

## Expression of C5a and its Receptor in Canine Spontaneous Tumours: A Preliminary Finding

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### ABSTRACT

A study of the development of spontaneous tumours in dogs gives many benefits in oncology research due to the similarity between dog and human cancer in terms of epidemiologic, biologic and clinical features. There is evidence that the complement component 5 anaphylatoxin (C5a) and its receptor are involved in the development of many types of tumour due to its inflammatory properties. The purpose of this study was to determine the expression of C5a on several types of canine spontaneous tumour i.e. mammary tumour, lung tumour, testicular tumour and melanoma. The expression of C5a in these tumours was compared with normal tissue from the breasts, lungs, testes and skin. The total of eight post-mortem canine tissues were collected from University Veterinary Hospital (UVH), University Putra Malaysia and stored in a preservative solution (RNAlater) to keep the RNA from degrading. The RNA was extracted using the Qiagen RNA Extraction Kit and a cDNA synthesis was carried out using a one-step PCR kit (Promega, USA). The expression of C5a was determined using reverse transcriptase PCR (RT-PCR) and Quantitative real-time PCR (qPCR) techniques. The results showed that all types of tumour gave higher expression of C5a compared to normal tissue. This means that the CT value for the tumours was below 30 cycles except for melanoma and the expression of C5a of normal tissues was above 30 cycles. This finding suggests that C5a and its receptor may be involved in the development of tumours in dogs and can be used as a tumour biomarker for both animals and humans in the future.

Nevertheless, further studies investigating the mechanisms of C5a and its receptor in canine spontaneous tumour are necessary.

**Keywords:** Canine spontaneous tumour, C5a, C5a receptor

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## INTRODUCTION

The choice of dogs as the animal model to study the role of the complementary protein C5a in the development of spontaneous tumours justifies its role as a good and reliable tool to gain further knowledge about spontaneous tumours and potential remedies. Dogs with spontaneous tumours may be used as good animal models based on several characteristics that have been studied by previous researchers (Rowell *et al.*, 2011; Pinho *et al.*, 2012). These similarities between canine and human tumours have been known to be related to genetics, treatment targets and physiology (Khanna *et al.*, 2006; Breen & Modiano, 2008; Paoloni & Khanna, 2008).

C5a is one of the proteins in the immune system that is involved in the inflammation reaction resulting in recruiting inflammatory cells to targeted regions in the body. Previous studies found that C5a was involved in many types of disease related to inflammation reaction (Dondorp *et al.*, 2005) and cancers (Kim *et al.*, 2005; Markiewski *et al.*, 2008; Hezmee *et al.*, 2011). However, the exact role of C5a in the body is still unclear due to lack of research and information about this complement.

Complement proteins, particularly of C5a, have been always thought to be one of the most vital substances in the host defence mechanisms. Nonetheless, many studies recently have implicated the role of complement activation in the development of multiple inflammatory and immunological diseases, including sepsis (Ward, 2004), acute respiratory distress syndrome (Robbins *et al.*, 1987), glomerulonephritis (Welch, 2002), ischemia-reperfusion injury (Arumugam *et al.*, 2004a), rheumatoid arthritis (Linton & Morgan, 1999) and asthma (Hawlich *et al.*, 2004).

C5a receptors (C5aR) can be located on the myeloid cells such as the phagocytic leukocytes (Chenoweth & Hugli, 1978; Kurimoto *et al.*, 1989; Gerard & Gerard, 1991; Werfel *et al.*, 1992; Revollo *et al.*, 2005), as well as those of a non-myeloid origin such as hepatocytes, lung vascular smooth muscle cells, bronchial and alveolar epithelial cells (Haviland *et al.*, 1995; Riedemann *et al.*, 2002), articular chondrocytes (Onuma *et al.*, 2002) and astrocytes (Gasque *et al.*, 1995). Previous studies showed that there was a 70-percent similarity of the C5a receptor between canines, humans, rats and bovines. Therefore, the canine C5a receptor is considered orthologous to the human C5a receptor, and this represents a surprisingly high interspecies variability compared with other G-protein-coupled-receptors (Perret *et al.*, 1992).

There are various functions of this receptor in the cells and tissues. One of the most important functions of this receptor is in the development of sepsis. It is achieved by the recruitment of neutrophils by C5a/C5aR signalling. C5a/C5aR signalling in neutrophils leads to activation of phosphatidylinositol 3-kinases (PI3K)/ a serine/threonine kinase (PI3K/Akt) pathways, which provide survival signals for neutrophils. Assembly of NADPH oxidase can be triggered by C5a/C5aR signalling, resulting in H<sub>2</sub>O<sub>2</sub> productions in organs and tissues. An increased number of neutrophils together with H<sub>2</sub>O<sub>2</sub> generation may be linked to tissue injury and multiple organ failure (Guo & Ward, 2005).

With this discovery, attention is shifted towards the prevention of the excessive complement activation that may lead to the development of multiple diseases. Therapeutic research has been conducted on laboratory animal models using trial antagonistic and agonist drugs to further understand the actions of complement proteins, particularly C5a. Therefore, the objective of

this preliminary study is to determine the availability of C5a receptors in the spontaneous tumour tissue of dogs. With the vast advancement of anti-complementary drugs available commercially, this study will provide a stepping stone for more detailed research into the use of these drugs to treat these tumours in dogs and, subsequently, in humans as well.

## MATERIALS AND METHOD

### *Canine Spontaneous Tumours and Sample Preparation*

A total of eight samples of different spontaneous canine tumours and normal tissues were obtained from excised tissues of diagnosed cases of canine spontaneous tumours from the University Veterinary Hospital (UVH), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). Table 1 shows the types of tumour based on their histopathological findings as diagnosed by the veterinary pathologist in Faculty of Veterinary Medicine, Universiti Putra Malaysia. A number of normal tissues were also obtained from pathological archives of the post mortem unit of Faculty of Veterinary Medicine for comparison. All the tissues were submerged in a sterile RNeasy<sup>®</sup> solution (Ambion, Inc., USA) for PCR analysis and sequencing of C5aR expression.

TABLE 1 : Types of Tumour Obtained

Lung carcinoma
Mammary carcinoma
Melanoma
Testicular tumour

The tissues that were submerged in the RNeasy<sup>®</sup> solution were treated for conversion to PCR products. The tissues were finely chopped into pieces of size 1-mm in diameter (50-100mg) and then transferred into a tube containing TRIzol<sup>®</sup> solution (Invitrogen<sup>™</sup>, USA) for preparation of PCR products.

### *RNA Isolation and cDNA Synthesis*

The tissues in the TRIzol<sup>®</sup> were homogenised using scalpel blade into much smaller pieces and vortexed vigorously. The digested tissues were left at 25 °C for 5 min. The insoluble materials were removed by centrifuge to obtain solutions containing the digested tissues at 13,000 rpm for 10 min at 4 °C. The supernatants were transferred into a new tube. Chloroform was then added to the supernatants and the tubes were shaken vigorously and left at 25 °C for 5 min and centrifuged at 13,000 rpm for 10 min at 4 °C. The clear supernatant was transferred to a fresh tube containing isopropanol solution. The solutions were left at 25 °C for 10 min and were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatants were discarded. The pelleted RNAs were washed with a 75% ethanol solution and the solutions were centrifuged at 9,000 rpm for 5 min at 4 °C. The RNA in the tubes was left to dry for 5 min. The process was continued with the RNA extractions. The pellets containing RNA products were re-suspended with nuclease-free water and heated at 55 °C for 15 min. DNaseI (Invitrogen<sup>™</sup>, USA) treatment

was conducted to remove traces of contaminating DNA. For the final preparation of the RNA extraction, cDNA syntheses of the RNA-treated cells were performed using the Universal RiboClone® cDNA synthesis system (Promega Corporation, USA). The RNAs from tumour tissues were used for C5a receptor detection and expression using a PCR machine.

#### *Cloning and Sequence Analysis of Canine C5aR (CD88)*

The primers for this study were designed according to the study by Perret *et al.* (1992). The C5aR cloning sequences and Hypoxanthine phosphoribosyltransferase (HPRT) genes used in this study were as follows:

C5aF = 5' GCT CAT CCT GCT CAA CAT GTA C 3'

C5aR = 5' CCG CGG AAG ATG AAC GA 3'

HPRTF = 5' TGC TCG AGA TGT GAT GAA GG 3'

HPRTR = 5' TCC CCT GTT GAC TGG TCA TT 3'

The primer sequences were obtained from BASE Life Sciences Holdings (Singapore). The primers were aliquot into smaller tubes and the leftover aliquot were stored in -80°C. One vial of each primer was used for this assay. Routine thermal reaction work out was conducted on the cDNA aliquots of 4µg of mammary tumour samples in 40 cycles in these conditions: pre-denaturing step for 10 min at 95 °C, denaturation step for 1 min at 94 °C, annealing temperature of 1 min at 50 °C, extension step for 1 min at 72 °C and extension procedure for 10 min at 72 °C. Other annealing temperature tested was 55 °C while HPRT primers were used to serve as canine housekeeping genes. Each PCR product from the thermal reaction was then subjected to gel electrophoresis through a 1.5% agarose gel and stained with ethidium bromide for screening purposes.

#### *Quantification of Target Gene Expression*

The quantification and negative control of C5aR gene transcripts were carried out by real time quantitative PCR using the Applied Biosystems (Victoria, Australia) and Sybr Green qPCR kit (Promega, USA). The PCR reaction mixture (20 µl) contained 10 µl of Sybr Green qPCR mix, 0.5 µl of the above-described primer mix 10 nM each, 1 µl RNAs inhibitor and 2 µl of cDNA template. The PCR protocol was: UNG enzyme incubation at 45 °C for 45 min; initial denaturation at 94 °C for 5 min; 40 cycles for primer annealing, 60 °C for 30 s for primer extension and an elevated temperature of 72 °C for 30 s for fluorescent data acquisition; and a final extension step at 80 °C for 2 s to allow the formation of fully duplexed DNA. To check the specificity of the amplified products, a melting curve analysis was performed immediately following the completion of the PCR. The melting protocol consisted of heating from 65 to 95 °C at a rate of 0.2 °C per step, and each step was held for 1 s for data acquisition. For each sample analysed, the mean Ct value based on the results of all experiments was calculated, together with that of the corresponding standard samples. The canine C5aR cDNA copy numbers were then normalised using the calculated HPRT cDNA copy numbers (the mean) of the same sample and run. This number was obtained by applying the respective Ct values for HPRT in the standard dilution curve of the same dilution.

## RESULTS AND DISCUSSION

The study of complement 5a (C5a) is a new field of study in Malaysia. It is important to study C5a due to lack of information regarding the exact role of C5a in the body and the involvement of this protein in various diseases. Many studies have shown that C5a is involved in many pathological states related to inflammatory disease and cancer (Dondorp *et al.*, 2005; Fitzpatrick, 2001; Hong *et al.*, 2010). The expression of C5a and its receptor can be defined using the reverse transcriptase Polymerase Chain Reaction (rt-PCR) and real time PCR (qPCR). These techniques are suitable for use in detecting the expression of C5a biology due to its properties, which are inexpensive, rapid response and high sensitivity in producing millions of copies of DNA even though the quality of the DNA may be poor (Erlich, 1989).

### *Expression of C5a Receptor Protein in Canine Tumours*

Fig.1 (A) shows the band for the housekeeping gene i.e. the HPRT gene. Meanwhile, Fig.1 (B) shows the single bands that were obtained from the C5a primer in tumour tissues in which Lane 1 is mammary carcinoma, lane 2 is lung carcinoma, lane 3 is melanoma and lane 4 is testicular tumour. The result indicates that C5a was expressed in all types of tumour except melanoma tissues while there was none in normal tissue (data not shown). The DNA ladder marker (Promega, USA) was used as a reference in which each band contributed to 100 bp size.

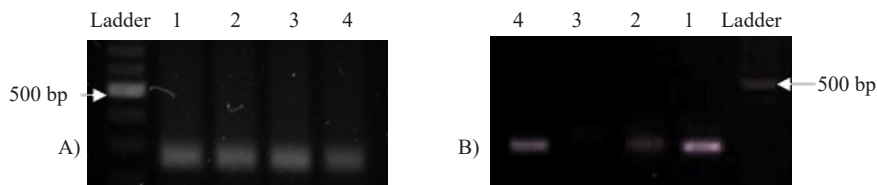


Fig.1: The expression of C5a receptor via Reverse Transcriptase PCR. Result of electrophoresis in 1.5% TBE-agarose gel of RT-PCR product using two sets of primers; A) HPRT primer as a housekeeping gene for each tumour tissues and B) C5a Primer with tumour tissues in which lane 1: Mammary carcinoma; lane 2: Lung carcinoma; lane 3: Melanoma and lane 4: Testicular tumour using 100bp ladder (Promega, USA).

The single band given for the PCR products showed that the polymerisation of C5aR using specific C5aR primers had occurred in the tissues. This result is a positive indicator for determining the role of the C5a and its receptor in the development of spontaneous cancer. It is due to the presence of PCR products in most of the tumour tissues; PCR products are absent in normal tissues. The presence of PCR products indicates that there was an abundance of C5a receptors in the tissues. This finding was consistent with previous studies that showed the involvement of C5a in many pathological states especially cancer (Smedegård *et al.*, 1989; Czermak *et al.*, 1999; Huber-Lang *et al.*, 2001; Huber-Lang *et al.*, 2006; Markiewski, 2008; Patel *et al.*, 2008). RT-PCR is the most sensitive procedure to detect RNA because it can detect and amplify much smaller samples (Kim & Kim, 2003) and also from a single cell. Housekeeping genes or internal control genes are normally used as a reference for the genes of interest (Suzuki *et al.*, 2000). It is constant in certain cell types but varies in other types of cell or tissue (Thellin *et al.*, 1999; Warrington *et al.*, 2000). Thus, the use of housekeeping genes is important in gene expression analysis.

*Determination of cDNA Sequencing of Canine C5aR (CD88)*

Nucleotide sequencing of canine C5aR cDNA revealed a coding region of 1357 bp. The deduced amino acid sequences of canine C5aR contained 657 residues. Sequencing of these digests showed that the recombinant was 97% homologous to the canine C5aR gene sequence deposited in GenBank under the accession no. X65860. The homologies of the cDNA and amino acid sequences among some mammalian species were calculated (Table 2).

TABLE 2: Nucleic Acid and Amino Acid Sequence Homologies of Canine C5aR, Compared with Other Mammalian Species

Dog	Human	Mouse	Rats	Sheep	Pig	Cattle
Nucleic acid (%)	91.0	91.0	92.0	85.0	88.0	85.0
Amino acid (%)	82.0	86.0	85.0	85.0	89.0	85.0

At the cDNA sequence level, canine C5aR was found to show high homologies with human C5aR (91%) and rats C5aR (92%). This result indicates that the role of C5a in the canine cells provided a direct indicator of the development of tumours in humans regarding the high sequence homology among them. Although rat models have a higher sequence homology for C5aR sequence, in terms of genome sequence, canine models have a higher similarity with human (79%) compared to rat (69%) models (Davis *et al.*, 2014). Besides that, the use of rats in research cannot always be faithfully extrapolated to human patients. Canines are more suitable for use in the study of cancer due to their characteristics of experiencing spontaneous disease with high frequency, developing the same types of cancer observed in humans and being receptive to the same therapeutic strategies (Pontius *et al.*, 2007). In addition, both canines and owners are susceptible to getting the same type of cancer due to exposure to the same environmental factors.

The use of dogs as a model of a variety of cancers in human has been suggested due to the fact that the characteristics and gene sequence of C5a in canines mimic those in humans (Rowell *et al.*, 2011; Pinho *et al.*, 2012). Hence, it is important to study the effects of C5a/c5aR on the development of tumours in canines. Unfortunately, due to the limited number of samples because of difficulty in getting spontaneous tumour samples from veterinary hospital in Malaysia, this study was unable to statistically exhibit these arguments on a much bigger scale. Nevertheless, this preliminary study provided a good platform for exploring the potential of using C5a/C5aR markers in clinical applications for diagnostic tools. It also highlighted prognostic indicators and potential targets for therapeutic and preventive strategies and supports efforts to evaluate clinical trials on the efficacy of C5a/C5aR-blockers in the treatment of canine tumours.

*Quantification of C5aR Gene Expression*

Four types of canine tumour were used in this study i.e. mammary carcinoma, lung carcinoma, melanoma and testicular tumour. Each tumour was compared to its normal tissue from the particular part of the body. The result showed that the mean CT value for the tumours was below 30 cycles except for melanoma and that the expression of C5a of normal tissues was

above 30 cycles. The differentiation of CT value between tumour tissues and normal tissues for mammary carcinoma, testicular tumour, lung carcinoma and melanoma were 15, 11, 5 and 2 cycles, respectively.

The CT value is a parameter used in detection and quantification of C5a expression in canine spontaneous tumours. The CT value form is in indirect proportion to the amount of PCR product in a reaction (Higuchi *et al.*, 1993). The higher expression of gene of interest gives a lower CT value due to the principle of RT-PCR i.e. the detection of samples depends on the fluorescent signal that is captured by computer software where the result is calculated based on cycle threshold (CT) value. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The result showed that the expression of C5a in canine spontaneous tumours was higher than in normal tissues due to the lower CT value of tumour tissues compared to normal issues.

These findings are supported by the previous study of Hezmee *et al.* (2011) in which the researchers found that the expression of C5a in canine malignant tumours was higher compared to that in benign tumours while there was no expression of C5a in canine normal mammary tissues. This suggests that C5a may have a direct biological effect on cancer cells. This finding is also in agreement with another study done on C5aR expression in bovine mammary tissues, in which C5aR was also expressed by inflammatory cells from bovine mammary tissues, but not from normal or inactive cells (Nemali *et al.*, 2008). Hence, this study suggested that activation of C5a receptors may have a consequence in tumour development. However, the use of a small sample size in this study means it is not clearly possible to make a proper correlation between the expression of C5aR and tumour types. Besides that, many precautionary steps starting from preparation protocol to analysing of results need to be emphasised due to the different type of tissues that can lead to a false positive result.

The Real Time Polymerase Chain Reaction (qPCR) is a method that can be used to replace the weakness of PCR (Erlich *et al.*, 1989; Whelan *et al.*, 2003). This system provides greater sensitivity for amplicon detection compared to conventional PCR that uses gel-based detection. It can be seen in the results, where conventional PCR gave a single band to most of the tumour tissues but there was no band for melanoma and normal tissues. This was due to very low expression of C5a in these tissues. However, when the samples were put through the real-time PCR technique, both tumours and normal tissues gave a CT value result. This was due to the capability of real-time PCR of detecting DNA up to 50 fg and its higher sensitivity, up to tenfold more, than that of conventional PCR. Based on the reverse transcriptase PCR and Real Time PCR, the results showed that the expression of C5a was up-regulated in all the tumour tissues except melanoma and down-regulated in all normal tissues (Fig.2).

Even though the role of C5aR in the development of canine tumours remains unclear, there are studies that suggest a link between chronic inflammatory response and the development of cancer (Coussens & Werb, 2002; Tan & Coussens, 2007). C5a and its receptors play an important role in various other disorders involving chronic inflammatory responses (Robbins *et al.*, 1987; Welch, 2002; Arumugam *et al.*, 2004a; Hawlisch *et al.*, 2004; Ward, 2004) and due to its property of being one of the most potent inflammatory peptides, excessive activation of complement proteins towards their receptors in tissues has been hypothesised to lead to the progression of tumour cells (Tan & Coussens, 2007).

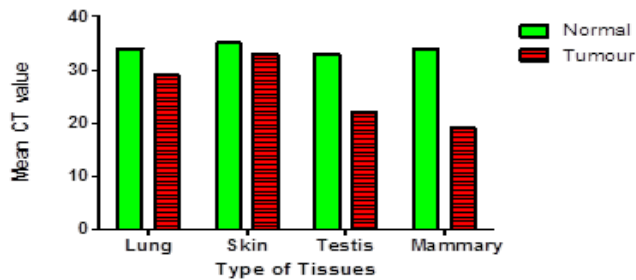


Fig.2: The magnitude of expression of C5a via Real Time PCR. The CT value recorded for C5a is higher in normal tissue compared to the tumour tissue. The CT values recorded is indirectly proportional with concentration of C5a in which the higher the concentration of C5a, the lower the CT values recorded. The HPRT gene was used as a housekeeping gene to validate the results. The red bars show that the tumour tissues started with lane 1: Lung carcinoma; lane 2: Melanoma; lane 3: Testicular tumour; and lane 4: Mammary tumour. The green bars show the normal tissues for each type of tumour starting with the lung tissue followed with skin, testis and mammary tissues.

The complex mechanism of humoral immunity of the host, which includes the activation of C5a and its receptors in tissue and cell surfaces, is still not fully understood; nevertheless, receptors for the Fc portion of IgG (especially FcγRIII) and complement factors (particularly C5a/C5aR) are recognised as co-dominant effectors in the process (Schmidt & Gessner, 2005). Tissues damaged by autoimmune disorders and by cancer have similar characteristics such as in chronic innate immune cell infiltration, tissue re-modelling, angiogenesis, altered survival pathways (Sapir & Shoefeld, 2005). It would seem there is a possibility that the same immune complex effector pathways are involved in pathogenesis of both disease (Tan & Coussens, 2007). Although this study did not provide any data regarding the treatment of these tumours, there are, however, scientifically tested and proven anti-complement drugs that are used to counter the effects of excessive and chronic C5a/C5aR activation (Short *et al.*, 1999; Arumugam *et al.*, 2002; Woodruff *et al.*, 2003; Arumugam *et al.*, 2004b).

## CONCLUSION

This preliminary study found that the expression of C5a was up-regulated in most of the spontaneous canine tissues and down-regulated in all normal tissues. This study gave a positive indicator of the role of C5a in the development of spontaneous tumours in canines as well as in humans due to high homology of the C5a sequence in both dogs and humans. However, further study is warranted to fully understand the mechanism of tumour development due to action of C5a/C5aR; data for a higher number of tumour-bearing animals and the effects of C5a/C5aR antagonist and agonist drugs should be included in such study.



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