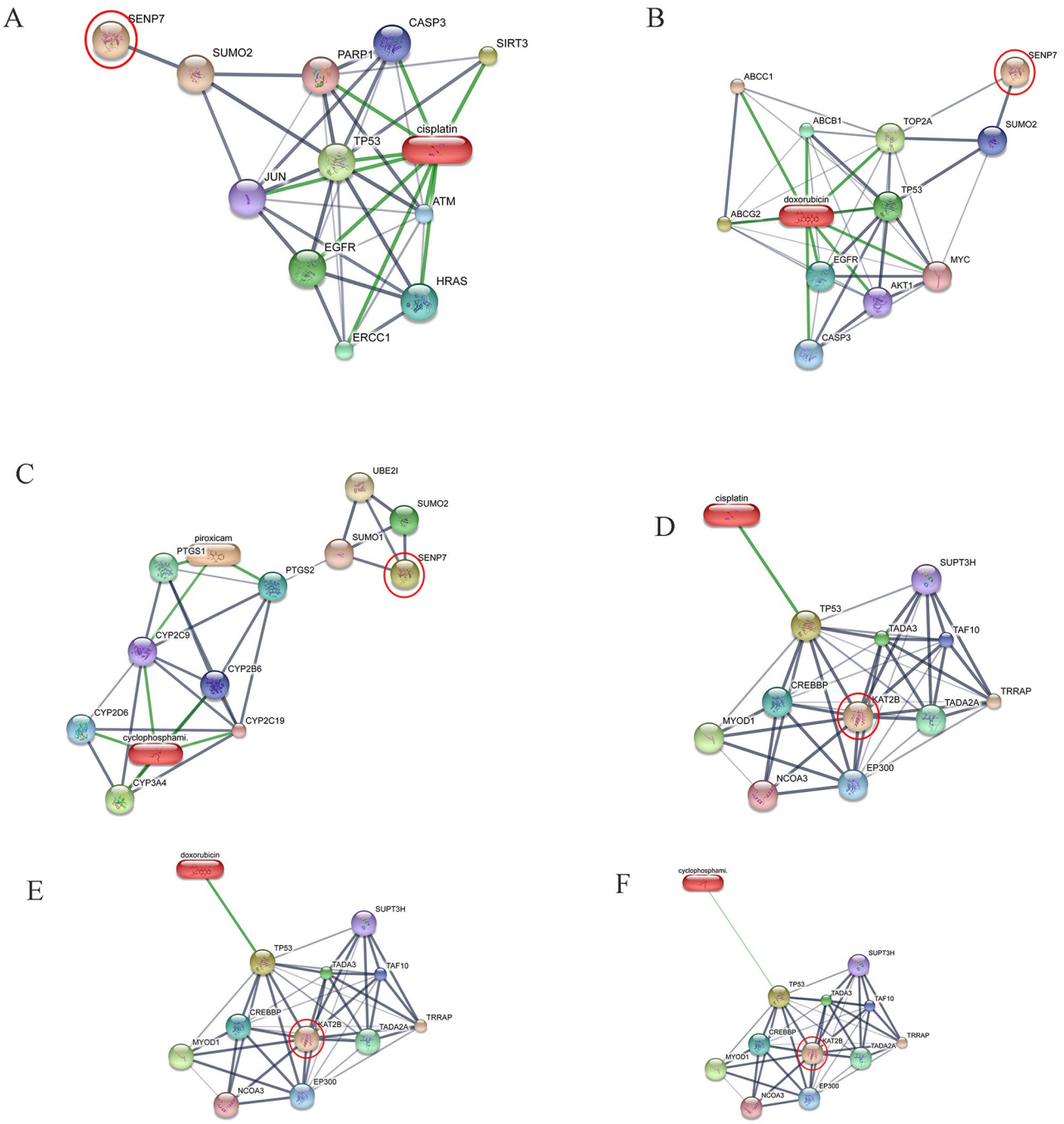

Fig 5. Involvement of sentrin-specific protease 7 (SENP7) and K(lysine) acetyltransferase 2B (KAT2B) in networks of protein–chemotherapy drug interactions, cisplatin, doxorubicin, cyclophosphamide and piroxicam. Interactions of SENP7 with cisplatin (A), SENP7 with doxorubicin **(B)**, SENP7 with cyclophosphamide and piroxicam **(C)**, KAT2B with cisplatin **(D)**, KAT2B with doxorubicin **(E)**, KAT2B with cyclophosphamide and piroxicam **(F)** were exhibited. Red circles: SENP7 and KAT2B. Abbreviations: ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1), ataxia telangiectasia mutated (ATM), ATP-binding cassette, sub-family C (CFTR/MRP), member 1 (ABCC1), ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2), v-akt murine thymoma viral oncogene homolog 1 (AKT1), caspase 3 (CASP3), c-jun (JUN), CREB binding protein (CREBBP), cytochrome P450 family 2 subfamily B member 6 (CYP2B6), cytochrome P450 family 2 subfamily C member 9 (CYP2C9), cytochrome P450 family 2 subfamily C member 19 (CYP2C19), cytochrome P450 family 2 subfamily D member 6 (CYP2D6), cytochrome P450 family 3 subfamily A member 4 (CYP3A4), E1A binding protein p300 (EP300), epidermal growth factor receptor (EGFR), v-myc myelocytomatosis viral oncogene homolog (MYC), DNA excision repair protein ERCC-1, endonuclease non-catalytic subunit (ERCC1), Harvey rat sarcoma viral oncogene homolog (HRAS), myogenic differentiation 1 (MYOD1), nuclear receptor coactivator 3 (NCOA3), poly [ADP-ribose] polymerase 1 (PARP-1), prostaglandin-endoperoxide synthase 1 (PTGS1), prostaglandin-endoperoxide synthase 2 (PTGS2), sirtuin 3 (SIRT3), solute carrier family 30 (zinc transporter),

member 6 (SLC30A6), suppressor of Ty 3 homolog (S. cerevisiae) (SUPT3H), SMT3 suppressor of mif two 3 homolog 1 (SUMO1), SMT3 suppressor of mif two 3 homolog 2 (SUMO2), transcriptional adaptor 2A (TADA2A), transcriptional adaptor 3 (TADA3), TAF10 RNA polymerase II (TAF10), topoisomerase (DNA) II alpha (TOP2A), transformation/transcription domain-associated protein (TRRAP), tumor protein p53 (TP53), ubiquitin-conjugating enzyme E2I (UBE2I).

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Statistical analysis

ClinProTools v. 3.0 and MASCOT softwares were used to analyze peak intensities of peptides in MALDI-TOF MS spectra and MASCOT LC-MS/MS scores, respectively. Western blot band intensity ratios were tested for normality and statistical differences were analyzed by ordinary

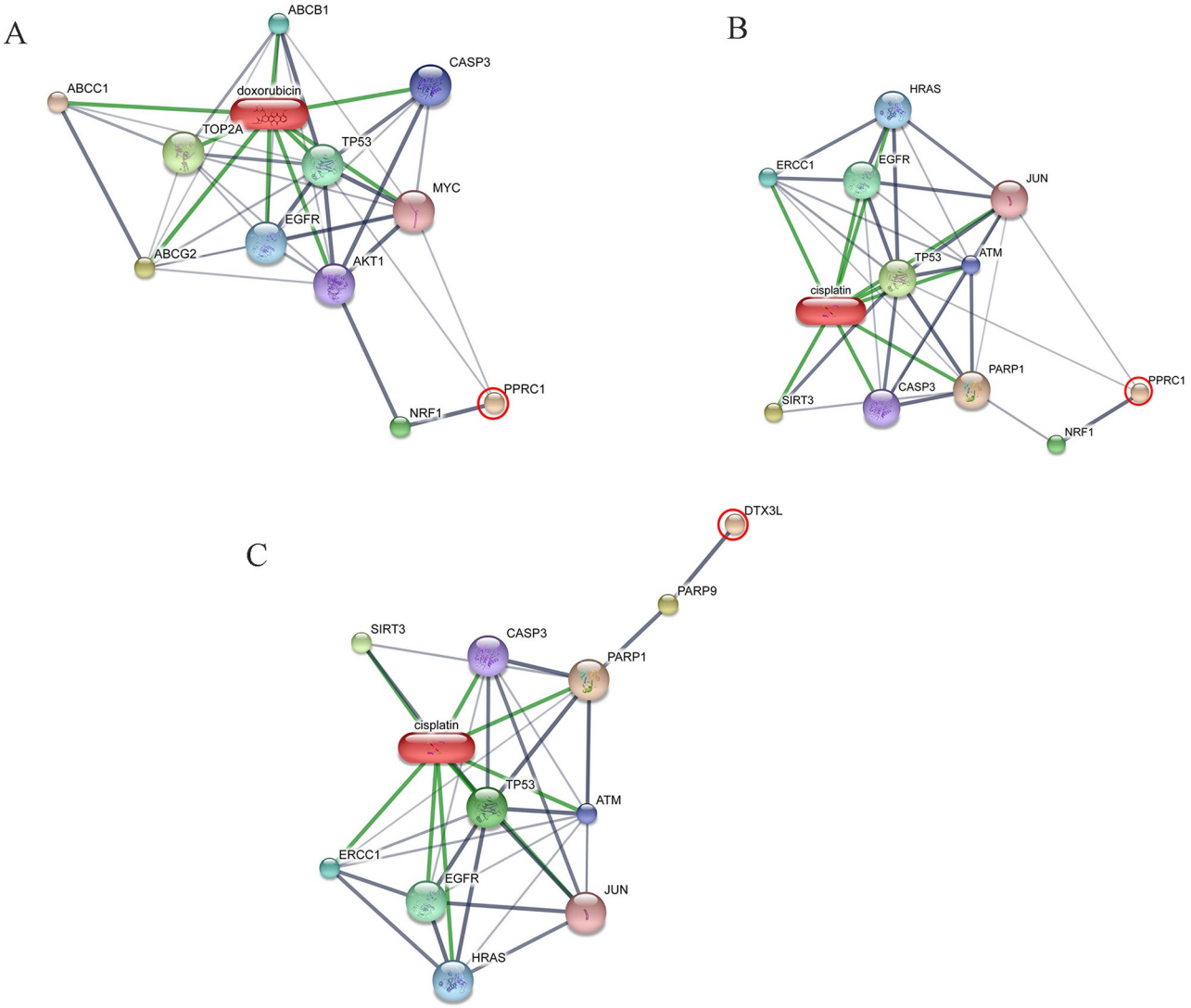

Fig 6. Involvement of peroxisome proliferator-activated receptor gamma, coactivator-related 1 (PPRC1) and deltex 3-like (DTX3L) in networks of protein–chemotherapy drug interactions, cisplatin and doxorubicin. Interactions of PPRC1 with doxorubicin **(A)**, PPRC1 with cisplatin **(B)** and DTX3L with cisplatin **(C)** are shown. Red circles: PPRC1 and DTX3L. Abbreviations: ABCB1, ATP-binding cassette, sub-family B, member 1; ABCC1, ATP-binding cassette, sub-family C, member 1; ABCG2, ATP-binding cassette, sub-family G, member 2; AKT1, v-akt murine thymoma viral oncogene homolog 1; ATM, ataxia telangiectasia mutated; CASP3, caspase 3; EGFR, epidermal growth factor receptor; ERCC1, excision repair cross-complementing rodent repair deficiency, complementation group 1; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; JUN, jun proto-oncogene; MYC, v-myc myelocytomatosis viral oncogene homolog; nuclear respiratory factor 1 (NRF1), PARP1, poly (ADP-ribose) polymerase 1; PARP9, poly (ADP-ribose) polymerase family, member 9; SIRT3, sirtuin 3; TOP2A, topoisomerase II alpha; TP53, tumor protein p53.

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one-way ANOVA with Bonferroni's multiple comparisons for TLR4 and NF-κB (pooled samples), ordinary one-way ANOVA with Tukey's multiple comparisons for SENP7 (pooled samples), and Kruskall Wallis with Dunn's multiple comparisons for TLR4, NF-κB and SENP7 (individual samples). Significance was accepted at the $p<0.05$ level.

Results

All 32 replicates in each pooled sample group demonstrated the homogeneity within the group. A 3-dimensional view of the PCA plot showed distinct clusters among the EOM, LOM, OSCC and BN groups, whereas periodontitis and healthy controls were shown to be in the same cluster and classified as a control (CP) group (Fig 1). The MALDI-TOF MS results had an accurate outcome with the 95% confidence interval. The cross-validation, calculated by ANOVA, in the CP, BN, EOM, LOM, and OSCC was 100%, 100%, 96.88%, 100% and 100%, respectively, and the recognition capability, calculated by QC/ Different Average, SNN, AD, TTA, W/KW and the Genetic Algorithm (GA) test in the CP, BN, EOM, LOM, and OSCC groups was all 100%, indicating that the results were of high reliability. Divergent PMFs of CP, EOM, LOM, OSCC and BN groups were observed, and peptide masses at 1000–5000 Da were selected by ClinProTools software and specific peptide sequences were analyzed by LC MS/MS. Seven peptide fragments appeared in the tumor groups (EOM, LOM, OSCC and BN) at 1096, 1208, 1322, 1794, 1864, 2354 and 2483 Da (SENP7 or KAT2B, PPRC1 or RMND1, DTX3L, ZNF699, MAP3K15 or ATP6V1E2, PLCL2 and COL12A1, respectively), two peptide fragments appeared in the LOM and OSCC groups at 2450 and 3492 Da (TNRC18 and TLR4, respectively), two peptide fragments appeared only in the CP controls at 2544 and 3026 Da (ZNF451 and CASPL4A2, respectively) (Figs 2–4). Candidate protein biomarkers were evaluated for biological processes and location in the cell by UniProtKB/Swiss-Prot (Table 1) [26]. Networks of protein–protein and protein–chemotherapy drug interactions were performed by the Stitch program, version 5.0 and pathways with high edge confidence scores (>0.700) represented the strength of the protein–protein interactions at the functional level (Figs 5–7) [27]. Several candidate proteins presented in this study showed a strong relationship with chemotherapy drugs, including KAT2B, PPRC1, DTX3L, ZNF699 and MAP3K15. Also, p53 was noticeable in all of these pathways as well as the pathways of SENP7-doxorubicin except

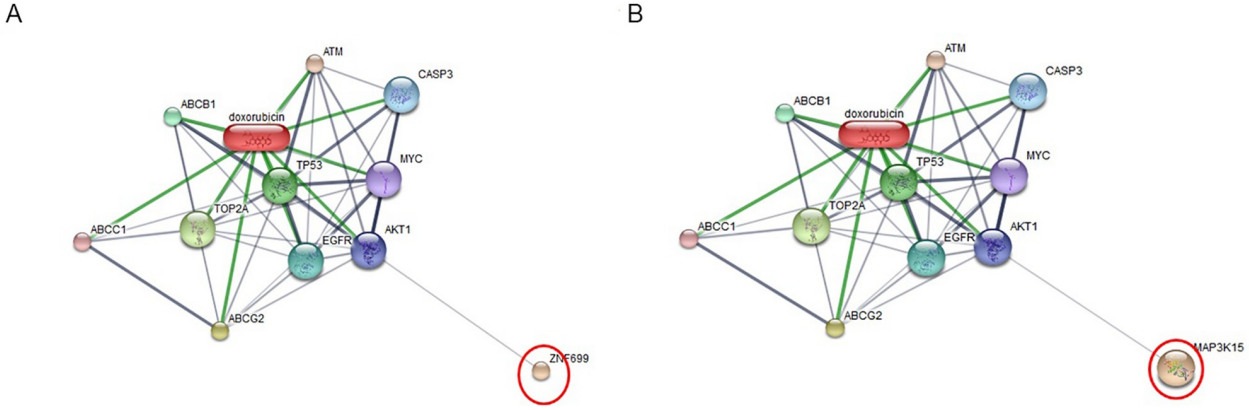

Fig 7. Involvement of zinc finger protein 699 (ZNF699) (A) and mitogen-activated protein kinase kinase kinase 15 (MAP3K15) (B) in networks of protein–chemotherapy drug interactions, doxorubicin. Red circles: ZNF699 and MAP3K15. Abbreviations: ABCB1, ATP-binding cassette, sub-family B, member 1; ABCC1, ATP-binding cassette, sub-family C, member 1; ABCG2, ATP-binding cassette, sub-family G, member 2; AKT1, v-akt murine thymoma viral oncogene homolog 1; ATM, ataxia telangiectasia mutated; CASP3, caspase 3; EGFR, epidermal growth factor receptor; MYC, v-myc myelocytomatosis viral oncogene homolog; TOP2A, topoisomerase II alpha; TP53, tumor protein p53.

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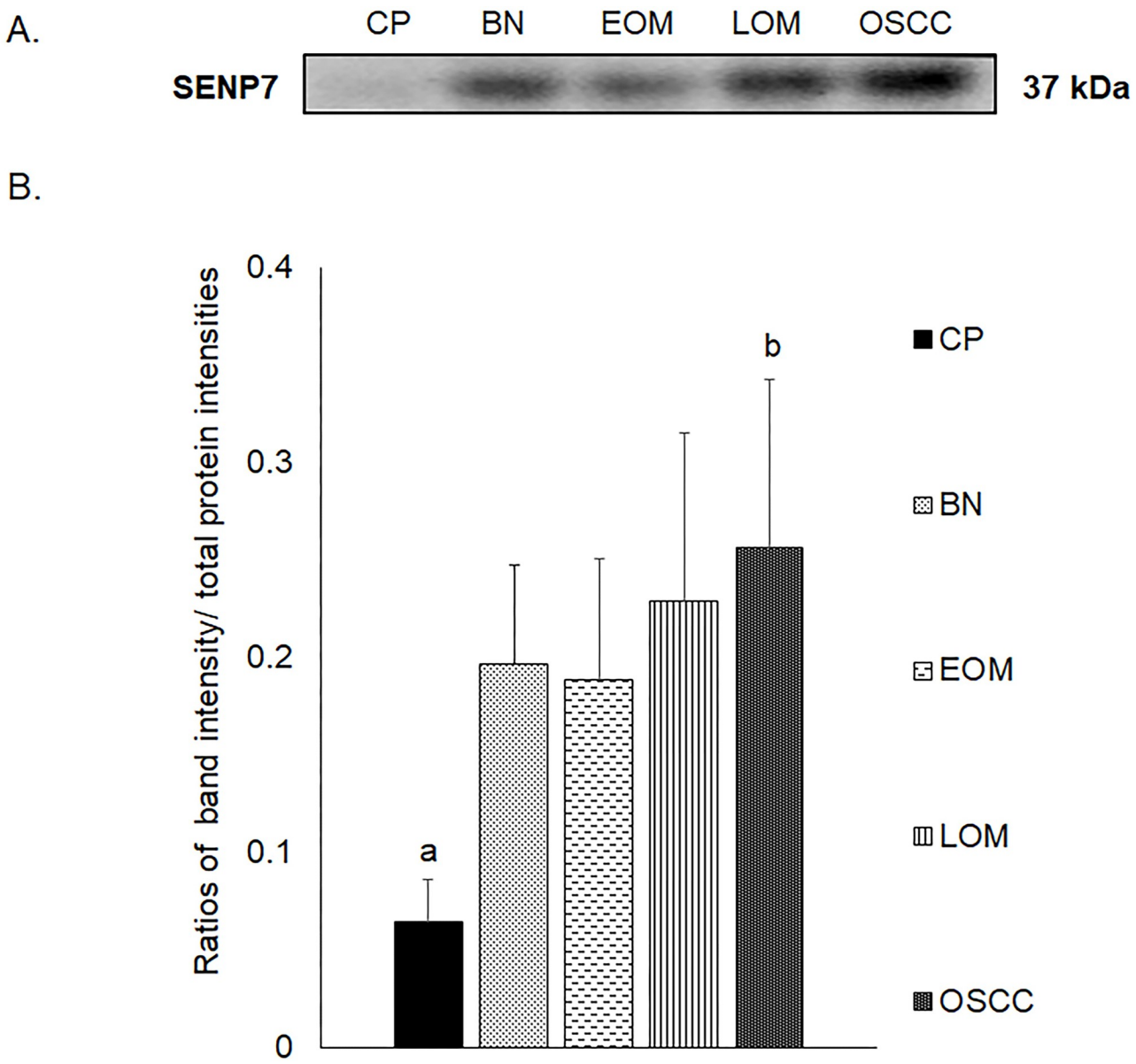

Fig 8. Western blot analysis of salivary sentrin-specific protease 7 (SENP7) of pooled saliva samples from dogs with periodontitis and normal controls (CP), benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively) and oral squamous cell carcinoma (OSCC). Representative western blot for SENP7 at 37 kDa **(A)** and bar graph of ratios of SENP7 protein intensity to total blotted protein intensities in each lane in the second half of a membrane* **(B)**. a–b denote a significant difference at $p<0.05$.

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SENP7-cyclophosphamide/piroxicam and SLC30A10-cyclophosphamide/piroxicam pathways which involved the cytochrome P450 family 2 (CYP2) family. We did not find an association of TLR4 with chemotherapy drugs. However, western blot analysis revealed protein expression of SENP7 in EOM, LOM, OSCC and BN, and TLR4 and NF-κB in LOM and OSCC (Figs 8–10, S1–S3 Figs and S1 Table). The protein bands of SENP7, TLR4 and NF-κB on the membranes were verified by LC-MS/MS. From the Mascot search results, MS/MS fragmentations of KFRKTLPR, NLRYLDISYTR and MLLAVQR were found to be matched with SENP7, TLR4, and NF-κB, respectively (Fig 11). Western blots were then performed in individual

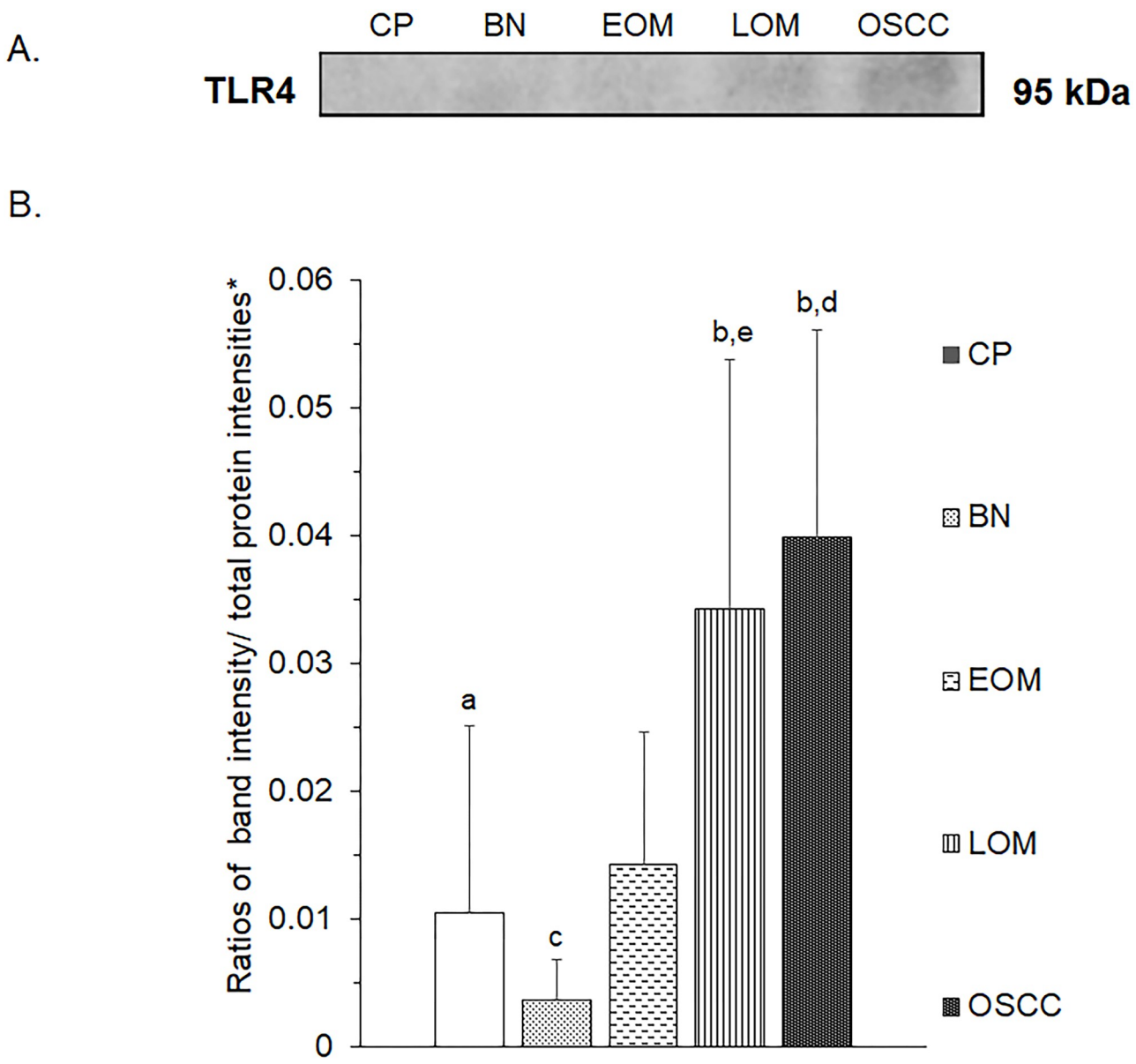

Fig 9. Western blot analysis of salivary toll like receptor 4 (TLR4) of pooled saliva samples from dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). Representative western blot for TLR4 at 95 kDa **(A)** and bar graph of ratios of TLR4 protein intensity to total blotted proteins in each lane in the first half of a membrane* **(B)**. a–b denote a significant difference at $p<0.05$. c–d denote a significant difference at $p<0.01$.

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samples. The results were shown in Figs 12–14 and S4–S6 Figs. The increased expression of SENP7, TLR4 and NF-κB was observed in LOM and OSCC compared with CP and BN.

Discussion

This study demonstrated the different salivary PMFs and clusters of EOM, LOM, OSCC, BN and CP groups by MALDI-TOF MS. In addition, unique protein expressions were observed by LC MS/MS and verified by western blotting. According to the discrete sample groups from the PCA, MALDI-TOF MS can possibly be used as a rapid and reliable method for detection

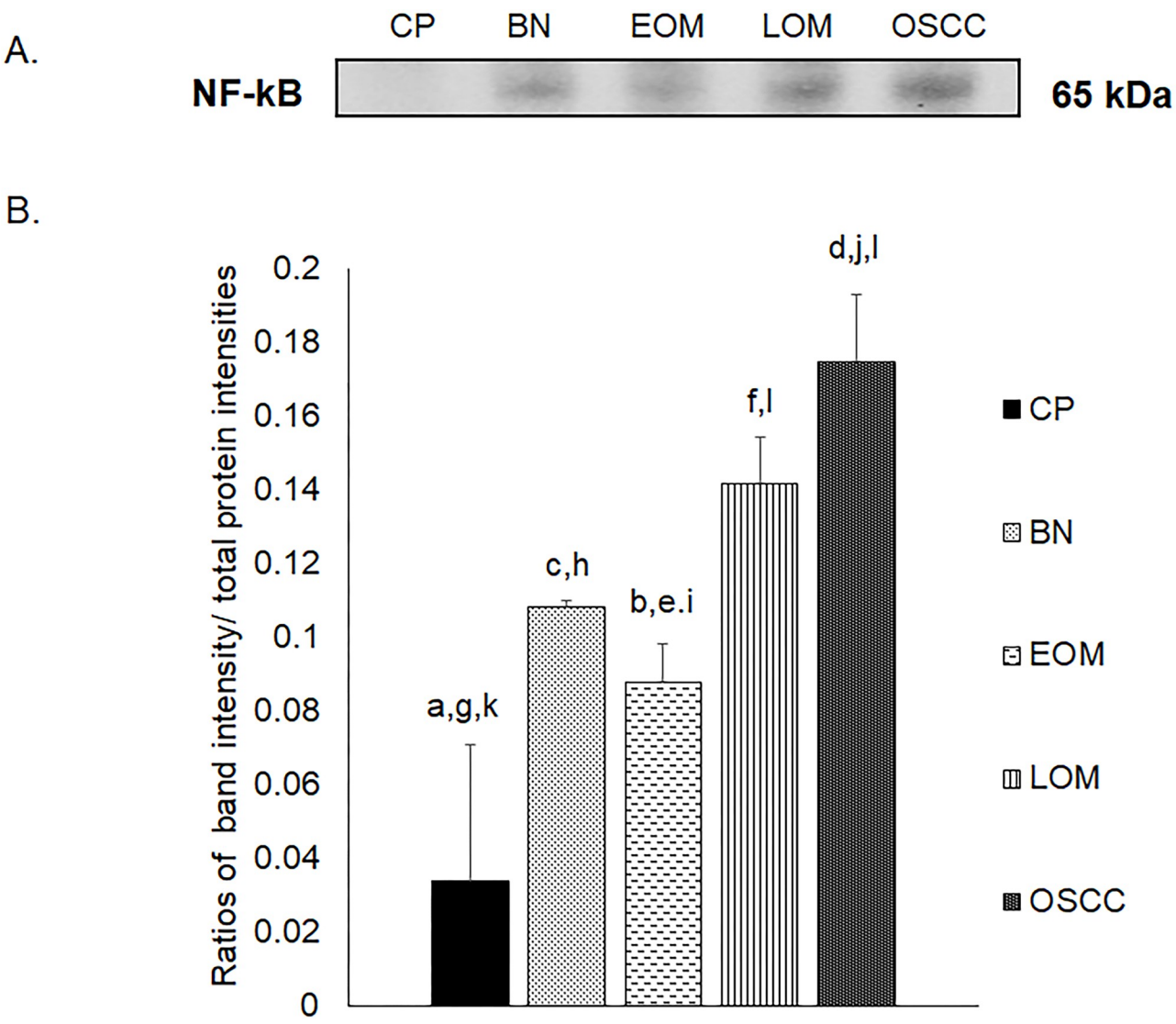

Fig 10. Western blot analysis of nuclear factor kappa B (NF-κB) of pooled saliva samples from dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). Representative western blot for NF-κB at 65 kDa **(A)** and bar graph of ratios of NF-κB protein intensity to total blotted proteins in each lane in the first half of a membrane* **(B)**. a–b, c–d and e–f denote a significant difference at $p<0.05$. g–h and i–j denote a significant difference at $p<0.01$. k–l denote a significant difference at $p<0.001$.

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of canine oral tumors. MALDI-TOF MS has been reported to be a potential tool to characterize human head and neck squamous cell carcinoma from oral brush biopsy, human OSCC from oral fluid and canine oral tumors from tissues [29–31]. Specific mass signals were found from saliva of oral lichen planus (OLP) patients as well as the discovery of a relationship between OLP and oral cancer [8]. Moreover, salivary PMFs showed a number of proteins that were differently expressed in the early-stage OSCC compared with healthy patient, using MALDI-TOF MS combined with magnetic beads [32]. The fast, high accuracy and high sensitivity of MALDI-TOF MS made it suitable for screening oral cancers from biological fluids, especially saliva, which was easy to collect. This could help reduce the recurrence of the disease in the future. However, more data from individual patients are required to set databanks of PMFs and PCA plots of the diseases.

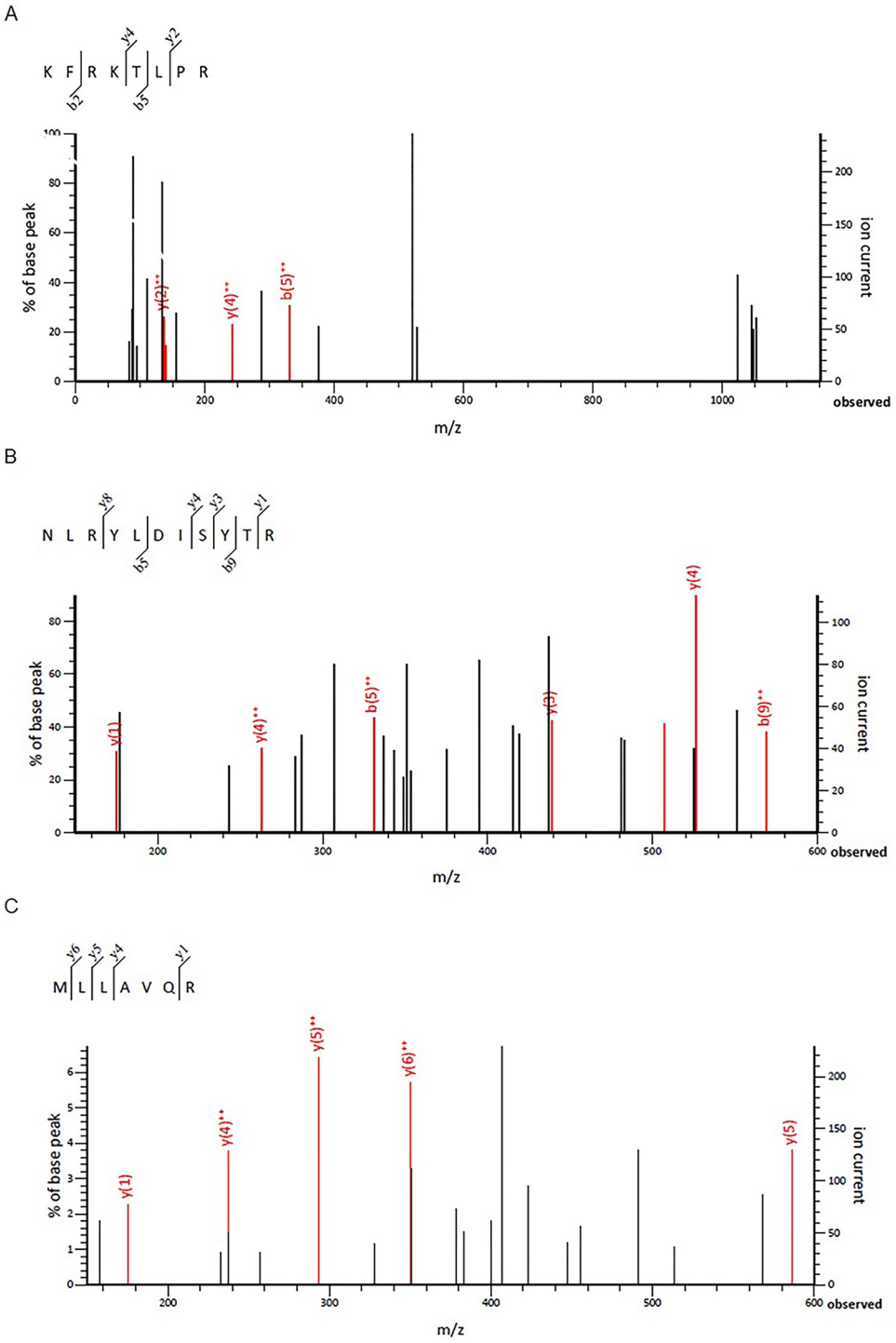

Fig 11. Verification of expressed protein sequences by LC-MS/MS. MS/MS fragmentations of KFRKTLPR found in SENP7 **(A)**, NLRYLDISYTR found in TLR4 **(B)**, and MLLAVQR found in NF-κB **(C)** were shown.

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Mass spectral peaks were analyzed for peptide and nominated protein identification. Differential protein expression has been revealed in canine oral tumors by MALDI-TOF MS coupled with LC-MS/MS and by in-gel digestion coupled with mass spectrometry (GeLC MS/MS) from tissues of oral tumors [33]. We did not find similar proteins to those from tumor tissues

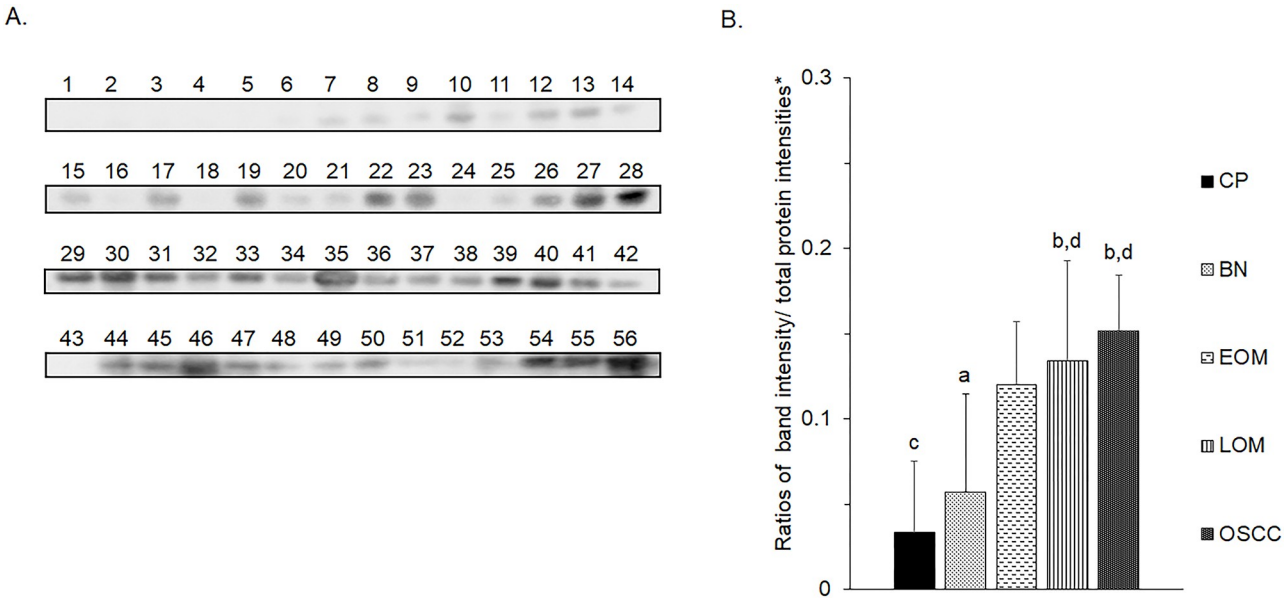

Fig 12. Western blot analysis of salivary sentrin-specific protease 7 (SENP7) of individual saliva samples from dogs with periodontitis and normal controls (CP), benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively) and oral squamous cell carcinoma (OSCC). Representative western blot for SENP7 at 37 kDa **(A)** and bar graph of ratios of SENP7 protein intensity to total blotted protein intensities in each lane in the second half of a membrane* **(B)**. a–b and c-d denote a significant difference at $p<0.05$ and $p<0.01$, respectively.

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which was probably due to the different source of samples, as proteins in saliva could be either secretary proteins from zygomatic, parotid, mandibular and sublingual salivary glands or proteins from oral tumors. Compared with the previously reported normal canine salivary proteomics, a number of different proteins in the CP group were observed [12–15]. This was possibly

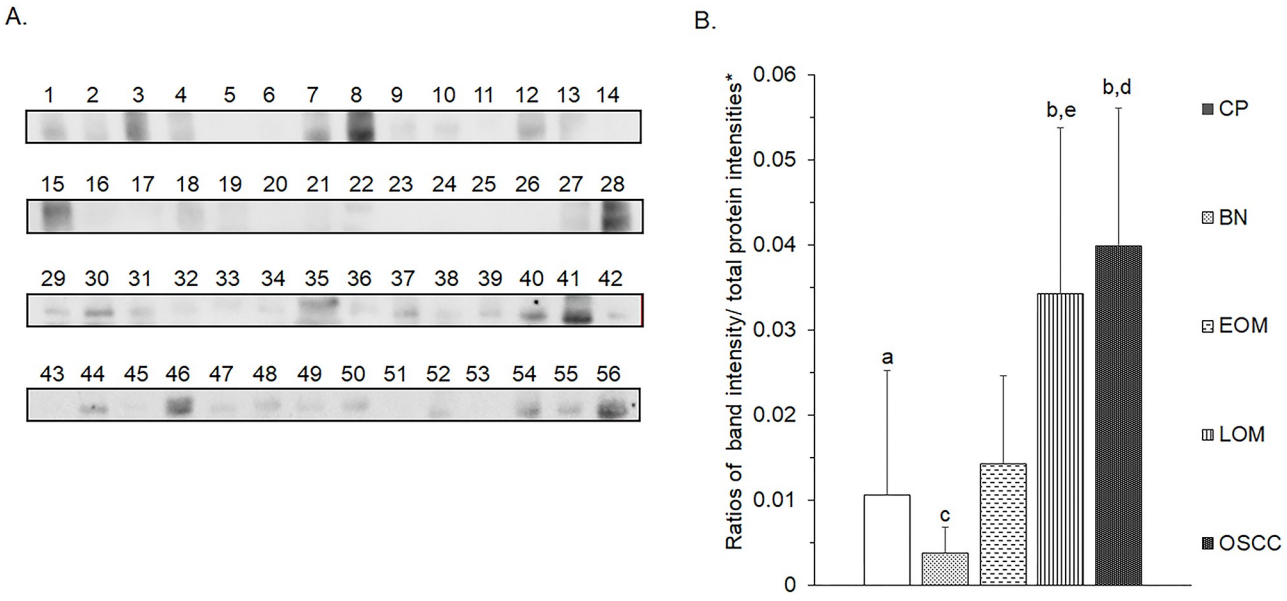

Fig 13. Western blot analysis of salivary toll like receptor 4 (TLR4) of individual saliva samples from dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). Representative western blot for TLR4 at 95 kDa **(A)** and bar graph of ratios of TLR4 protein intensity to total blotted proteins in each lane in the first half of a membrane* **(B)**. a–b, c-d and c-e denote a significant difference at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

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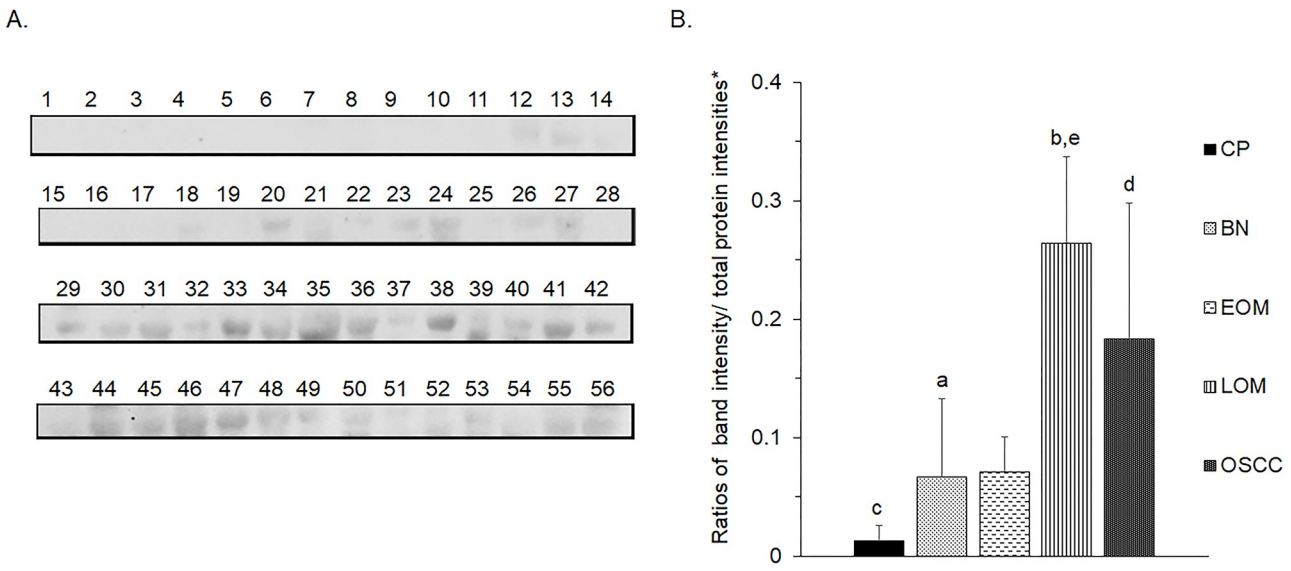

Fig 14. Western blot analysis of nuclear factor kappa B (NF-κB) of individual saliva samples from dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP). Representative western blot for NF-κB at 65 kDa **(A)** and bar graph of ratios of NF-κB protein intensity to total blotted proteins in each lane in the first half of a membrane* **(B)**. a–b, c-d and c-e denote a significant difference at $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

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owing to different groups of dogs in the study as we combined periodontitis and healthy dogs as a CP group. In addition, different dog breeds and environments as well as different proteomic approaches could affect the results [13–15].

Our study showed for the first time elevated SENP7 in canine oral tumors. SENPs are small ubiquitin-like modifier (SUMO)-specific protease. SENP7 functioned to deconjugate SUMO from cellular substrates [34]. SENP7 is in the same family as SENP3. Overexpressed SENP3 leads to the imbalance of SUMO homeostasis and to development and progression of a number of cancers including prostate, ovarian, lung, rectum, and colon [34]. The long SENP7 transcript has been reported to promote epithelial–mesenchymal transition and decrease the cell adhesion molecule E-cadherin (CDH1) in breast cancer cell line, which could lead to the metastasis of the disease [35]. Decreased mRNA expression of cell adhesion molecules [*CDH1*, syndecan-1 (*SDC1*) and *NECTIN4*] has been reported in canine OM [36]. SENP7 also showed a strong relationship with the chemotherapy drugs (Fig 5A–5C). The networks of SENP7 as well as other targets, KAT2B and DTX3L, with chemotherapy drugs showed the strong relationship with TP53 protein, a biomarker of oral cancers [(Figs 5 and 6) [37, 38]. The association of target proteins with TP53 and chemotherapy drugs, especially carboplatin and doxorubicin, should be further investigated.

TLR4 expression has been reported in several cancers, including human head and neck cancer [39], human laryngeal and oral cancer cell line and melanoma [40–42]. In fact, TLR4 has been reported to play divergent roles, either as a pro- or an anti-tumor agent [43]. Our study demonstrated that TLR4 and NF-κB expressions were elevated in LOM and OSCC, which displayed intimate clusters in a PCA plot; hence, TLR4 promoted cancer progression and possibly served as a prognostic factor. In addition, TLR4 has been reported to regulate the inflammatory response by activating NF-κB either via the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway or the Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway [39, 44]. The TLR4–NF-κB pathway has been intensively studied in several cancers such as laryngeal carcinoma and ovarian

carcinoma as prognostic markers and inhibiting of the pathway might serve as a potential treatment of the cancers. Suppressor of cytokine signaling 1 (SOCS1), a regulator of cytokine-mediated innate and adaptive immunity, has been reported to inhibit the TLR4–NF-κB pathway in laryngeal carcinoma. In addition, decreased TLR4 expression has been observed in ovarian carcinoma after treating with NF-κB inhibitor [45].

Conclusions

The present study revealed the discrete clusters of EOM, LOM, OSCC, BN and CP groups, using salivary MALDI-TOF MS. With the combination of MALDI-TOF MS and LC MS/MS, potential protein candidates associated with the diseases were identified. Western blot analysis could verify SENP7, TLR4 and NF-κB as potential salivary biomarkers of canine oral tumors. Further studies of the role of TLR4 and NF-κB as prognostic markers of progressive tumors and of the signal transduction of SENP7 and TLR4–NF-κB pathways and the mechanisms to inhibit the pathways should be performed.

Supporting information

S1 Fig. Representative western blot of pooled samples for SENP7 at 37 kDa. Lane 1: Periodontitis and normal controls; Lane 2: Benign oral tumors; Lane 3: Early-stage oral melanoma; Lane 4: Late-stage oral melanoma; Lane 5: Oral squamous cell carcinoma.
(TIF)

S2 Fig. Representative western blot of pooled samples for TLR4 at 95 kDa. Lane 1: Periodontitis and normal controls; Lane 2: Benign oral tumors; Lane 3: Early-stage oral melanoma; Lane 4: Late-stage oral melanoma; Lane 5: Oral squamous cell carcinoma.
(TIF)

S3 Fig. Representative western blot of pooled samples for NF-κB at 65 kDa. Lane 1: Periodontitis and normal controls; Lane 2: Benign oral tumors; Lane 3: Early-stage oral melanoma; Lane 4: Late-stage oral melanoma; Lane 5: Oral squamous cell carcinoma.
(TIF)

S4 Fig. Representative western blot of individual samples for SENP7 at 37 kDa. CTRL, 6 normal controls; PD, 3 Periodontitis; EOM, 5 Early-stage oral melanoma; BN, 11 Benign oral tumors; OSCC, 9 Oral squamous cell carcinoma; LOM, 22 Late-stage oral melanoma.
(TIF)

S5 Fig. Representative western blot of individual samples for TLR4 at 95 kDa. CTRL, 6 normal controls; PD, 3 Periodontitis; EOM, 5 Early-stage oral melanoma; BN, 11 Benign oral tumors; OSCC, 9 Oral squamous cell carcinoma; LOM, 22 Late-stage oral melanoma.
(TIF)

S6 Fig. Representative western blot of individual samples for NF-κB at 65 kDa. CTRL, 6 normal controls; PD, 3 Periodontitis; EOM, 5 Early-stage oral melanoma; BN, 11 Benign oral tumors; OSCC, 9 Oral squamous cell carcinoma; LOM, 22 Late-stage oral melanoma.
(TIF)

S1 Table. The ratios of target band intensities from pooled samples to the total proteins in each lane in the first or second half of a membrane according to the sizes of target proteins.
(XLSX)

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Formal analysis: Gunnaporn Suriyaphol.

Funding acquisition: Sekkarin Ploypetch, Gunnaporn Suriyaphol.

Investigation: Sekkarin Ploypetch.

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Visualization: Sekkarin Ploypetch.

Writing – original draft: Gunnaporn Suriyaphol.

Writing – review & editing: Sekkarin Ploypetch, Gunnaporn Suriyaphol.

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In-gel digestion coupled with mass spectrometry (GeLC-MS/MS)-based salivary proteomic profiling of canine oral tumors

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Abstract:	Background: Various types of oral tumors, either benign or malignant, are commonly found in dogs. Since saliva directly contacts the tumors and saliva collection is non-invasive, easily accessible and cost effective, salivary biomarkers are practical to be used for the diagnosis and/or prognosis of these diseases. However, there is limited knowledge of protein expression in saliva for canine oral tumors. The present study aimed to investigate novel biomarkers from the salivary proteome of dogs with early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC), benign oral tumors (BN), and periodontitis and healthy controls (CP), using an in-gel digestion coupled with mass spectrometry (GeLC-MS/MS). The relationships between protein candidates and chemotherapy drugs were explored and the expression of potential salivary biomarkers was verified by western blot analysis. Results: Increased expression of protein tyrosine phosphatase non-receptor type 5 (PTPN5) was shown in all tumor groups compared with the CP group. Marked expression of PTPN5 was also observed in LOM and OSCC compared with that in BN and EOM. In addition, tumor protein p53 (p53), which appeared in the PTPN5–drug interactions, was exhibited to be expressed in all tumor groups compared with that in the CP group. Conclusions: PTPN5 and p53 were proposed to be potential salivary biomarkers of canine oral tumors.
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In-gel digestion coupled with mass spectrometry (GeLC-MS/MS)-based salivary proteomic profiling of canine oral tumors

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Abstract

Background: Various types of oral tumors, either benign or malignant, are commonly found in dogs. Since saliva directly contacts the tumors and saliva collection is non-invasive, easily accessible and cost effective, salivary biomarkers are practical to be used for the diagnosis and/or prognosis of these diseases. However, there is limited knowledge of protein expression in saliva for canine oral tumors. The present study aimed to investigate novel biomarkers from the salivary proteome of dogs with early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC), benign oral tumors (BN), and periodontitis and healthy controls (CP), using an in-gel digestion coupled with mass spectrometry (GeLC-MS/MS). The relationships between protein candidates and chemotherapy drugs were explored and the expression of potential salivary biomarkers was verified by western blot analysis.

Results: Increased expression of protein tyrosine phosphatase non-receptor type 5 (PTPN5) was shown in all tumor groups compared with the CP group. Marked expression of PTPN5 was also observed in LOM and OSCC compared with that in BN and EOM. In addition, tumor protein p53 (p53), which appeared in the PTPN5–drug interactions, was exhibited to be expressed in all tumor groups compared with that in the CP group.

Conclusion: PTPN5 and p53 were proposed to be potential salivary biomarkers of canine oral tumors.

Keywords: Dog, in-gel digestion coupled with mass spectrometry (GeLC-MS/MS)., Oral tumors, tumor protein p53 (p53), protein tyrosine phosphatase non-receptor type 5 (PTPN5)

Background

Head and neck tumors account for approximately 7% of all tumors in dogs. Among these, oral melanoma (OM) and oral squamous cell carcinoma (OSCC) are most commonly found [1]. The World Health Organization (WHO) has classified the clinical staging system for tumors of the oral cavity in dogs into 4 stages according to their primary sizes and metastatic profile, the tumor, node and metastasis (TNM). Stages I (a < 2 cm diameter tumor) and II (a 2 to < 4 cm diameter tumor) are defined as early clinical stages with no metastasis, whereas stages III (a ≥ 4 cm tumor and/or lymph node metastasis) and IV (a tumor with distant metastasis) are late clinical stages [2]. The latter are most frequently observed in the animal hospital owing to the difficulty in routinely examining tumors in dogs' mouths [3, 4]. After surgical resection, patients with late clinical stage are normally treated with chemotherapy drugs such as carboplatin, a derivative of the anticancer drug cisplatin, doxorubicin (or Adriamycin®), cyclophosphamide and piroxicam. With a high rate of metastasis and recurrence of oral cancer, novel biomarkers are important for early clinical diagnosis, screening and prognosis of the diseases [5]. Saliva proteins have high potential to be appropriate biomarkers because saliva makes direct contact with an oral mass, and saliva collection is non-invasive and not difficult to manipulate [6]. Novel salivary proteome biomarkers have been discovered in human oral tumors [7–10]. However, the study of salivary proteomics of dogs with oral diseases is still limited [6]. The present study aimed to explore novel suitable biomarkers in saliva of dogs with early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC), benign oral tumors (BN), periodontitis (P) and healthy controls (C) (CP group), using in-gel digestion coupled with mass spectrometry (GeLC-MS/MS). Associations of disease-related proteins with the chemotherapy drugs cisplatin,

cyclophosphamide, piroxicam and doxorubicin were exhibited. The candidate protein expressions were verified by western blot analysis.

Results

GeLC-MS/MS results

A total of 3726 proteins were identified. The distribution of the individual and overlapped proteins in EOM, LOM, OSCC, BN and CP groups was illustrated by a Venn diagram (Fig. 1). In addition, the molecular function, biological process, cellular component and the relative expression levels of the proteins uniquely expressed in each group and commonly expressed in all cancerous groups was analysed using the PANTHER software tools (Tables 1 and 2 and Supplementary Table S1). For the networks of protein–protein and protein–chemotherapy drug interactions, analysed by the Stitch program, version 5.0, edge confidence scores demonstrated the strength of the interactions at the functional level. Pathways with high edge confidence scores (>0.700) were presented as thick lines. The associations of protein tyrosine phosphatase non-receptor type 5 (PTPN5) and tumor protein p53 (p53) with cisplatin and doxorubicin drugs were shown. Additionally, the correlation of PTPN5 and cyclophosphamide was demonstrated (Fig. 2).

Western blot analysis results

Western blot analysis unveiled an enhanced expression of PTPN5 and p53 in tumor groups compared with that in the CP group (Figs. 3 and 4). In addition, the expression of PTPN5 in LOM and OSCC was augmented compared with that in BN and EOM (Fig. 3). Peptide sequences of PTPN5 and p53 western blot analysis were verified by LC-MS/MS (Fig. 5).

Discussion

In the present study, GeLC-MS/MS was used to identify novel salivary biomarker candidates in canine oral tumors. PTPN5 and p53 were plausibly shown to be candidates in LOM and OSCC. PTP is a group of protein tyrosine phosphatases that have divergent functions, either promoting or suppressing cancer. Several oncogenic PTPs have been reported to be highly expressed in human breast cancer [24]. In contrast to receptor-type PTPs that localized to the plasma membranes, the non-receptor type PTPs, PTPNs, are located in the cytosol. PTPN5 is in the same non-receptor Cys-based classical PTPs as PTPN1 and PTPN11, which promoted tumorigenesis in ovarian cancer, gastric cancer, prostate cancer, breast cancer, leukaemia, colorectal cancer and uveal melanoma [25-31]. To the best of our knowledge, this study presented for the first time the association of PTPN5 expression and canine oral cancers, particularly LOM and OSCC. Likewise, PTPN1 has been reported to be increased in canine oral cancer tissues by MALDI-TOF MS plus LC-MS/MS [15], indicating that expression of PTPNs might play a role in canine oral cancer. PTPN1 functioned via Src/Ras/Erk and PI3K/Akt pathways, whereas PTPN11 functioned via EGFR/Ras/MAPK pathways [27, 29, 32–34]. Further study should be carried out on the PTPN5 signalling pathway. Since most families of PTPs served as biomarker targets of several anticancer drugs, including PTPN11, PTPN6 and PTP1B, potential inhibitors of PTPN5 as candidate anticancer drugs for oral tumors should be investigated [35].

In the present study, we also exhibited the enhanced expression of p53, in tumor groups, particularly in LOM and OSCC. Likewise, p53 was found in the interaction networks of PTPN5 and the chemotherapy drugs cisplatin and doxorubicin. p53 is a tumor suppressor protein; however, mutant p53 protein has been shown to be a biomarker in several cancers, such as human breast cancer, colorectal cancer, ovarian cancer, oesophageal squamous cell carcinoma, non-small cell lung cancer, and a prognostic marker in breast cancer, oesophageal squamous cell carcinoma,

colon cancer, non-small cell lung cancer and B cell lymphoma [36–43]. In human head and neck squamous cell carcinoma, p53 mutation played an important role in tumorigenesis and progression. It has been used not only as a risk and prognostic biomarker, but also as a predictive biomarker in the clinical response to chemotherapy treatments [44–48]. Several studies, aiming to treat cancer in humans, have investigated the promoting function of wild-type p53 and degradation of mutant p53 [39, 49, 50]. Further investigation of p53 in canine oral tumors for potential prognostic and therapeutic biomarkers should be performed.

In our previous study of salivary proteomics of canine oral tumors using MALDI-TOF MS and LC-MS/MS, expression of sentrin-specific protease 7 (SENP7) was found to be increased in saliva of dogs with LOM and OSCC [6]. SENP7 functions to edit the poly-small ubiquitin-related modifier (SUMO) chains during SUMOylation, a post-translational modification of target proteins involving in several carcinogenic mechanisms [51]. In the present study, increased expression of another protein involved in the SUMOylation process, RanBP2, was noted in a cancerous group (Table 2). RanBP2 regulated translocation of p53, a well-known target of SUMOylation, to the cytoplasm, leading to poor prognosis and prostate cancer progression [52]. In conclusion, the present study used GeLC-MS/MS and western blotting to reveal the potential salivary biomarkers of canine oral tumors, PTPN5 and p53. The network interactions between the candidate proteins and chemotherapy drugs were also demonstrated**.** For future work, signalling pathways and potential inhibitors of the target proteins should be investigated as potential anticancer drugs for canine oral tumors.

Materials and Methods

Animals

Saliva samples were recruited from 5 EOM, 24 LOM, 10 OSCC and 11 BN dogs (age range 7–14 years) scheduled for surgical excision at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University and private animal hospitals. Patients were diagnosed without previous treatments with chemotherapy and/or radiotherapy. The staging of OM and OSCC were determined according to the WHO, whereby EOM and LOM include stages 1–2 and 3–4, respectively [11, 12]. Regional lymph nodes were examined cytologically for metastasis. Tumor spreading to abdominal organs was checked by ultrasonography. Skull-to-abdomen radiography was performed by a Brivo DR-F digital X-ray system (GE Healthcare, Chicago, IL, USA) or an Optima CT660 64-slice CT scanner (GE Healthcare). Seven samples were obtained from healthy dogs with no history or clinical signs of oral cavity or cancerous problems (age range 7–8 years). A chronic periodontitis group contained 5 dogs showing gingivitis, dental tartar and/or periodontal attachment loss (age range 7–13 years). The sample collection protocol was approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand (Approval number 1631042). The samples were obtained with the consent of all dog owners.

Sample collection and preparation

Saliva was collected on the day of surgery without mechanical and chemical stimulation. Dogs were fasted for at least 1 h before saliva collection. Mouths were cleaned with 0.9% sterile normal saline solution [9]. Whole saliva (0.5–1.0 mL) was collected for 5–10 min using a sterile cotton swab. Samples were centrifuged at 2600 × g for 15 min at 4°C [13]. Halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) was added to 200 µL of supernatant and samples were kept at –20°C until analysis. Total protein concentrations were determined by the Lowry method, using bovine serum albumin as a protein standard [14]. According to our previous peptide

profiles obtained from MALDI-TOF MS data, showing the control and chronic periodontitis in the same cluster, control and chronic periodontitis samples were consequently combined as a CP group [6].

Analysis of salivary peptides by GeLC-MS/MS

Salivary peptides were analysed by GeLC-MS/MS as previously described with some modifications [15]. Briefly, 50 µg of pooled samples in each group (CP, BN, EOM, LOM and OSCC) were mixed with loading buffer [0.5M dithiothreitol (DTT), 10% w/v SDS, 0.4 M Tris-HCl pH 6.8, 50% v/v glycerol, 0.1 mg/ml Bromophenol Blue] and boiled at 90°C for 5 min prior to separating on 12.5% SDS-PAGE (Atto, Tokyo, Japan). Gels were fixed using 50% methanol, acetic acid and 37% formaldehyde and stained with silver nitrate solution, before being scanned using a GS-710 scanner (Bio-Rad Laboratories, Benicia, CA, USA) and stored in 0.1% acetic acid. After that in-gel tryptic digestion was performed where protein bands in each lane were divided into 17 segments and chopped into 1 mm^3 pieces. Gel pieces were dehydrated using 100% acetonitrile (ACN) and dried. Cysteines were reduced and alkylated by 10 mM DTT in 10 mM ammonium bicarbonate and 100 mM iodoacetamide in 10 mM ammonium bicarbonate, respectively, prior to dehydrating twice in 100% ACN. Trypsin digestion was performed in 50 mM NH_4HCO_3 (pH 7.8) overnight at 37ºC. The tryptic peptides were extracted from the gels using 50% ACN in 0.1% formic acid (FA). Pooled samples were submitted to a reversed-phase high performance liquid chromatography (HPLC). The gradient-eluted peptides were analysed using an Ultimate 3000 LC System coupled to an HCTUltra PTM Discovery System (Bruker Daltonics, Bremen, Germany). Peptides were separated on a PepSwift monolithic column (100 µm internal diameter × 50 mm) (Thermo Fisher Scientific). Peptide separation was achieved with a linear

gradient at a flow rate of 1000 nL/min from 4% ACN, 0.1% FA to 70% ACN, 0.1% FA for 7.5 min with a regeneration step at 90% ACN, 0.1% FA and an equilibration step at 4% ACN, 0.1% FA. The entire process took 20 min. Peptide fragment mass spectra were acquired in a data-dependent Auto MS mode with a scan range 400–1500 m/z. However, in the case of having more than 5 precursor fragments, peptides would be selected from the MS scan at 200–2800 m/z. CompassXport software (Bruker Daltonics) was used to convert data from LC-MS/MS into the mzXML format. Protein quantitation was performed using DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare) [16, 17]. The peptide sequences were searched against the NCBI mammal database for protein identification using MASCOT software, version 2.2 (Matrix Science, London, UK) [18]. Database interrogation included taxonomy (mammals), enzyme (trypsin), variable modifications (oxidation of methionine residues), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (1.2 Da), fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and maximum number of missed cleavages. Proteins were identified from one or more peptides with an individual MASCOT score corresponding to $P < 0.05$. Proteins were annotated by UniProtKB/Swiss-Prot entries (http://www.uniprot.org/) and classified according to their molecular function, biological process and cellular component using the PANTHER classification system, version 8.1 (www.pantherdb.org/) [19]. Protein list comparison among different sample groups was displayed using jvenn diagram (http://bioinfo.genotoul.fr/jvenn/example.html) [20]. The interaction network of candidate proteins and chemotherapy drugs was explored using the Stitch program, version 5.0 (http://stitch.embl.de/) [21].

Validation of MS results by western blot analysis

Protein concentrations of pooled saliva samples were determined by Lowry assay, SDS-PAGE and western blotting as described previously [6, 22]. Briefly, samples (10 µg) were mixed with loading dye, heated and applied to a pre-cast NuPAGE 4–12% (w/v) Bis-Tris gel (Thermo Fisher Scientific) using RunBlue MES Run Buffer (Expedeon, Heidelberg, Germany) at 200 V for 90 min. Protein standard marker was PageRuler prestained protein ladder (molecular weight range 10–180 kDa) (Thermo Fisher Scientific). After that, the proteins were transferred to TranBlot Turbo nitrocellulose membranes (Bio-Rad Laboratories) at 25 V for 14 min using Trans-Blot Turbo 5× transfer buffer (Bio-Rad Laboratories). Detection of total protein band intensities in each lane was performed by a Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes (Thermo Fisher Scientific) according to the manufacturer's instructions. Non-specific protein binding was blocked with 5% bovine serum albumin (BSA) (GoldBio, St Louis, MO, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) at 25°C overnight. After washing with TBST, primary antibodies diluted at 1:1000 were incubated with a membrane at 4°C overnight, including mouse monoclonal anti-human PTPN5 (Santa Cruz Biotechnology, Dallas, TX, USA) and Novocastra liquid mouse monoclonal anti-human p53 (D-07) (Leica Biosystems, Newcastle upon Tyne, UK). Membranes were washed with TBST and then incubated with 1:10 000 horseradish peroxidase conjugated-rabbit anti-mouse IgG secondary antibody (Abcam, Cambridge, UK) for 1 h at 25°C. The proteins of interest were visualized with ECL western blotting detection reagents (GE Healthcare). Western blot imaging was performed using a ChemiDoc Touch Imaging System (Bio-Rad Laboratories). Protein bands intensities were analysed by Image Lab 6.0.1 software (Bio-Rad Laboratories). Total protein normalization was performed with the modification of Aldridge *et al.* (2008) [6, 23]. The ratios of target band

intensities to the total proteins in each lane in the first or second half of a membrane were calculated according to the sizes of target proteins. The western blotting was performed in triplicate.

Verification of expressed protein sequences by LC-MS/MS

LC-MS/MS was utilized to confirm PTPN5 and p53 (or TP53) protein identities as described previously [6]. Briefly, blotting membranes were incubated with Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific) for 15 min and washed 4 times with TBST. Protein bands were cut and incubated with 10 mM DTT in 10 mM ammonium bicarbonate overnight. Samples were then trypsinized at 37°C for 3 h and applied to the LC-MS/MS as mentioned above.

Statistical analysis

ANOVA statistical analysis, incorporated into the DeCyder MS differential analysis software, and MASCOT software, version 2.2 were used to analyse significantly different peptide peak intensities and MASCOT LC-MS/MS scores, respectively. Western blot analysis was performed by ordinary one-way ANOVA with Tukey's multiple comparisons for PTPN5 and p53. Statistical analyses of protein expression data were conducted using GraphPad Prism, version 8.0.1 (GraphPad Software, La Jolla, CA, USA). Significance was accepted at the $P < 0.05$ level.

Abbreviations

ACN: acetonitrile; Akt: Protein Kinase B; BN: benign oral tumors; BSA: bovine serum albumin; CU-ACUC: The Chulalongkorn University Animal Care and Use Committee; CP: periodontitis and healthy controls; CT: Computer tomography; CPC: chromosomal passenger complex; DTT: dithiothreitol; EGFR: epidermal growth factor receptor; EOM: early-stage oral melanoma; Erk: Extracellular-signal-regulated-kinase; FA: formic acid; GeLC-MS/MS: In-gel digestion coupled

with mass spectrometry; HCTUltra: high-capacity ion trap mass spectrometry; HPLC: high performance liquid chromatography; i.d.: Inside diameter; IAA: iodoacetamide; IgG: Immunoglobulin G; LC: liquid chromatography; LOM: late-stage oral melanoma; MALDI-TOF MS: matrix-assisted laser desorption ionization mass spectrometry; MAPK: Mitogen-activated protein kinase; MES buffer: 2-(*N*-morpholino)ethanesulfonic acid buffer; MS: mass spectrometry; m/z: mass per charge ratio; NCBI: National Center for Biotechnology Information; NH_4HCO_3: ammonium bicarbonate; OSCC: oral squamous cell carcinoma; p53: tumor protein p53; PI3K: phosphoinositide-3 kinase; PTM: Post-Translation Modification; PTPN1: protein tyrosine phosphatase non-receptor type 1; PTPN5: protein tyrosine phosphatase non-receptor type 5; PTPN6: protein tyrosine phosphatase non-receptor type 6; PTPN11: protein tyrosine phosphatase non-receptor type 11; PTP1B: protein tyrosine phosphatase 1B; RanB2: E3 SUMO-protein ligase RanBP2; Ras: Ras protein; SDS: sodium dodecyl sulfate; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SENP3: SUMO specific-isopeptidase; SENP7: Sentrin-specific protease 7; Src: Proto-oncogene tyrosine-protein kinase; SUMO: small ubiquitin-like modifier; TBST: Tris buffered saline buffer containing 0.1% Tween 20; TNM stage: stages according to their primary sizes and metastatic profile, the tumor, node and metastasis; Topoll: Targeting DNA topoisomerase II; Tris-HCl: Tris hydrochloride; WHO: World Health Organization

Declarations

- **Ethics approval**

All experimental protocols were approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Faculty of Veterinary Science, Chulalongkorn University (Approval

number 1631042). All procedures were performed in accordance with the relevant guidelines and regulations. The owners gave consent for their animals to be part of this study.

- **Consent to publish**

Written informed consent was obtained from dog's owners for publication of this manuscript.

- **Availability of data and materials**

Not applicable.

- **Competing interests**

The authors declare that they have no competing interests.

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- **Author contributions**

G.S. and S.R. designed the study. S.P., C.K. and A.R. collected samples. S.P. and N.P. performed the experiments and analyses. G.S. and S.P. drafted the manuscript. G.S. and S.R. finalized the manuscript. All authors read and approved the final manuscript.

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Table 1 Overexpressed proteins uniquely found in normal controls and periodontitis, benign oral tumors, early-stage oral melanoma, late-stage oral melanoma and oral squamous cell carcinoma based on biological process involvement and protein score

Database	Protein name	Protein ID score	Peptides	Biological process	Subcellular distribution
Normal controls and periodontitis					
XP_016007048.1	Semaphorin-4B isoform X1	13.9	QLVASYCPK	1. Negative chemotaxis 2. Semaphorin–plexin signalling pathway	1. Extracellular space 2. Integral component of plasma membrane
XP_011988340.1	Visual system homoeobox 1 isoform X2	16.98	FPGRPLPSAARQK	1. Multicellular organism development 2. Regulation of transcription	1. Nucleus 2. Cytoskeleton
XP_013973434.1	CDK5 regulatory subunit-associated protein 2 isoform X1	12.52	FTNQGKR	Microtubule organizing center	

XP_002689199.3	Olfactory receptor 2M5	26.19	MCWQVAAMSWAGGAR	Olfaction	Plasma membrane
XP_008048855.1	Potassium voltage-gated channel subfamily Q member 1	34.67	LNIEDFR	1. Potassium ion export across plasma membrane 2. Cellular response to cAMP	1. Endoplasmic reticulum 2. Endosome 3. Plasma membrane
XP_007125871.1	GLIPR1-like protein 1	14.03	AHNEAR	Single fertilization	Plasma membrane
EHB15707.1	Transient receptor potential cation channel subfamily M member 5	26.14	TVAPKSLLFR	Ion transmembrane transport	Plasma membrane
Benign oral tumors					
KFO21119.1	Germ cell-less protein-like 1	7.86	KAVAAR	Cell differentiation	Nucleus
XP_004629194.1	Poly [ADP-ribose] polymerase 12	21.09	KLGMSSELVHR	Protein auto-ADP-ribosylation	Nucleus
XP_015289690.1	Lamin tail domain-	8.98	GLLPPMSSGK	Cell population proliferation	1. Cytoskeleton 2. Nucleus

	containing protein 2				
XP_012868232.1	Telomeric repeat-binding factor 2-interacting protein 1	16.48	AEPDPEAAESVEPQTK	1. Negative regulation of DNA recombination at telomere 2. Positive regulation of NF-κB transcription factor activity	Nucleus
XP_012373519.1	Myb-related protein B	16.69	MLPGRYVPGGGVGAR	1. Mitotic cell cycle 2. Regulation of cell cycle	Nucleus
XP_012865682.1	Erythrocyte membrane protein band 4.2	12.59	QWSAVVEDR	1. Cell morphogenesis 2. Hemoglobin metabolic process cell differentiation	Cytoskeleton 1. Cytoplasm 2. Membrane
XP_005371197.1	Long-chain-fatty-acid–CoA ligase ACSBG2	5.91	APGTGFLTEMLR		
Early-stage oral melanoma					

XP_011760132.1	Putative protein SSX6	12.53	GGNMPGPTGCVR	Regulation of transcription, DNA-templated	Nucleus
XP_004326275.1	Bromodomain testis-specific protein-like	14.28	DNAKPMNYDEKR	Chromatin remodelling	Nucleus
XP_006868797.1	Zinc finger protein GLI2-like	16.61	GGSLENSSIPDLSR	Nucleic acid binding	Nucleus
Late-stage oral melanoma					
EPQ15807.1	Transformation/ transcription domain-associated protein	9.28	AMAILTPAVPAR	1. DNA repair 2. Histone deubiquitination	1. Golgi apparatus 2. Nucleus
XP_009240233.1	Glutathione S-transferase-like	20.93	ARISHILTINK	Glutathione transferase activity	Cytoplasm
XP_011282224.1	Protein FAM186A	32.14	SVEQSFLELLIEEDR	No data	1. Nucleus 2. Cytoplasm
XP_004412391.1	Deleted in lung and oesophageal cancer protein 1	7.49	AGPPKNK	Negative regulation of cell population proliferation	Cytoplasm

Oral squamous cell carcinoma					
XP_007944568.1	Ankyrin repeat domain-containing protein 26-like	6.56	ADIKENMVIDMQANCMILXK	Protein interaction	Cytoplasm
XP_012392091.1	Cytohesin-4 isoform X2	9.84	YPGELSSGEAEELQR	Regulation of ARF protein signal transduction	Nucleus
XP_007532207.2	Probable C-mannosyltransferase DPY19L4	17.69	KPKSSGNK	Protein C-linked glycosylation via 2'-alpha-mannosyl-L-tryptophan	Membrane
EHB17858.1	Dynein heavy chain 11, axonemal	3.80	ATSEMR	Determination of left/right symmetry	Cytoskeleton
XP_004275614.1	Fanconi anaemia-associated protein of 100 kDa	7.99	XGMDDR	Interstrand cross-link repair	Nucleus
OBS77059.1	Protein A6R68_16468	7.01	DQVSDDVSVQSSGPNCQR	Regulation of transcription by RNA polymerase II	Nucleus

Table 2 Overexpressed proteins commonly found in early-stage oral melanoma, late-stage oral melanoma and oral squamous cell carcinoma based on biological process involvement and protein score

Database	Protein name	Protein ID score	Peptides	Biological process	Subcellular distribution
XP_005376885.1	ATP synthase subunit s, mitochondrial isoform X1	4.77	HQTMLFGK	ATP biosynthetic process	Mitochondria
XP_004411845.1	Carbonic anhydrase 12 isoform X1	33.40	SLHAAAVLLLLCFK	Carbonate dehydratase activity	Integral component of membrane
XP_015354861.1	Cell division cycle-associated protein 2	17.63	RSFCAPTLSSK	Cell cycle cell division	Nucleus
XP_004625867.1	dihydroorotate dehydrogenase (quinone), mitochondrial	17.17	IPIIGVGGVSSGQDAMDK	‘de novo’ UMP biosynthetic process	Mitochondrion inner membrane
XP_014948096.1	Hermansky–Pudlak syndrome 3 protein isoform X1	9.93	ACPPISMDVCALR	Organelle organization, pigmentation	Cytosol
XP_004644982.1	KN motif and ankyrin repeat domain-containing protein 3	14.22	FALNQNLPDLGGSR	Negative regulation of	Cytoplasm

				actin filament polymerization	
XP_008158631.1	Leucocyte immunoglobulin-like receptor subfamily A member 6	3.43	EPAEVEELK	Adaptive immune response	Membrane
XP_003787787.1	Negative elongation factor C/D	7.47	SNFIMMN	Transcription by RNA polymerase II	Nucleus
XP_011285357.1	Neurexin-2-β	13.66	VVVVLGGQGSSG	Neuron cell–cell adhesion signal transduction	Membrane
XP_005629058.1	Origin recognition complex subunit 1 isoform X1	6.66	SRPTPSHPATPRAK	DNA replication, mitotic cell cycle	Nucleus
XP_006896914.1	Phosphoenolpyruvate carboxykinase, cytosolic [GTP] isoform X1	18.32	ARVSQM	Gluconeogenesis	Cytosol
XP_004620060.1	Phospholipase B1, membrane-associated-like	11.55	RMENNSGINFNEDWK	Phospholipase activity	Integral component of membrane

XP_012626009.1	Progesterone receptor isoform X2	17.75	VLLLLNTTR	DNA-binding transcription factor activity	Nucleus
XP_008151988.1	Secernin-2	13.13	QGGITAEAMMDILRDK	Exocytosis	Extracellular exosome
XP_007489730.1	Sodium/iodide cotransporter	6.99	DSKEYPQEVK	Cellular response to cAMP	Membrane
XP_016811442.1	T-box transcription factor TBX18 isoform X2	12.54	MYSGELGPI	DNA-binding transcription factor activity	Nucleus
XP_004045865.1	Uncharacterized protein LOC101132572	12.64	RFTLSLDAPAPTQGVCK	Unknown	Unknown
XP_006190947.1	Zinc finger protein ZIC 3	8.6	THTGKGEGGR	Cell differentiation	Nucleus
XP_011744397.1	28S ribosomal protein S14, mitochondrial	16.97	KNTXLPK	Mitochondrial translational elongation and translation	Mitochondria

XP_007505382.1	3-hydroxyisobutyrate dehydrogenase, mitochondrial isoform X1	8.97	SMASKTPVGFVGLGNMGNPMAK	3-hydroxyisobutyrate dehydrogenase activity	Mitochondria
XP_004448347.1	α-ketoglutarate-dependent dioxygenase alkB homolog 4 isoform X1	7.08	LVSLNLLSSTVLSMSR	Demethylation	Mitochondria
XP_005065718.1	Ankyrin repeat domain-containing protein 34B	20.75	QKALMTTNGPK	Unknown	Nucleus
NP_036833.1	β1 adrenergic receptor	13.02	QGFSSESK	Adenylate cyclase-activating adrenergic receptor signalling pathway	Endosome, plasma membrane
ELK12127.1	Cytochrome b-c1 complex subunit 2, mitochondrial	11.51	DNMAYTGEGLR	Aerobic respiration	Mitochondria
XP_006883886.1	E3 SUMO-protein ligase RanBP2	11.07	LSQSGHMLINLSRGK	centrosome localization	Nucleus

BAD96349.1	Heme oxygenase (decyclizing) 2 variant	11.2	KSSGALEK	Heme oxygenase (decyclizing) 2 variant	Endoplasmic reticulum
OBS70980.1	Pyrroline-5-carboxylate reductase	9.86	LTAFXPAPK	L-proline biosynthetic process	Mitochondria
XP_015976454.1	Laminin subunit α1	15.83	YXNGTWYK	Cell adhesion	Extracellular region or secreted
KFO28259.1	Mitochondrial import receptor subunit TOM20 like protein	10.02	LFSVQMPLAKLPTTGQR	Protein import into mitochondrial matrix	Mitochondria
EAW72809.1	Signal sequence receptor, delta (translocon-associated protein delta), isoform CRA_c	3.09	APTQAPMR	Regulate the retention of ER resident proteins	Endoplasmic reticulum
XP_006865897.1	Tyrosine-protein phosphatase non-receptor type 5	21.9	AEGLRGSHR	Cellular response to cytokine stimulus	Endoplasmic reticulum

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Figure Legends

Fig. 1 Venn diagram of proteins differentially expressed in early-stage OM (EOM), late-stage OM (LOM), oral squamous cell carcinoma (OSCC), benign oral tumors (BN) and normal and periodontitis (CP). Circles indicate overexpressed proteins uniquely found in each group and commonly found in all cancerous groups.

Fig. 2 Involvement of tyrosine-protein phosphatase non-receptor type 5 (PTPN5) and tumor protein p53 (TP53) in networks of protein chemotherapy drug interactions, cisplatin and doxorubicin, analysed by Stitch, version 5.0; **a** Interactions of PTPN5 and TP53 with cisplatin; **b** Interactions of PTPN5 and TP53 with doxorubicin; c Interactions of PTPN5 and TP53 with cyclophosphamide

Red circles: PTPN5 and TP53. Abbreviations: ataxia telangiectasia mutated (ATM), breast cancer 4721, early onset (BRCA1), cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), cyclin-34473 dependent kinase inhibitor 2A (CDKN2A), CREB binding protein (CREBBP), E1A binding 474 protein p300 (EP300), K(lysine) acetyltransferase 2B (KAT2B), mitogen-activated protein kinase 4758 (MAPK8), Mdm2 (MDM2) and sirtuin 1 (SIRT1).

•

Fig. 3 Western blot analysis of salivary tyrosine-protein phosphatase non-receptor type 5 (PTPN5) of dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP); **a** Representative western blot for PTPN5 at 57–68 kDa; **b** bar graph of ratios of PTPN5 protein intensity to total blotted proteins in each lane in a membrane; a-b denote a significant difference at $P < 0.05$; c-d denote a significant difference at $P < 0.001$.

Fig. 4 Western blot analysis of salivary tumor protein p53 (p53) of dogs with benign oral tumors (BN), early- and late-stage oral melanoma (EOM and LOM, respectively), oral squamous cell carcinoma (OSCC) and periodontitis and normal controls (CP); **a** representative western blot for P53 at 53 kDa; **b** bar graph of ratios of P53 protein intensity to total blotted proteins in each lane in a membrane; a-b denote a significant difference at $P < 0.05$; a-c denote a significant difference at $P < 0.01$; a-d denote a significant difference at $P < 0.001$.

Fig. 5 Verification of expressed protein sequences by LC-MS/MS; **a** MS/MS fragmentations of LRVISLR found in salivary tyrosine-protein phosphatase non-receptor type 5 (PTPN5); **b** ALPPSTSSSPPQK found in salivary tumor protein p53 (p53).

Supporting information

Supplementary Table S1. The relative expression levels of proteins found in normal controls and periodontitis (CP), benign tumors (BN), early-stage oral melanoma (EOM), late-stage oral melanoma (LOM) and oral squamous cell carcinoma (OSCC) as $\log_2$ intensities.

(XLSX)

Figure 1

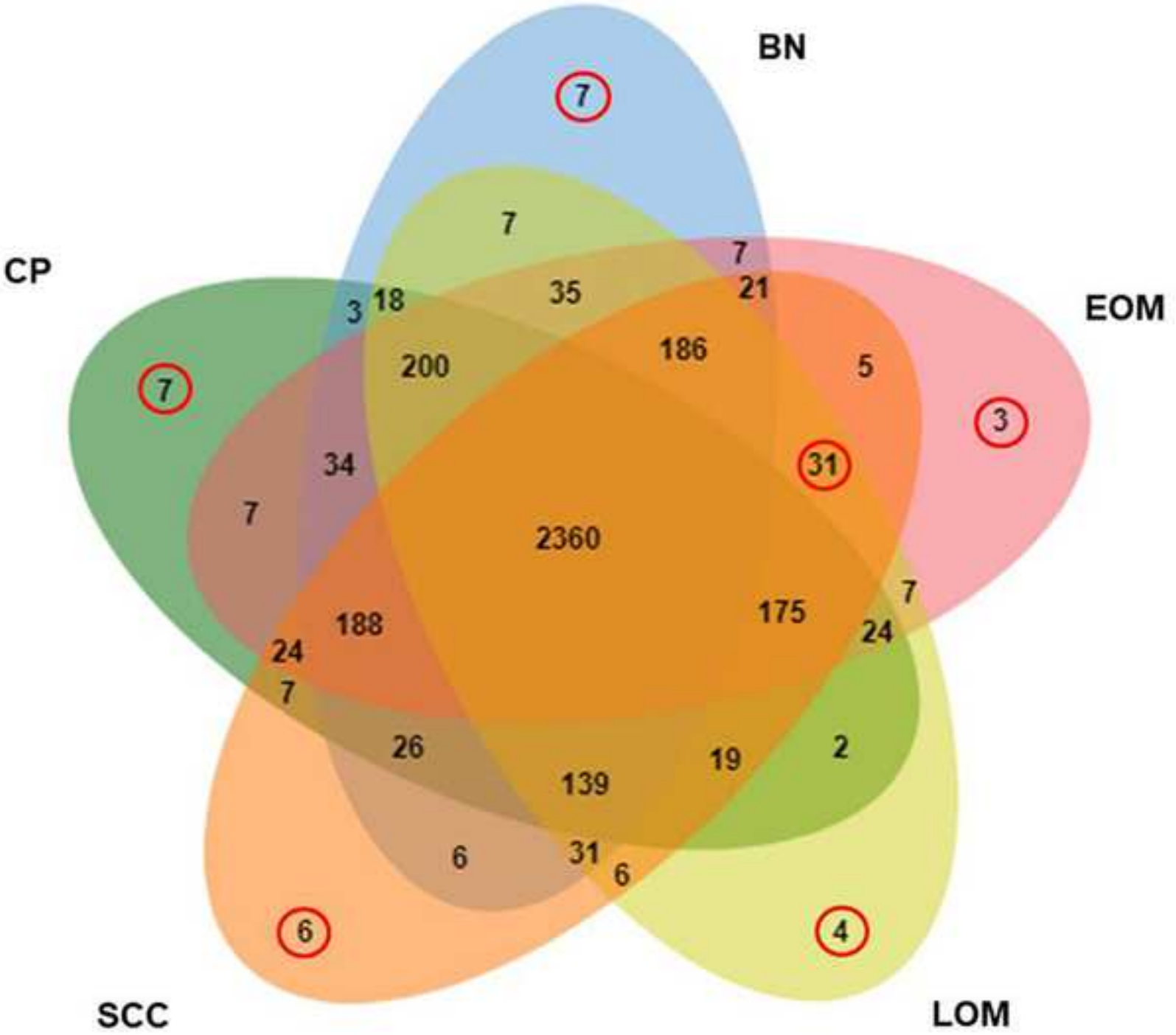


Figure 2a

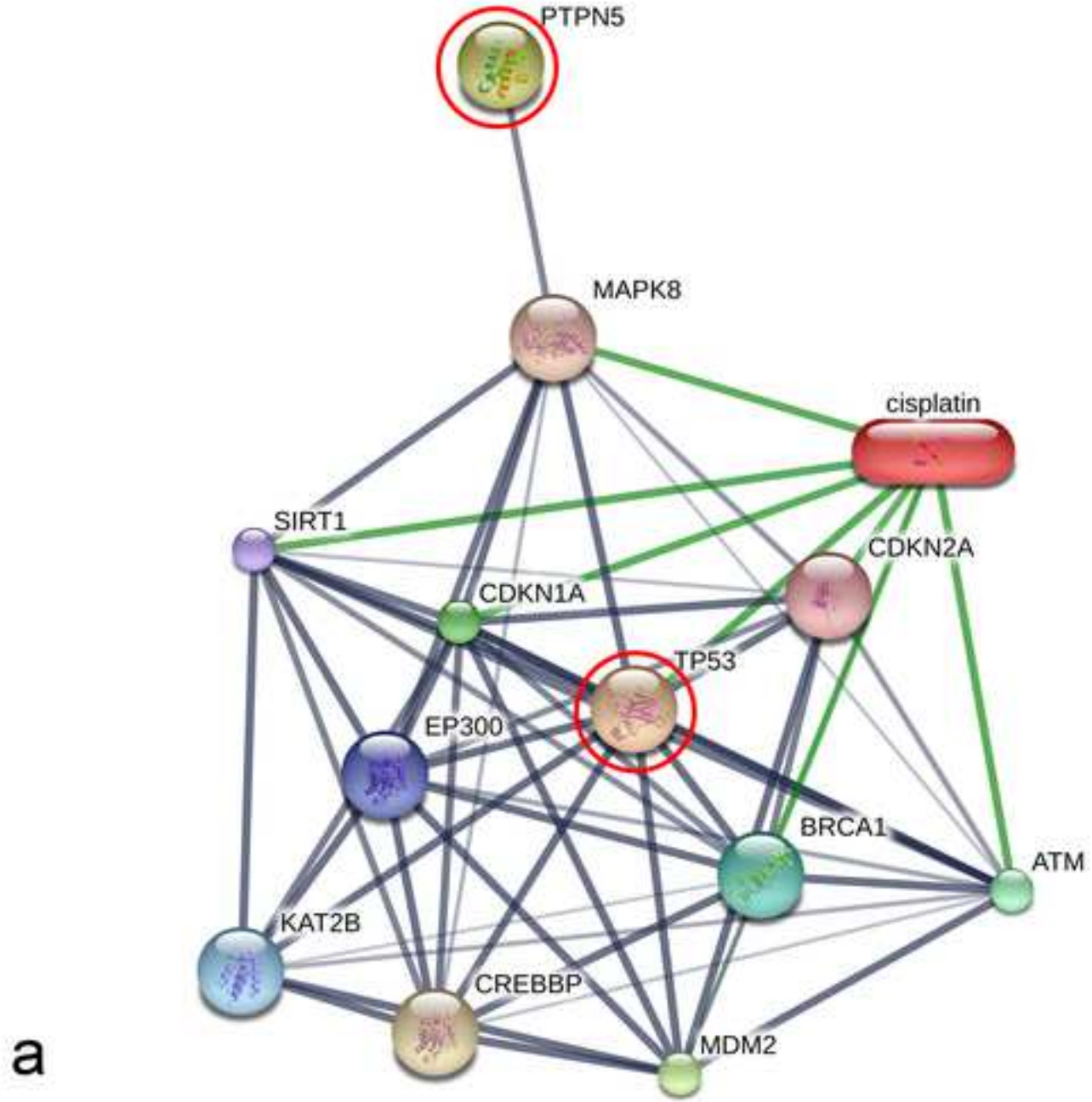


Figure 2b

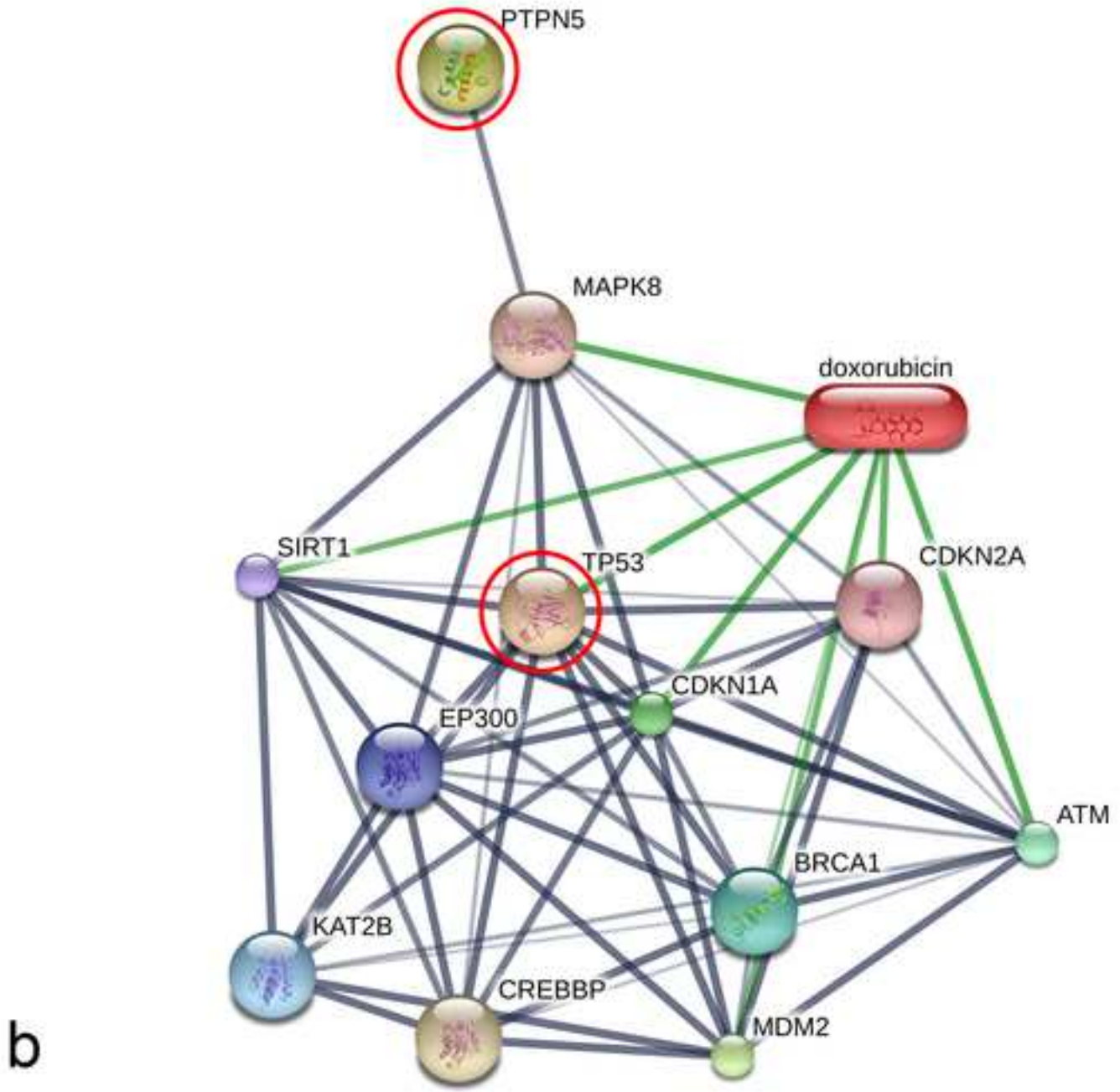


Figure 2c

Click here to access/download;Figure;Fig. 2c - PTPN5 VS. cyclo.tif

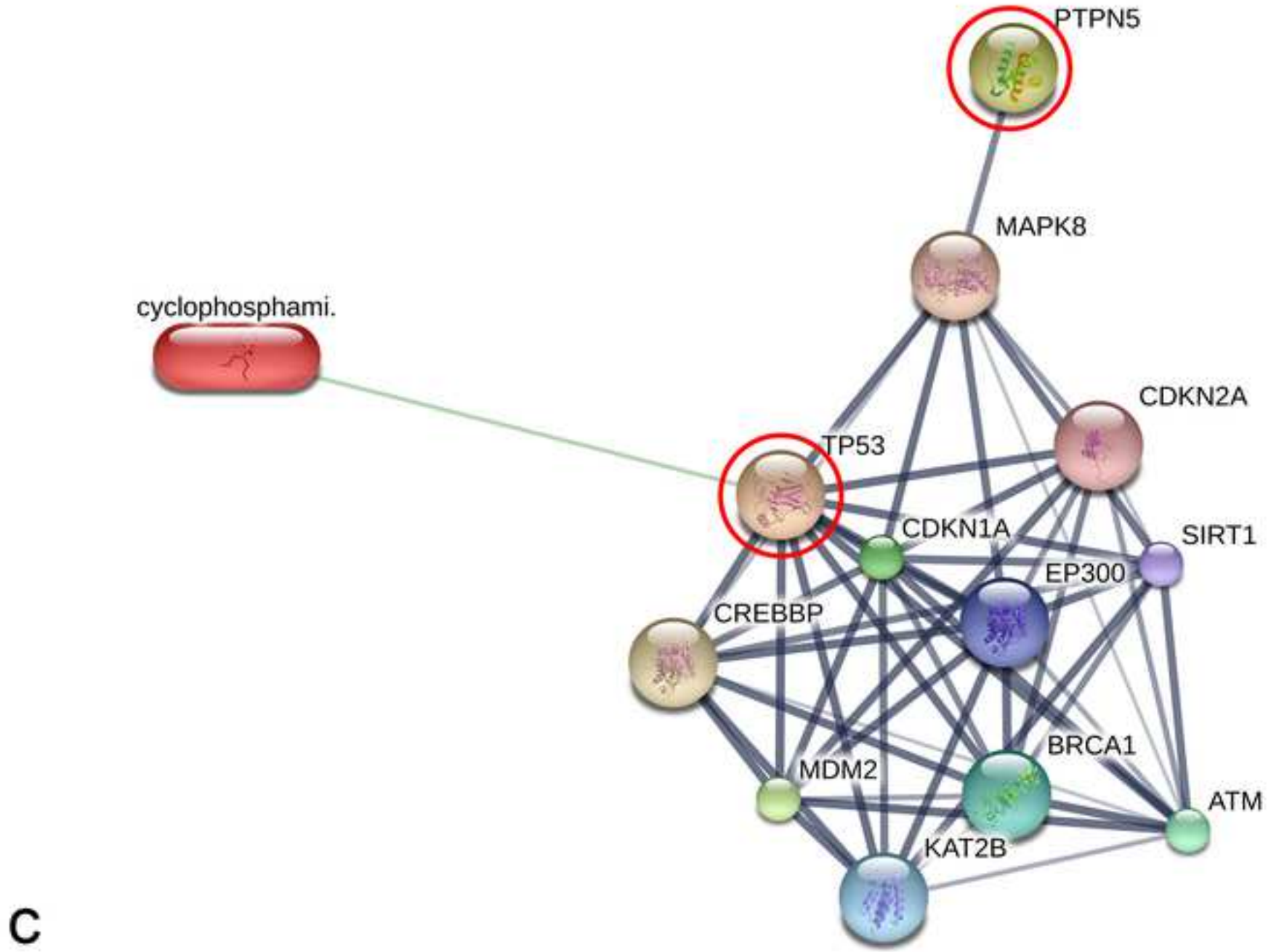


Figure 2c

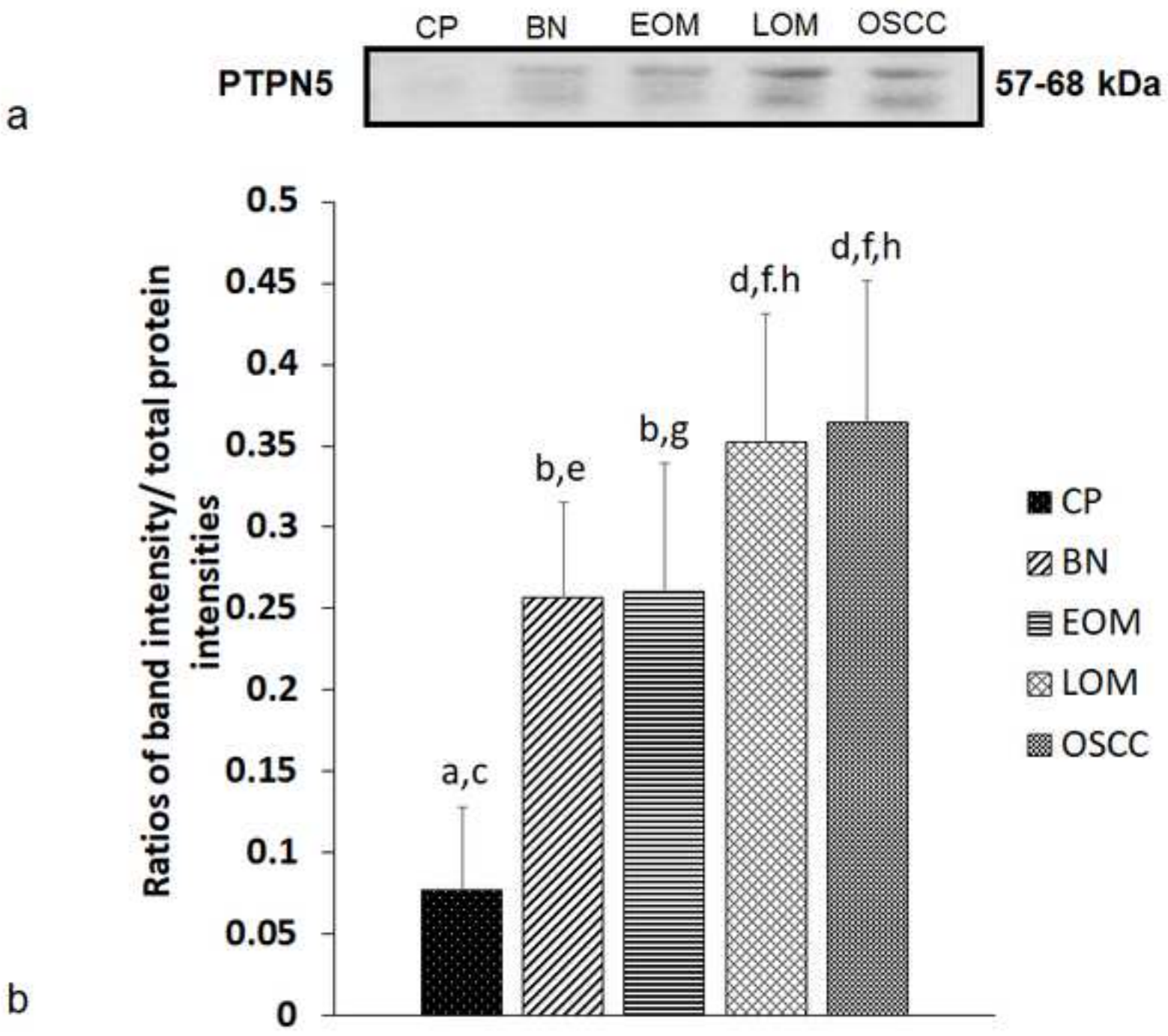


Figure 4

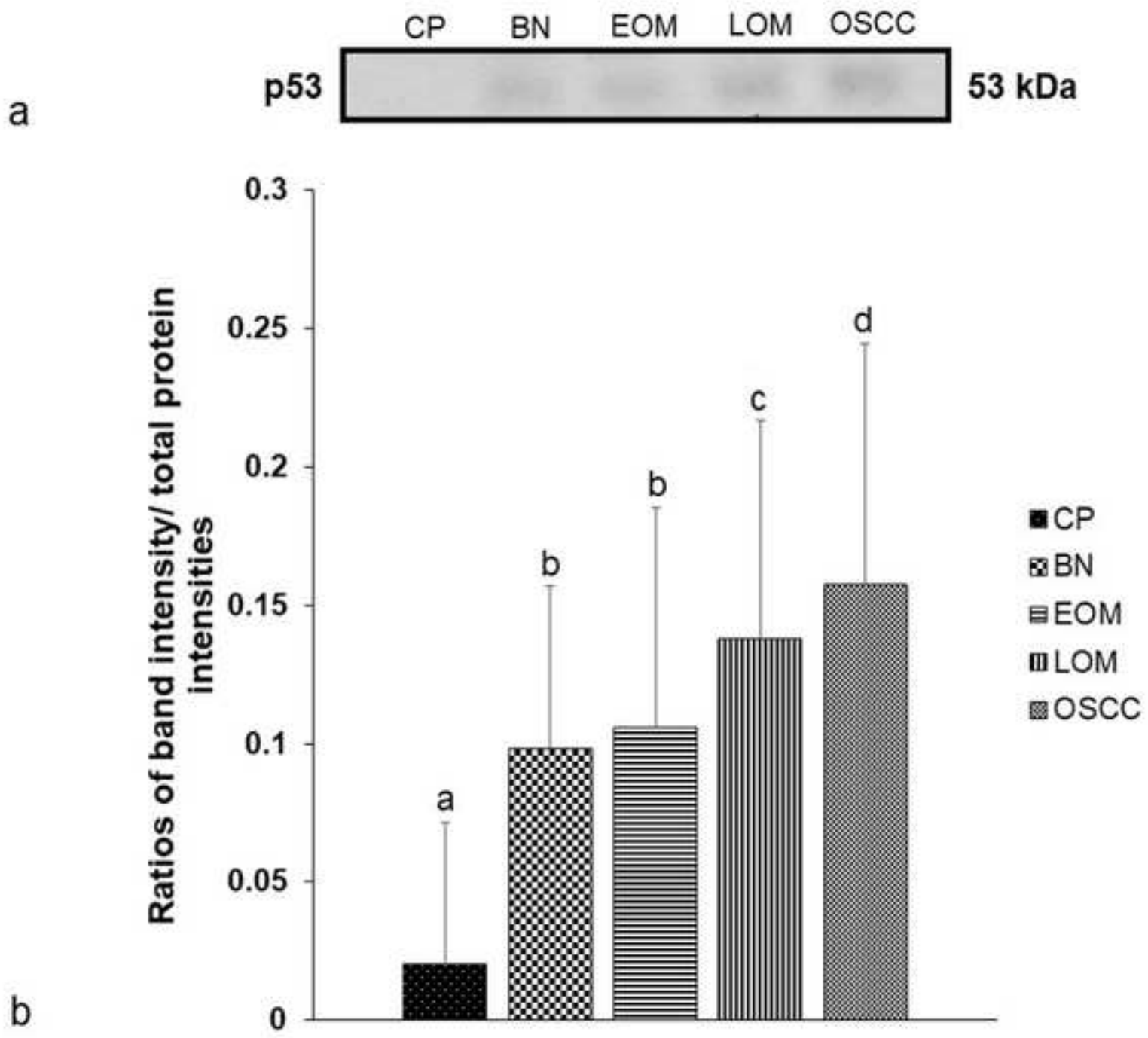
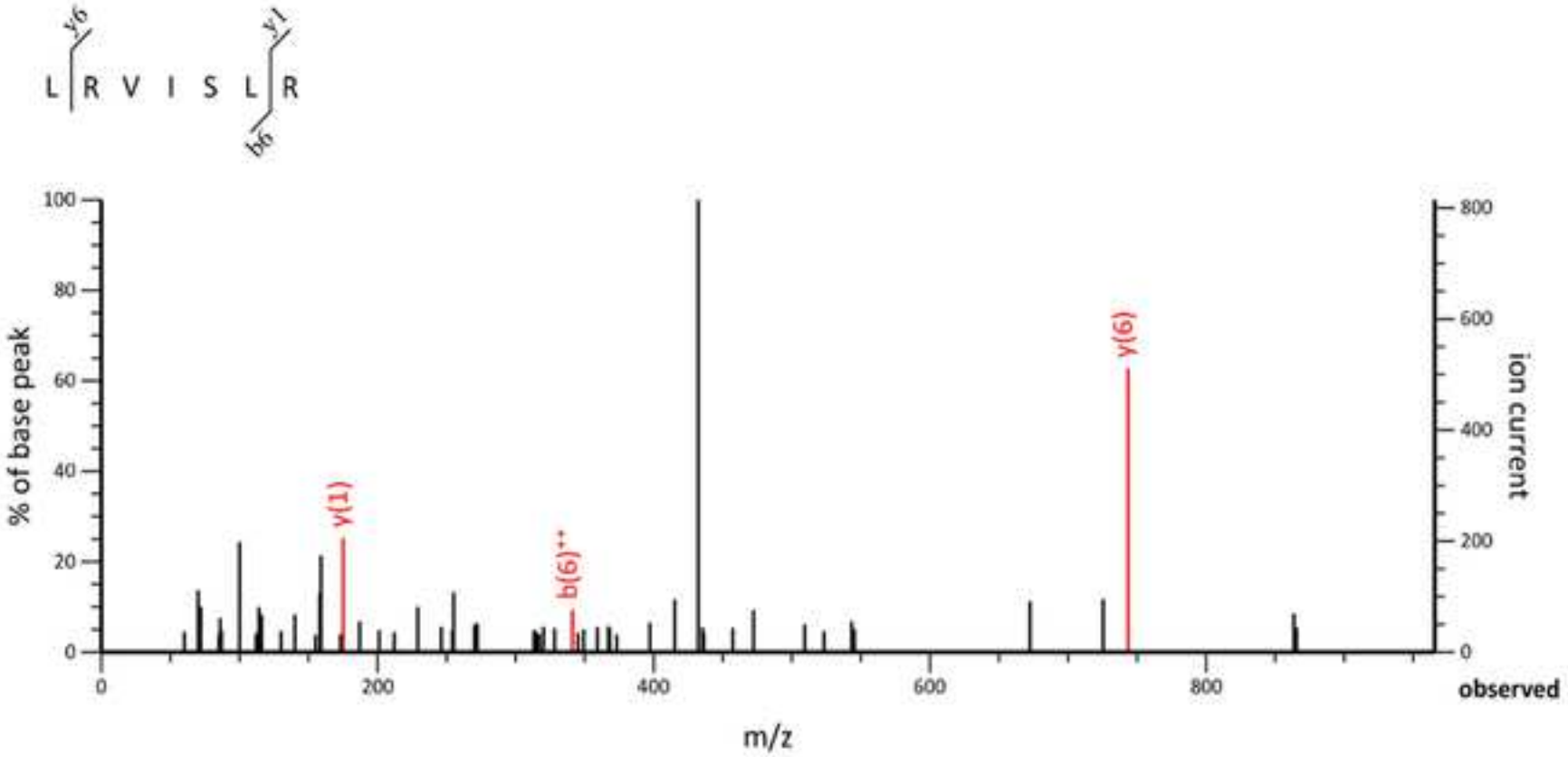


Figure 5a

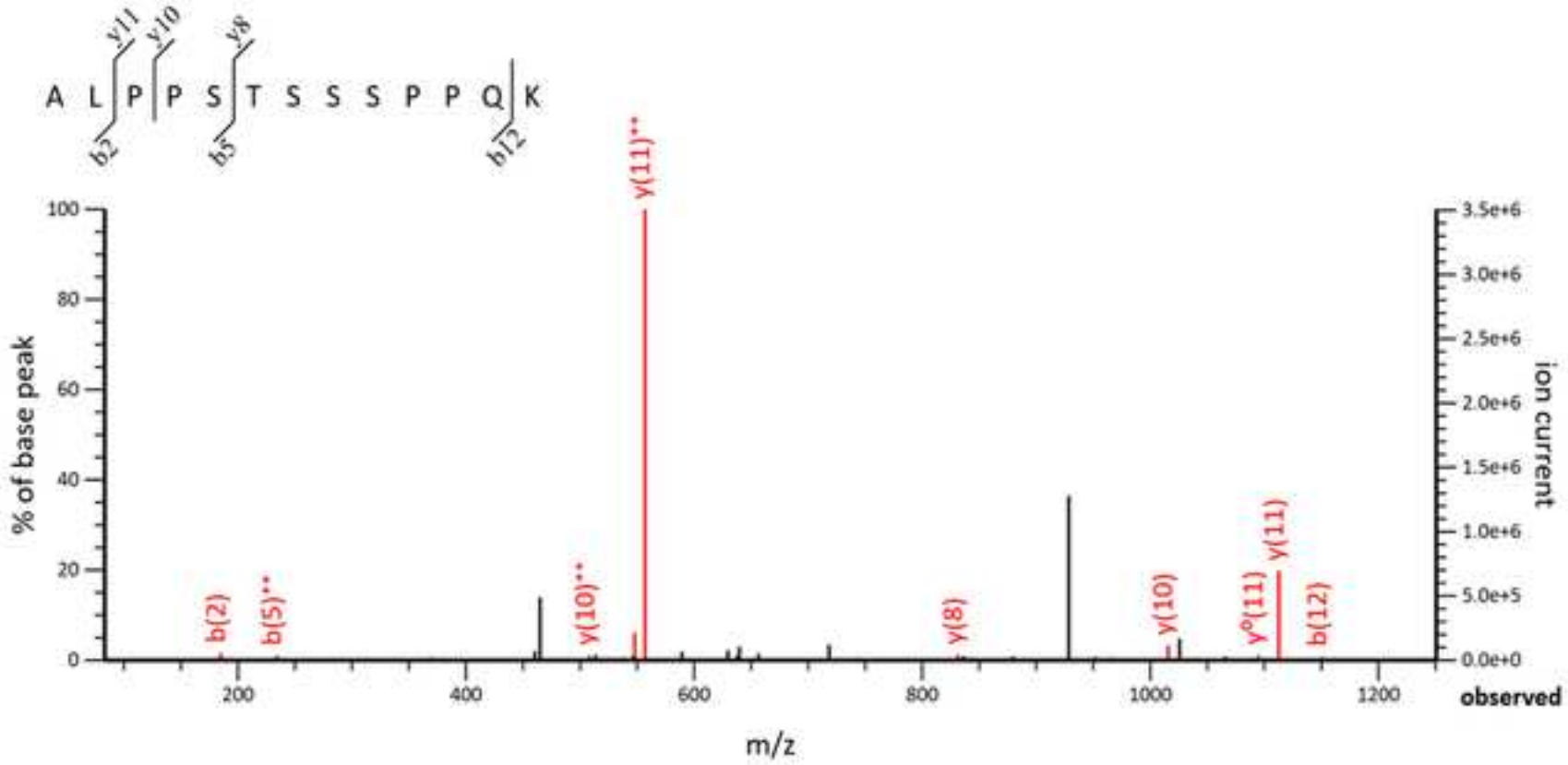
Click here to access/download;Figure;Fig. 5a - PTPN5.tif



a

Figure 5b

Click here to access/download;Figure;Fig. 5b - P53.tif



b

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Supplementary Material

Supplement table.xlsx

Salivary proteomics in monitoring therapeutic response of canine oral melanoma

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Abstract

Saliva biomarkers are suitable for monitoring therapeutic response of canine oral melanoma (COM) because saliva directly contacts to the tumor and saliva collection is non-invasive, convenient and cost effective. The present study aimed to investigate novel biomarkers from the salivary proteome of COM treated with surgery and a chemotherapy drug, carboplatin, for 1-7 times, using a liquid chromatography-tandem mass spectrometry. The expression of potential salivary biomarker, ubiquitin D (UBD), was verified by western blot analysis. In OM dogs with short-term survival (STS) (less than 12 mos after surgery), a significantly increased ratio of free UBD (fUBD) to conjugated UBD (cUBD) was shown in the pre-surgery stage (PreS) compared with that after being treated with surgery, followed by carboplatin for 2, 4 and 5 times (AT2, AT4 and AT5). The ratio was also shown to be augmented in PreS with STS compared with that in PreS with long-term survival (more than 12 mos after surgery). The expression of fUBD was enhanced in PreS compared with that of AT2 in STS group. In conclusion, this study revealed that a ratio of fUBD to cUBD in PreS was plausibly shown to be a potential prognostic biomarker for survival in dogs with OM.

Keywords: Dog, Liquid chromatography-tandem mass spectrometry (LC-MS/MS), Oral melanoma, Treatment monitoring, Ubiquitin D (UBD)

Canine oral melanoma (COM) is one of the most common head and neck tumors in dogs[1]. The clinical staging system for the disease is classified into 4 stages as follows: stages I (a < 2 cm diameter tumor) and II (a 2 to < 4 cm diameter tumor), defined as early-clinical stages with no metastasis, whereas stages III (a ≥ 4 cm tumor and/or lymph node metastasis) and IV (a tumor with distant metastasis) were late-clinical stages (LOM)[2]. Patients with LOM are most commonly

found owing to the difficulty in routinely examining tumors in dogs' mouths[3,4]. They are normally treated with surgical resection in combination with chemotherapy drugs, carboplatin, doxorubicin or cyclophosphamide and piroxicam[5,6]. Several factors can lead to failures of treatments such as the nature of OM with high metastasis and high recurrence, owners' decision not pursuing chemotherapy after surgical resection, cancer drug resistance. Tumor biomarkers of the cancers will be assistant tools for owners' decision to pursue full-course chemotherapy treatments. Several tumor biomarkers have been used to help diagnosis, prognosis and surrogate endpoints, and monitoring treatment response and/or recurrence of the diseases[7]. It is noteworthy that mass spectrometry (MS)-based proteomics has been widely used to study novel expressed proteins in several cancers in large scale such as in tissues and saliva of COM, oral squamous cell carcinoma, benign tumors and chronic periodontitis, in tears of canine cancers, in lymph nodes and serum of canine lymphoma[8-12]. There remain knowledge gaps in proteome profiles of COM therapeutic response. The objective of the present study was to investigate novel biomarkers from the salivary proteome of dogs with OM during pre-surgery (PreS), post-surgery (PostS) and after treating with chemotherapy drugs, carboplatin, for 1-7 times (AT1-AT7), using an in-solution digestion coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The candidate protein expression, ubiquitin D (UBD), was affirmed by western blot analysis.

Results

Survival times. Samples of LOM were divided into 2 groups according to the survival times – group 1: a short-term survival (with median survival time less than 12 mos) (STS) and group 2: a long-term survival (with median survival time more than 12 mos) (LTS). The STS and LTS had

median survivals of 3 and 14.5 m, respectively. The two survival curves were illustrated with p = 0.0046 (Fig. 1).

LC-MS/MS results. A total of 132, 29 and 74 proteins were commonly found in individuals of PreS, PostS, and R and M, respectively (Supplementary Tables S1–S3). Proteins matched predicted UBD of *Rousettus aegyptiacus* and predicted transient receptor potential cation channel subfamily M (melastatin) member 8 (TRPM8) channel-associated factor 2 of *Monodelphis domestica* appeared in all samples in every group of chemotherapy treatment, excluding the PreS, PostS, R and M groups.

Western blot analysis and LC-MS/MS verification results. The expression of fUBD and cUBD in STS and LTS samples was illustrated in Figs. 2-3. In dogs with STS, a significantly increased ratio of fUBD to cUBD was shown in PreS compared with that of AT2, AT4 and AT5 (Fig. 4). The ratio was also shown to be significantly augmented in PreS group with STS compared with that with LTS ($p < 0.01$). In addition, the expression of fUBD was enhanced in PreS compared with that of AT2 (Fig. 5). UBD sequence was confirmed by LC-MS/MS (Fig. 6).

Discussion

In the present study, LC-MS/MS and western blot were used to identify novel salivary biomarker candidates of COM during pre-operation, post-operation, after treatments with carboplatin for 1-7 times, metastasis and recurrence, and checking up. In-gel digestion coupled with mass spectrometric analysis (GeLC-MS/MS) and LC-MS/MS have been used to compare salivary proteomes of healthy dogs with different breeds and between healthy dogs and human[13-16]. One publication has reported the salivary proteome in dogs infected with Leishmania infantum, using

LC-MS/MS[17]. For canine oral tumor proteomics, matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry (MALDI-TOF MS) has been used to analyze peptide mass fingerprints, three-dimensional principal component analysis scatterplots and potential protein candidates in saliva and tissues of dogs with early-stage OM, late-stage OM, oral squamous cell carcinoma, benign oral tumors and healthy controls[8,9]. To the best of our knowledge, this study has shown for the first time of salivary proteomics of OM in dogs for monitoring surgery and chemotherapy responses.

From LC-MS/MS results, the expression of UBD appeared in all samples with chemotherapy treatment. The expression of UBD in different forms was further confirmed by western blot analysis. Ratios of fUBD to cUBD in PreS of LOM with STS were significantly higher than those in AT in contrast to the trends of those of LOM with LTS, possibly showing the potential prognostic biomarker for survival of LOM. In patients with stage IIB-IIC colon cancer, the expression of UBD has been identified as a recurrent risk and associated with STS after surgery[18,19]. UBD was overexpressed in cervical squamous cell carcinoma tissues and associated with tumor size and lymphatic metastasis[20]. Silenced expression of UBD, regulated by miR-24-1-5p could enhance autophagy and apoptosis of human skin melanoma cells[21]. In fact, ubiquitination (UBQ) or the conjugation of Ub to target proteins leads to the protein degradation by 26S proteasome[22]. In our study, according to the higher ratios of fUBD to cUBD in dogs with STS treated with chemotherapy, the increased cUBD after therapy in individuals with STS and the increased fUBD in individuals with LTS in PreS group, it might be implied that the lower fUBD expression (or the higher UBQ) during treatment associated with the STS (S4 Table). Since several formula of chemotherapy drugs and treatment have been currently used in treating canine oral cancers, other suitable drugs or treatment might be considered for treating the STS group with high

ratios of fUBD to cUBD, regarding to the concept of precision medicine for canine oral cancer[5,6,23]. However, with the limit samples in this study, the study should be further investigated in larger populations. In addition, misregulated expression of several ubiquitin-conjugating enzymes used in UBQ, contributes to eccentric expression of NFκB and TGFβ and their signaling, leading to angiogenesis, increased invasiveness, chemotherapy resistance and metastasis of several cancers[24] In fact, expression of NFκB has been reported in saliva of canine LOM and OSCC and the expression of sentrin-specific protease 7 (SENP7), small ubiquitin-like modifier (SUMO)-specific protease 7, has been reported in saliva of dogs with oral squamous cell carcinoma[9]. The link of NFκB with Ub should be further investigated. In conclusion, the present study has proposed for the first time a ratio of fUBD to cUBD in PreS as a candidate prognostic biomarker for survival in dogs with LOM. For the future work, the study of expression of ubiquitin-conjugating enzymes used in UBQ and proteins involving in autophagy and apoptosis should be performed in larger populations. Suitable drugs or treatment might be reconsidered for treating the STS group with high ratios of fUBD to cUBD.

Materials and Methods

Animals. Saliva samples were collected from dogs with OM without previous treatment either chemotherapy or radiotherapy. They were appointed for surgical excision and chemotherapy at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. The staging of OM was determined according to World Health Organization (WHO)[25]. Dogs were examined for an oral, regional lymph node, and physical condition; moreover, the regional lymph nodes were required to rule out metastasis by cytological examination. Skull to abdomen

radiography was evaluated by Brivo DR-F Digital X-rays system (GE Healthcare, Little Chalfont, UK) or Optima 64-slice helical CT unit CT-scan (GE Thailand). Ultrasound was performed OM metastasis to abdominal organ. Tumor diagnosis was done by cytology and histopathology.

Short-term survivors (STS) and long-term survivors (LTS) were defined as patients with late-clinical stage OM and living shorter than 12 mos or longer than 12 mos after surgery resection, respectively. Patient histories and Patient treatment histories were shown in Tables 1-2, respectively.

Sample collection and preparation. Saliva was collected without mechanical or chemical stimulation as previously described[9]. Briefly, the patients were fasted and mouths were cleaned with 0.9% sterile normal saline solution before saliva collection. Samples were obtained at the initial visit for surgical excision (PreS group) and at 14 days after operation (PostS group). Adjuvant chemotherapeutic protocol, carboplatin, was given at a dosage of 250 mg/m^2 of a 3-week interval for 6-7 treatments. Saliva was collected post chemotherapy treatments for 1-7 times (AT1-AT7) and during following-up for 1 or 2 month-intervals for 1-4 times after treatment ends (C1-C4). Saliva samples were centrifuged. Halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) was added and the supernatant was stored at -20°C until use. The study was approved by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand (Protocol No. 1631042). Written informed consents were obtained from all dog owners.

Preparation of saliva samples for LC-MS/MS analysis. Total protein of samples was measured by Lowry's assay[26]. Each sample was prepared to 1.5 µg/µL in 10 mM ammonium bicarbonate. Disulfide bonds were reduced by 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate for 1 h at room temperature and alkylated in 100 mM iodoacetamide (IAA) in 10 mM ammonium

bicarbonate for 1 h at room temperature in the dark. After that, the protein in each sample was digested with the sequencing grade modified trypsin (Promega, Madison, WI) of 50% acetonitrile (ACN) in 10 mM ammonium bicarbonate for overnight. Then, the solvent was removed. Finally, each sample was dissolved with 20 µl of 0.1% formic acid and centrifuged 10,000 rpm for 5 min before LC-MS/MS analysis. Spike BSA as internal standard was prepared by using 1.5 µg/µL in 10 mM ammonium bicarbonate.

LC-MS/MS Analysis and Data Processing. The samples were analyzed using an Ultimate 3000 LC System coupled to an HCTUltra PTM Discovery System (Bruker Daltonics, Bremen, Germany) linked to a reversed-phase high performance liquid chromatography (HPLC) for separation. The gradient-eluted peptides were injected onto a PepSwift monolithic column (100 µm internal diameter × 50 mm) (Thermo Fisher Scientific). Peptides was applied to separate with a linear gradient from 4% ACN, 0.1% formic acid (FA) to 70% ACN, 0.1% FA for 7.5 min with a regeneration step at 90% ACN, 0.1% FA and an equilibration step at 4% ACN, 0.1% FA at a flow rate of 1000 nL/min. The process took 20 min per sample. Peptide mass spectra were acquired in the positive ion mode with a scan range of 400 to 1500 m/z. However, in the case of having more than 5 precursor fragments, peptides would be selected from the MS scan at 200-2800 m/z. MS spectra data were analyzed as previous described[9,27-28]. The peptide sequences were searched against the NCBI mammal database for protein identification using MASCOT software, version 2.2 (Matrix Science, London, UK)[29]. Database interrogation included taxonomy (mammals), enzyme (trypsin), variable modifications (oxidation of methionine residues), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (1.2 Da), fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and maximum number of missed cleavages. Proteins were identified from one or more peptides with an individual MASCOT score

corresponding to $p < 0.05$. Proteins were annotated by UniProtKB/Swiss-Prot entries (http://www.uniprot.org/) and compared among different sample groups was displayed using jvenn diagram (http://bioinfo.genotoul.fr/jvenn/example.html)[30]. The interaction network of candidate proteins and chemotherapy drugs was explored using the Stitch program, version 5.0 (http://stitch.embl.de/)[31].

Western blot analysis. To validate the MS results, Fifteen micrograms of samples were mixed with loading dye, heated and applied to a pre-cast NuPAGE 4–12% (w/v) Bis-Tris gel (Thermo Fisher Scientific) using NuPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific) at 200 V for 60 min. Protein standard marker was PageRuler prestained protein ladder (molecular weight range 10–180 kDa) (Thermo Fisher Scientific). After that, the proteins were transferred to Trans-Blot Turbo nitrocellulose membranes (Bio-Rad Laboratories) at 25 V for 7 min using Trans-Blot Turbo 5X transfer buffer (Bio-Rad Laboratories). Detection of total protein band intensities in each lane was performed by a Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes (Thermo Fisher Scientific) according to the manufacturer's instructions. Non-specific protein binding was blocked with 5% bovine serum albumin (BSA) (GoldBio, St Louis, MO, USA) in phosphate-buffered saline containing 0.1% Tween 20 (PBST) at 4°C overnight. After washing with PBST, primary antibodies diluted at 1:1000 were incubated with a membrane at 4°C overnight, including mouse monoclonal anti-human ubiquitin (Ub) (A-5) (Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were washed with PBST and then incubated with 1:15,000 horseradish peroxidase conjugated-rabbit anti-mouse IgG secondary antibody (Abcam, Cambridge, UK) for 1 h at 25°C. The proteins of interest were visualized with ECL western blotting detection reagents (GE Healthcare). Western blot imaging was performed using a ChemiDoc Touch Imaging System (Bio-Rad Laboratories). Protein bands intensities were

analysed by Image Lab 6.0.1 software (Bio-Rad Laboratories). Total protein normalization was performed with the modification of Aldridge et al. (2008)[9,32]. The ratios of target band (free Ub and conjugated Ub) intensities to the total proteins in each lane was calculated. The western blotting was performed in triplicate.

Verification of expressed protein sequences by LC-MS/MS. LC-MS/MS was utilized to confirm Ub protein identities as described previously[9]. Briefly, blotting membranes were incubated with Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific) for 15 min and washed 4 times with PBST. Protein bands were cut and incubated with 10 mM DTT in 10 mM ammonium bicarbonate overnight. Samples were then trypsinized at 37ºC for 3 h and applied to the LC-MS/MS as mentioned above.

Statistical analysis. ANOVA statistical analysis, incorporated into the DeCyder MS differential analysis software, and MASCOT software, version 2.2 were used to analyse significantly different peptide peak intensities and MASCOT LC-MS/MS scores, respectively. Western blot analysis was performed by Kruskall-Wallis and Mann-Whitney tests for ratios of fUBD to cUBD, fUBD and cUBD expression. Kaplan-Meier survival curves were performed using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon analytic methods. Statistical analyses of protein expression data were conducted using GraphPad Prism, version 8.3.0 (GraphPad Software, La Jolla, CA, USA). Significance was accepted at the $p < 0.05$ level.

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Author Contributions

G.S., S.P. and S.R. designed and planned the experiments. S.P., P.T., J.J., N.P., and S.K. conducted the experiments. G.S., S.P. and S.R. analysed the results. G.S. and S.R. supervised the experiments. G.S. and S.P. coordinated the project and handled funding. G.S. and S.P. wrote the original draft. G.S. finalized the manuscript. All authors approved the manuscript.

Additional Information

Supporting information

Supplementary information

Supplementary Table S1. Common proteins found in all individuals with OM during pre-surgery (PreS).

Supplementary Table S2. Common proteins found in all individuals with OM during post-surgery (PostS).

Supplementary Table S3. Common proteins found in all individuals with OM during recurrence (R) and metastasis (M).

Competing Interests: The authors declare no competing interests.

Table Legends

Table 1. Patient history

	ID	Age at initial treatment (m)	Breed	Sex	Clinical stage	Survival time (m)
1	10	9 y 4 m	German shepherd	F	III	8 m 23 d
2	11	10 y 4 m	Poodle	F	III	6 m 9 d
3	16	11 y 5 m	Poodle	M	III	3 m 19 d
4	44	10 y 9 m	Mixed	Fs	III	1 m 10 d
5	46	7 y 7 m	Golden retriever	M	III	3 m 22 d
6	31	12 y 8 m	Shih tzu	F	III	24 m 24 d
7	71	12 y 3 m	Terrier	M	III	14 m 12 d
8	72	10 y	Mixed	Fs	III	13 m 12 d
9	86	13 y 1 m	Poodle	M	III	15 m 21 d

*M = Male, Mc = Male castration, F = Female, Fs = Female spray

322 **Table 2**. Patient treatment history

No.	PreS	PostS	AT1	AT2	AT3	AT4	AT5	AT6	AT7	R	M1	M2	C1	C2	C3	C4	Remarks
1	•	•	•	•	•	•	•				•	•					Metastasis
2	•				•	•	•	•					•	•			Recurrence
3	•	•	•		•	•											Recurrence
4		•	•	•													Metastasis
5		•	•	•	•												Recurrence
6	•	•		•	•	•		•					•	•	•	•	Seizures
7	•	•	•	•	•	•											Metastasis
8		•	•	•	•	•											Metastasis
9	•	•	•	•	•	•											Recurrence

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Figure Legends

Fig. 1. Overall survival of patients with short-term survival (STS) (less than 12 months after surgery) and long-term survival (STS) (more than 12 months after surgery)

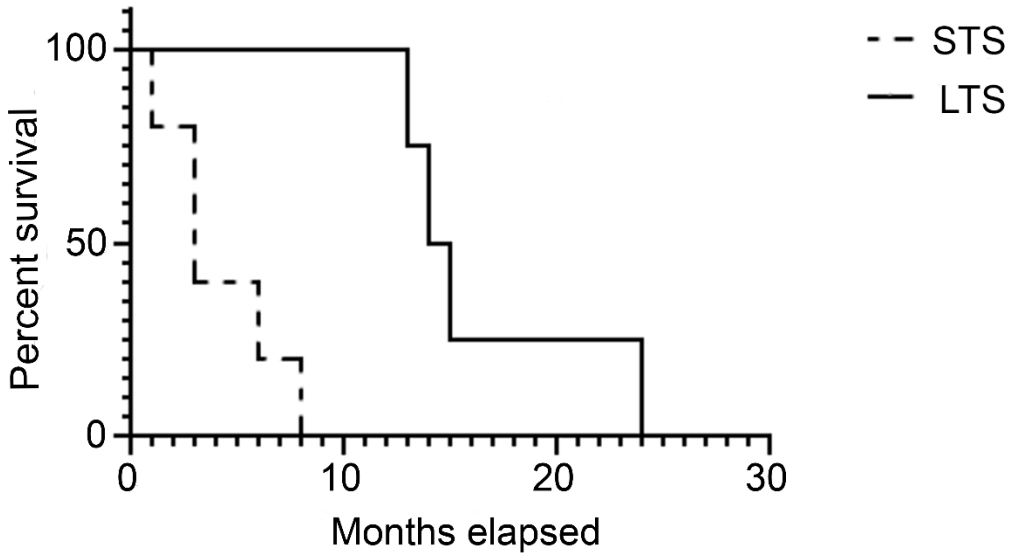


Fig. 2. Representative western blot of patients with short-term survival for free ubiquitin D (fUBD) at 9 kDa and conjugated ubiquitin D (cUBD) in saliva. Lane 1: Pre-surgery (PreS); Lane 2: Post-surgery (PostS); Lane 3: After treating with chemotherapy drug for 1 time; Lane 4: After treating with chemotherapy drug for 2 times; Lane 5: After treating with chemotherapy drug for 3 times.

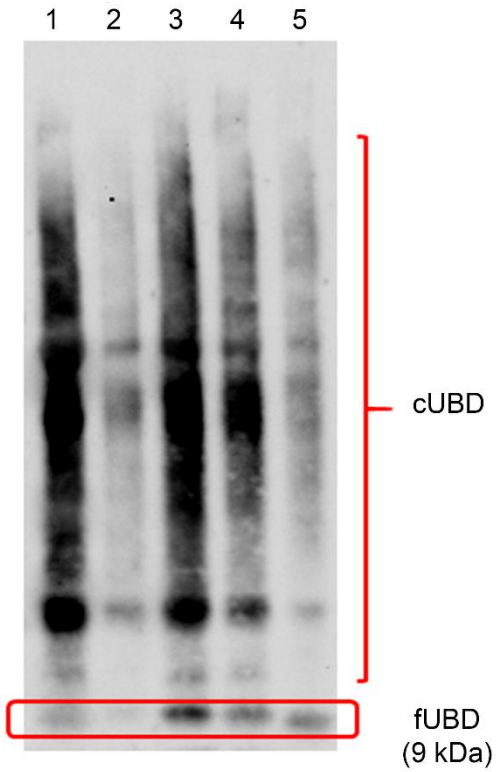


Fig. 3. Representative western blot of patients with long-term survival for free ubiquitin D (fUBD) at 9 kDa and conjugated ubiquitin D (cUBD) in saliva. Lane 1: Pre-surgery (PreS); Lane 2: Post-surgery (PostS); Lane 3: After treating with chemotherapy drug for 2 times; Lane 4: After treating with chemotherapy drug for 3 times; Lane 5: After treating with chemotherapy drug for 4 times; Lane 6: After treating with chemotherapy drug for 6 times; Lane 7: 1st Checking up after treating with chemotherapy drug; Lane 8: 2nd Checking up after treating with chemotherapy drug; Lane 9: 3rd Checking up after treating with chemotherapy drug; Lane 10: 4th Checking up after treating with chemotherapy drug.

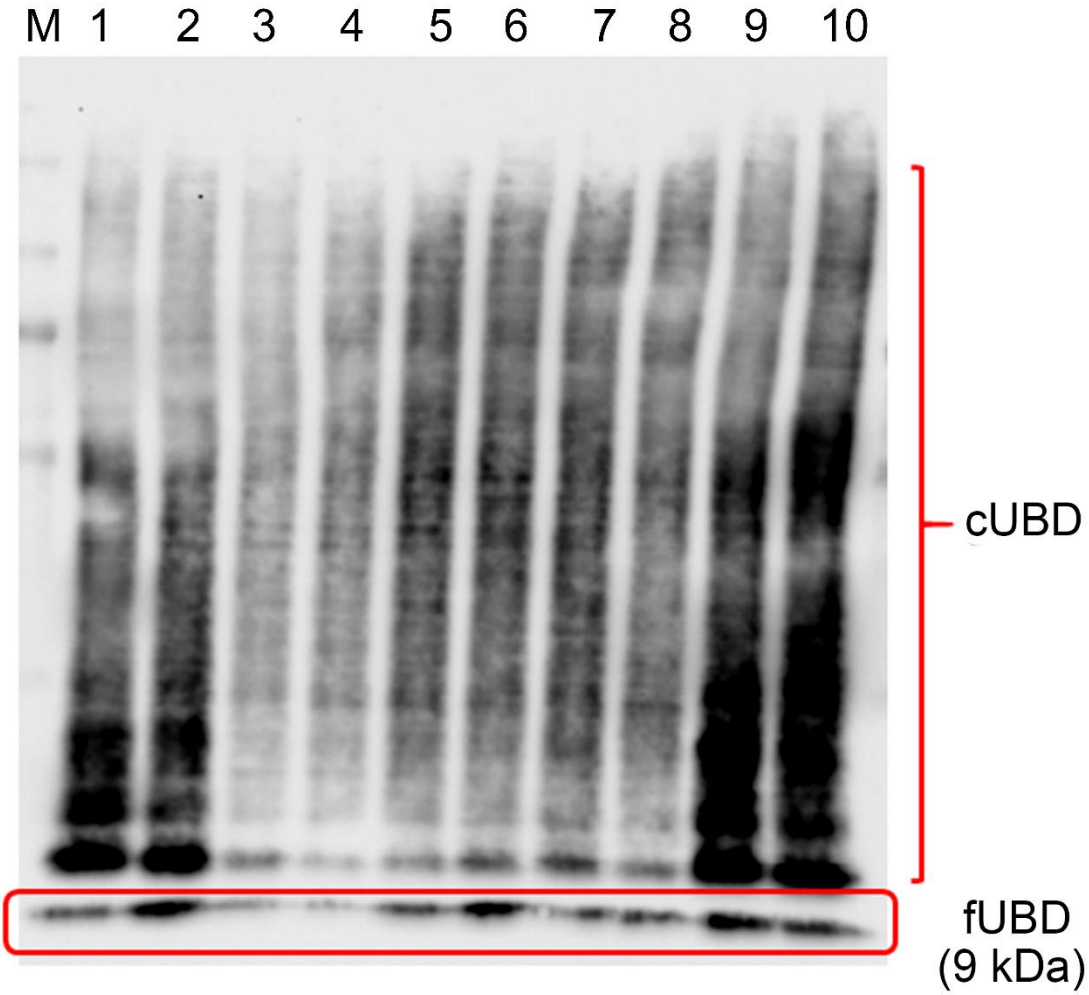


Fig. 4. Western blot analysis of ratios of free ubiquitin D (fUBD) to conjugated ubiquitin D (cUBD) in short-term survival and long-term survival groups. a–b denote a significant difference at $p < 0.05$. c–d denote a significant difference at $p < 0.01$.

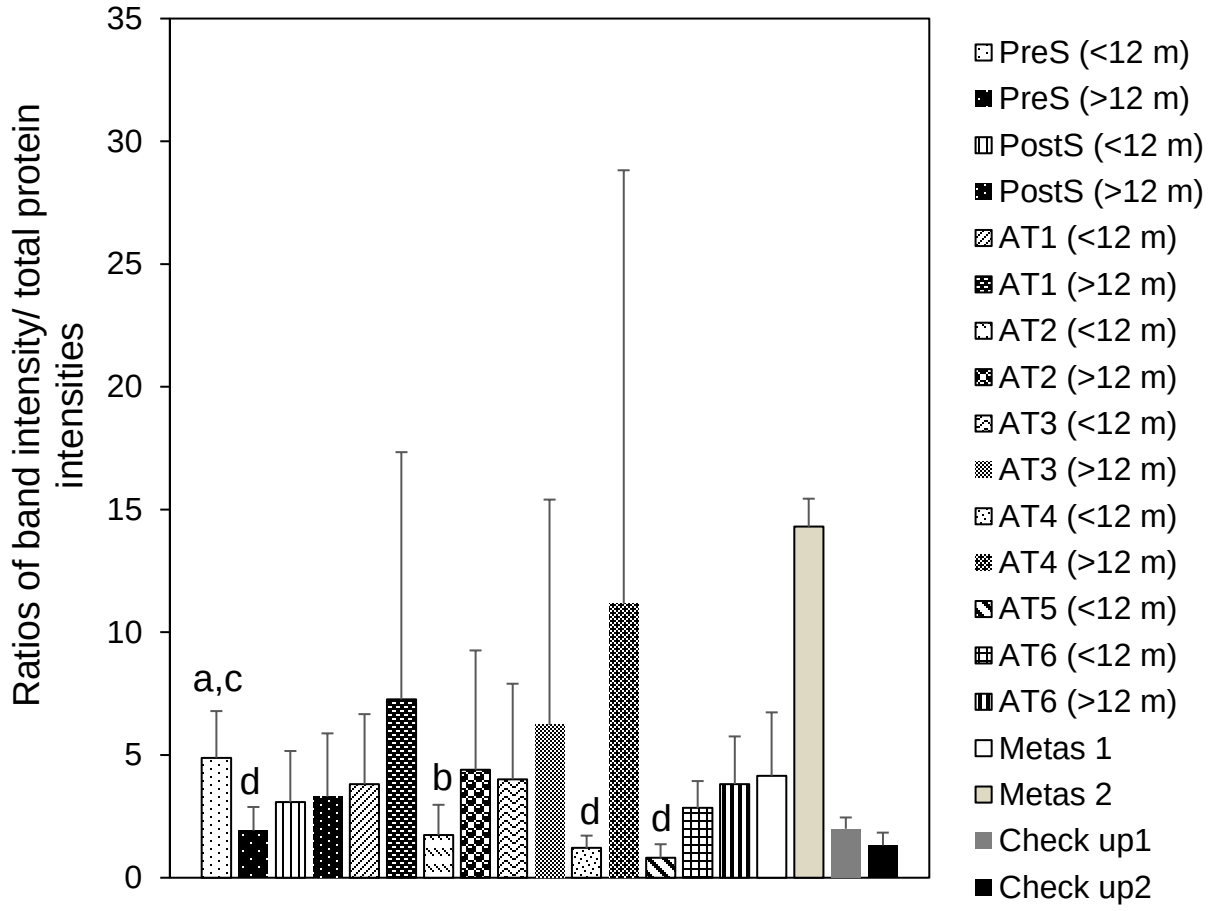
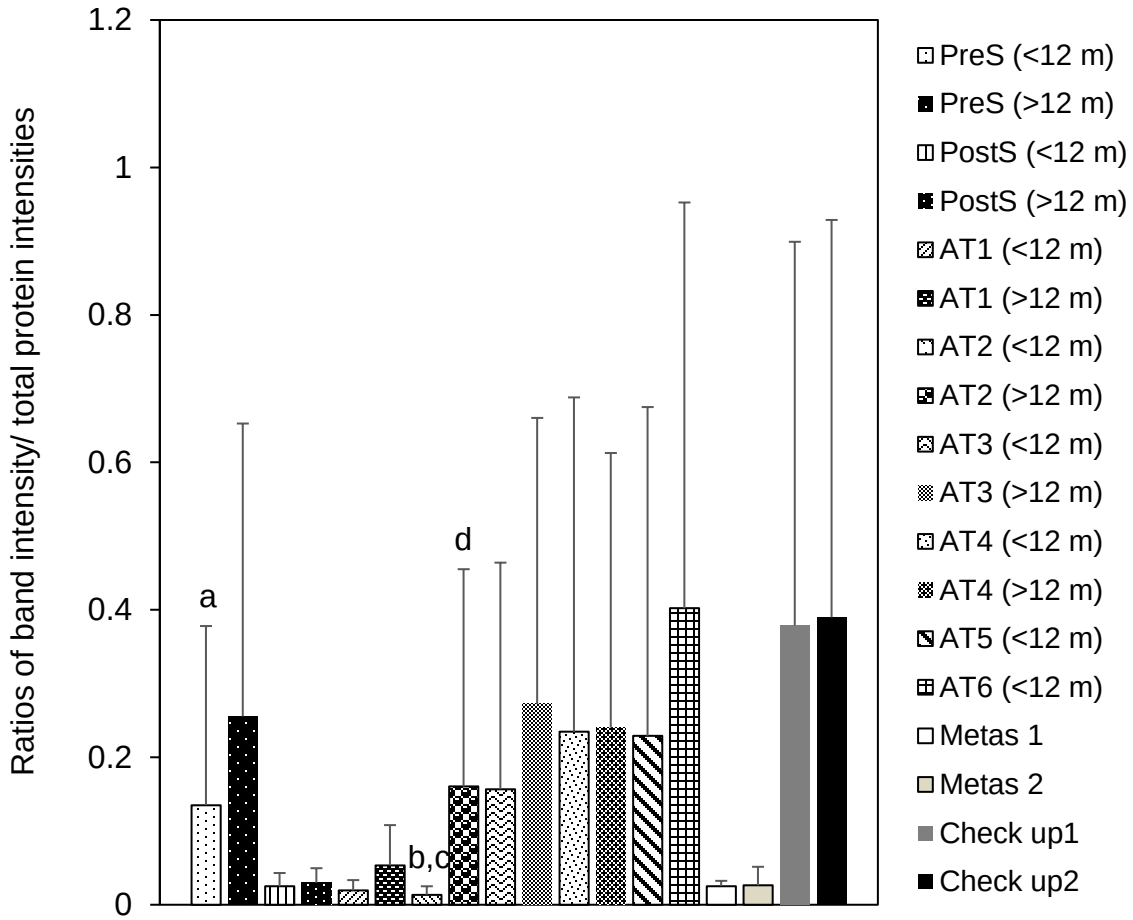
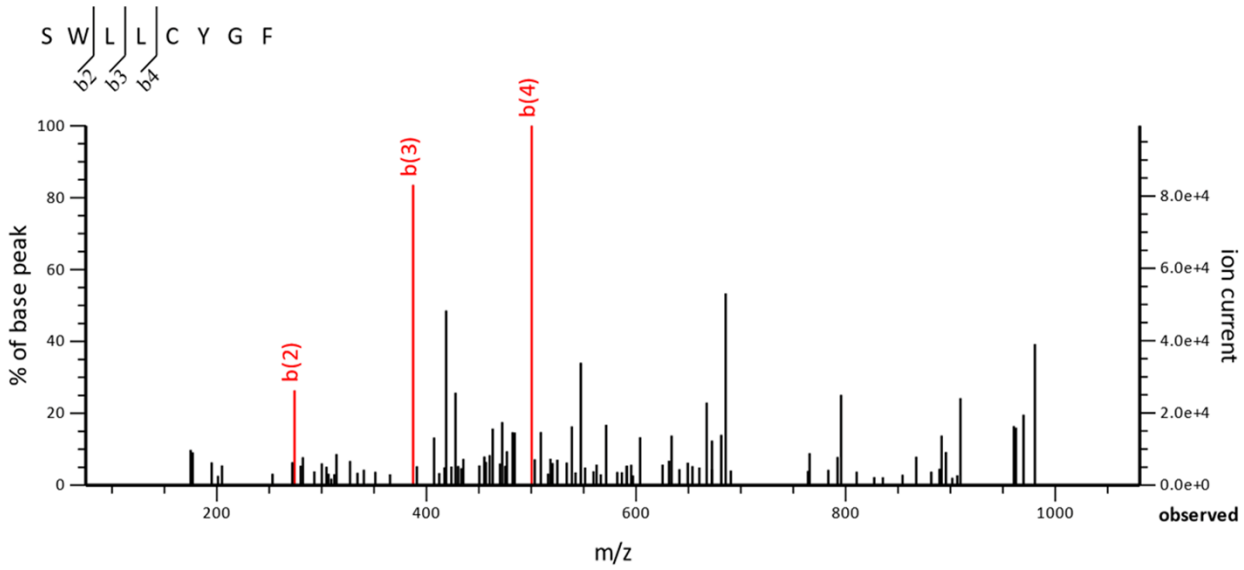


Fig. 5. Western blot analysis of free ubiquitin D (fUBD) in short-term survival and long-term survival groups. a–b and c-d denote a significant difference at $p < 0.05$.



365 **Fig 6.** Verification of UBD sequence by LC-MS/MS**.** MS/MS fragmentation of **SWLLCYGF**

366 found in free ubiquitin D (fUBD) was shown.



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Serum Proteomic Analysis by MALDI-TOF MS for Rapid Screening of Canine Oral Melanoma at Different Clinical Stages

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Abstract

Background

Oral melanoma (OM) is the most aggressive canine oral tumors with high recurrence and/or metastasis after surgery (Verstraete, 2005). Cancer screening and early detection can increase the opportunity of successful treatment. This study focused on detection of biomarkers in serum because it is less invasive and easy to be collected. Peptide mass fingerprint (PMF) by matrix absorbed laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) is commonly used to identify unique protein fingerprints in patients. MALDI-TOF MS is a rapid technique with high sensitivity and high specificity (Hortin, 2006). This study aimed to determine PMF in the canine OM serum samples at early and late clinical stages by MALDI-TOF MS, supporting the utilization of this technique for rapid canine OM screening.

Methods

Nine serum samples (3 early-stage OM, 3 late-stage OM and 3 normal oral health controls) were recruited in the study. The clinical staging of OM was determined according to World Health Organization (WHO) staging scheme (Owen, 1980). PMFs were characterized using Ultraflex III TOF/TOF in a linear positive mode with a mass range of 0 - 5,000 Da. MS spectra were analyzed by Flex Analysis.

Results

Distinct fingerprint spectra were observed among early- (3,900-4,000 Da) and late-stage OM (2,600-2,700 Da) and controls (1,800-1,900 and 2,100-2,200 Da) (Fig. 1).

Conclusion

This study proposed the potential utilization of PMF as serum biomarker for rapid screening in the early- and late-stage OM. The results of different sample groups showed specific mass signals and distinct peptide patterns. Since peak patterns of serum peptides might vary in individuals, pool serum samples might be analyzed for representation and classification of OM.

Keywords

canine oral melanoma; clinical stages; peptide mass fingerprint; MALDI-TOF mass spectrometry; serum

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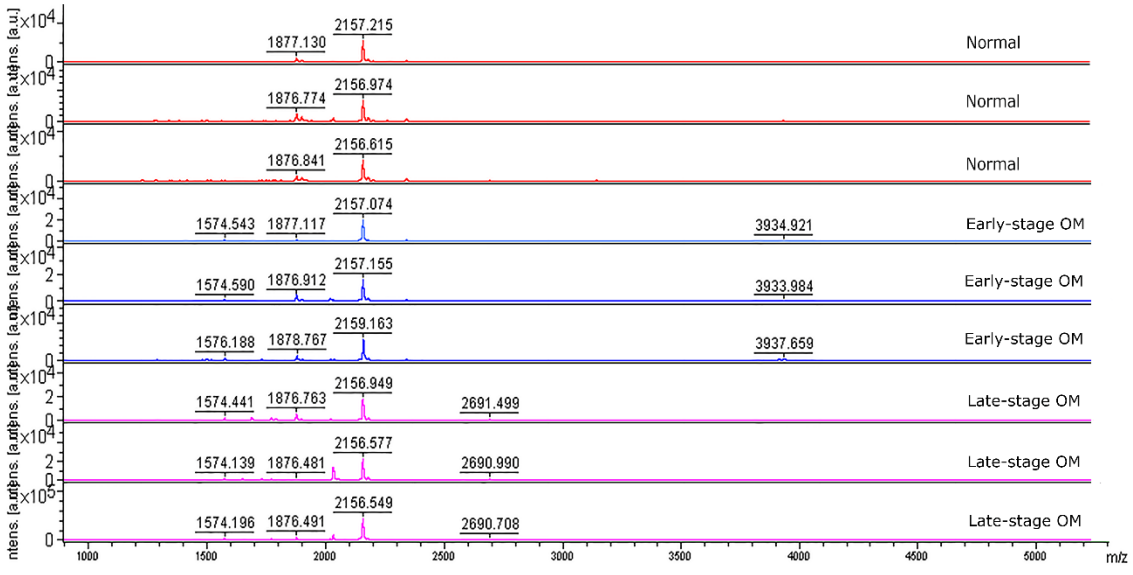

Figure 1. Peptide mass fingerprint of the early-stage oral melanoma (OM), the late-stage OM and normal gingiva tissues in range of 1,000 – 5,000 Da

Saliva Peptide Finger Print Analysis of Canine Oral Squamous Cell Carcinoma by MALDI-TOF Mass Spectrometry

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Keywords: canine oral squamous cell carcinoma, peptide mass fingerprint; MALDI-TOF mass spectrometry; saliva

Introduction

Canine OSCC was the second most common oral cancer, accounting for 17 - 25% (1). Biopsy technique is used as the gold standard to evaluate the oral lesion but it is an invasive technique and not suitable for cancer screening and monitoring (2). Rapid screening test and early diagnosis can improve survival rate of dogs with OSCC after treatment (1). Saliva is selected as an alternative for diagnostic or therapeutic purposes due to its non-invasiveness and easy collection (3). Matrix absorbed laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) is a rapid technique commonly used to identify peptide mass finger print (PMF) in patients. MALDI- TOF MS provides high throughput, high reproducibility and high resolution (4). This is the first study to identify salivary PMF by MALDI-TOF MS for rapid screening of canine OSCC.

Materials and Methods

Six saliva samples (3 OSCC and 3 normal oral health controls) were provided in the study. The histological results were determined by pathologist. Ultraflex III TOF/TOF was used to classify PMF in a linear positive mode with a mass range of 1,000-20,000 Da. Principle component analysis (PCA), a multivariate method, was used to analyze the variance of a data set MS spectra and the PCA were analyzed by Flex Analysis and ClinPro Tool software, respectively.

Results and Discussion

PMF results showed distinct MS spectra peaks in OSCC (4,070 – 4,080, 5,180 – 5,190, 7,320 – 7,330, 10,360 – 10,370 and 14,640 – 14,650 Da) from controls (3,160 – 3,170 and 6,870 – 6,880 Da) (Fig. 1). Some individual variations were also found in this study. The PCA of OSCC samples were clustered together and separated from that of the controls (Fig 2). For the future work, selected peaks should be analyzed by MALDI-TOF MS/MS to identify protein biomarkers.

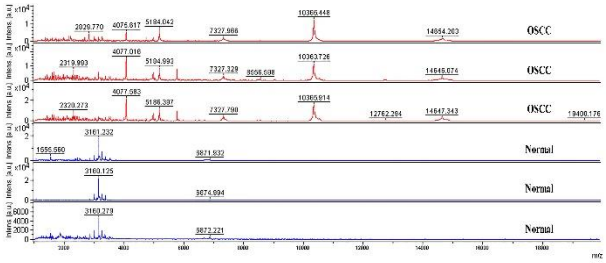

Figure 1 Peptide mass fingerprint of saliva in OSCC and normal gingiva in range of 1,000 – 20,000 Da

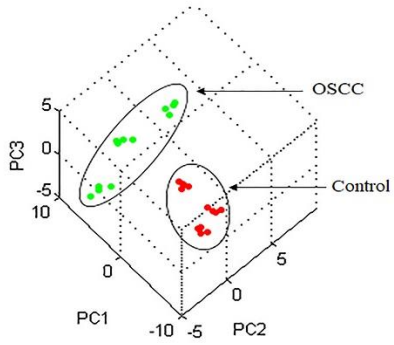

Figure 2 The 3-dimensional principal component analysis (3D PCA.) scatterplot of oral squamous cell carcinoma (OSCC) and control groups

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Salivary Proteomic Analysis of Canine Oral Melanoma at Different Clinical Stages by MALDI-TOF Mass Spectrometry

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Keywords: Canine oral melanoma, MALDI-TOF MS, Proteomics, Saliva

Introduction

Canine oral melanoma (COM) is the most common oral cancer in dog[1]. Methods that could help early diagnosis, disease screening and disease monitoring, play an important role in effective treatment. Salivary biomarker is preferred because saliva directly contacts with oral mass. Moreover, saliva collection is non-invasive and uncomplicated. Recently, matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been widely used to study proteome profiling and protein biomarkers in veterinary oncology[2,3] Peptide mass fingerprint (PMF) and proteomic profiles have previously been demonstrated in canine oral tumor tissues, including early- (OM with non-metastasis, EOM) and late-clinical stages (OM with metastasis, LOM), oral squamous cell carcinoma and oral benign tumors[2]. Moreover, the saliva proteomes of dog and human showed different protein- expression that related to tumorigenesis and apoptotic pathways [4].

The aim of the present study was to investigate PMF of EOM and LOM. The application of this knowledge for developing a clinical diagnosis should be further investigated.

Materials and methods

Saliva was obtained from 3 each of EOM and LOM and 3 healthy dogs, Samples were centrifuged and supernatants were collected. Salivary peptides were analyzed by Ultraflex III TOF/TOF in a linear positive mode with a mass range of 0–10,000 Da. Peptide mass fingerprint (PMF) and principal component analysis (PCA) of mass spectra was determined using t-test/ANOVA (TTA), and Wilcoxon/Kruskal-Wallis (W/KW) statistical algorithms incorporated in the ClinProTools software version 3.0.

Results and discussion

Specific salivary PMFs were demonstrated in each group (Fig 1a). Five peptide fragments were significantly increased in LOM or controls as showed in Table 1. Fig. 1b showed percentage of mass intensities at 4076 Da, which uniquely appeared in the LOM. A 3D view of PCA plot showed distinct clusters among EOM, LOM and control groups (Fig 1c). This study revealed distinct salivary PMF and clusters among EOM, LOM, and controls by MALDI-TOF Mass spectrometry. Further research is required analyzed the protein profiles and unique peak at 4076 Da for representation and classification of COM.

Figure 1. Distinct salivary PMFs from early-stage oral melanoma (EOM), late-stage OM (LOM) and controls (a). Percentages of interpretable mass signals at 4076 Da from early-stage oral melanoma (EOM), late-stage OM (LOM) and controls (b). Three-dimensional principal component analysis (3D PCA) scatterplot of early-stage oral melanoma (EOM), late-stage OM (LOM) and controls (c).

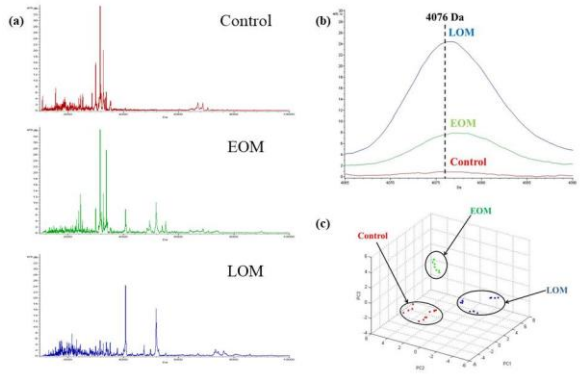

Table 1. Different peptide fragments determined by ClinProTools software.

Total average mass	statistical algorithms	High Intensity Group
3002	TTA	Control
3345	TTA	Control
4076	TTA W/KW	**LOM**
4099	TTA	LOM
6875	TTA	Control

Acknowledgements

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