



Genetic characterization and phylogenetic analysis of triose phosphate isomerase (TPI) gene amplified from a case of canine mammary tumour showing TPI over-expression and high titre anti-TPI antibodies

RICHA ARORA¹, SONAL SAXENA², SAMEER SHRIVASTAVA³, PIYUSH KUMAR SINGH⁴, SHAHID HUSSAIN⁵, MANOJ KUMAR⁶, SUBAS CHANDRA JENA⁷, MANOJ KUMAWAT⁸, RAJKUMAR JAMES SINGH⁹, PURNIMA GOGOI¹⁰, MONALISA SAHOO¹¹ and NAVEEN KUMAR¹²

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

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Canine mammary tumour (CMT) is the most common malignancy of female dogs, comprising about 52% of all neoplasms in unspayed female dogs (Macewen and Withrow 1996). Majority of the CMTs have poor clinical outcomes with three times higher mortality rates than human breast cancer (Davidson 2003, Egevall *et al.* 2005, Shafiee *et al.* 2013). High mortality, high incidence, higher chances of recurrence and similarities with human breast cancer highlights the importance of CMTs. The glycolytic pathway plays an important role in the neoplastic cells which survive under hypoxic conditions through production of ATP using glycolytic enzymes. Triose phosphate isomerase (TPI) encodes for a glycolytic enzyme which is essential for cell growth and maintenance. Gene alterations in tumour associated antigens has been suggested as a mechanism for the production of mutated proteins, which are recognized as foreign by the host cells, resulting in production of autoantibodies. Triose phosphate isomerase (TPI), a normal glycolytic enzyme, has been reported to be highly overexpressed in cancer cells. Autoantibodies to TPI are also present in various types of human cancers (Desmetz *et al.* 2009, Valera *et al.* 2010), and canine mammary cancer (Zamani-Ahmadmhamudi *et al.* 2014). Various other theories such as loss of tolerance, and alterations in antigen expression, as well as their altered exposure or presentation, decreased degradation, post-translational modifications (PTMs), and their aberrant location etc have been proposed for the production of autoantibodies in cancer cells (Zaenker *et al.* 2016).

Present address: ^{2,3}Senior Scientist (sonalvet@gmail.com, sameer_vet@rediffmail.com), Veterinary Biotechnology Division; ^{1,4,5,6,7,8,9,10}Research Scholar (richaarora5521@gmail.com, rahul.piyush@hotmail.com, shahidhussainsofi@live.com, 0711mworld@gmail.com, drsubasvet@gmail.com, pmanoz59@gmail.com, jamesalmighty36@gmail.com, gogoipurnima17@gmail.com, Veterinary Biotechnology Division; ¹¹Scientist (vety.lisa@gmail.com), Veterinary Pathology Division; ¹²Principal Scientist (naveen.ivri1961@gmail.com), Veterinary Surgery Division.

Since TPI gene is highly overexpressed in CMTs, and autoantibodies to TPI had also been reported in CMTs, the present study was taken up to analyse any mutations in the TPI gene sequence in dog suffering from CMT and showing TPI over expression and high titre TPI auto-antibodies.

CMT tissues and serum samples used in this work were obtained from clinical cases of CMT presented to the Referral Veterinary Polyclinic, Indian Veterinary Research Institute (IVRI), Izatnagar. Tumour cases were confirmed by histopathological examination of H & E stained tissue sections. Anti-TPI antibodies in the serum samples were confirmed using Indirect ELISA. The samples which showed OD value above the cut-off value [average OD of healthy animals (50) + 2 SD] were considered as positive for TPI autoantibodies. Based upon the results of ELISA, a clinical case which showed high titre anti-TPI antibodies in the sera sample was selected for further study. The tumour tissue from the selected CMT case, histologically classified as adenocarcinoma, was obtained by surgical removal of the tumour mass.

Overexpression of TPI in the tumour tissue sections was examined by immunohistochemistry (IHC) using rabbit polyclonal antibodies against TPI protein (Santa Cruz) at 1:50 dilution. IHC was performed using SuperPicture™ polymer detection kit (ThermoFischer Scientific, USA) following manufacturer's recommendations.

Total RNA was isolated from tumour tissue sample by RNeasyplus™ mini kit (Qiagen, Hilden, Germany). Briefly, 30 mg of tumour tissue was homogenized in RLT buffer supplied with the kit and processed further as per manufacturer's protocol. Two microgram of the total RNA isolated was used for preparation of cDNA using M-MuLV RT Reverse transcriptase (Fermentas, USA) and Oligo (dT) primers.

The primers were designed for amplification of full length TPI gene coding region using Premier 5.0 software (National Bioscience). Sites for restriction enzymes *XbaI* and *PstI* sites were incorporated at the 5' ends of forward

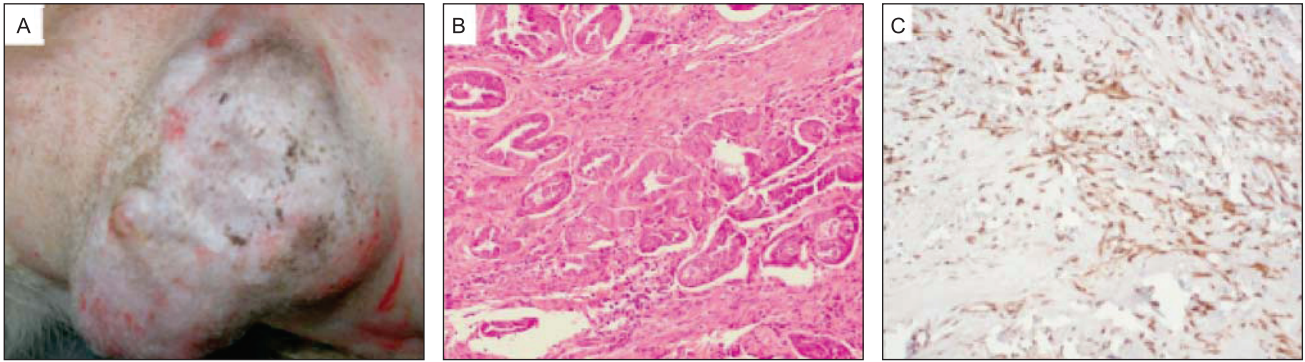


Fig. 1(A-C). Gross (A), histopathological (B) and immunohistochemical (C) examination of the tumour tissue used for amplification of TPI gene.

and reverse primer, respectively. The cDNA from CMT tissue was used as a template for amplification of TPI gene. Cycling conditions for amplification of the gene included, initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 98°C for 20 sec, annealing at 60°C for 15 sec and extension at 72°C for 15 sec with final extension at 72°C for 10 min. The TPI PCR product was further cloned in pH6HTN His 6 HaloTag® T7 vector using *XbaI* and *PstI* restriction endonucleases (NEB, England). Recombinant plasmid was further confirmed by plasmid DNA sequencing. Analysis of gene sequence was done using DNA-STAR sequence analysis software and basic local alignment search (BLAST) tool.

Sequences similar to the sequence of the isolated TPI gene were identified by performing BLAST against NCBI

nucleotide database. The sequences were aligned by Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm in MEGA7 software. The phylogeny was then inferred by the maximum-likelihood method upon assessment of best-fit substitution model.

Pictures for gross appearance and histopathological examination of the tumour are represented in Figs 1A,B respectively. Histologically the tissue was classified as adenocarcinoma as per Goldschmidt *et al.* (2011). Upon immunohistochemical staining, using TPI specific antibodies, the tumour tissue exhibited overexpression of TPI as shown in Fig. 1C.

The amplified product showed expected band corresponding to DNA of size of 800 bp on agarose gel electrophoresis (Fig. 2A). The amplified product was cloned

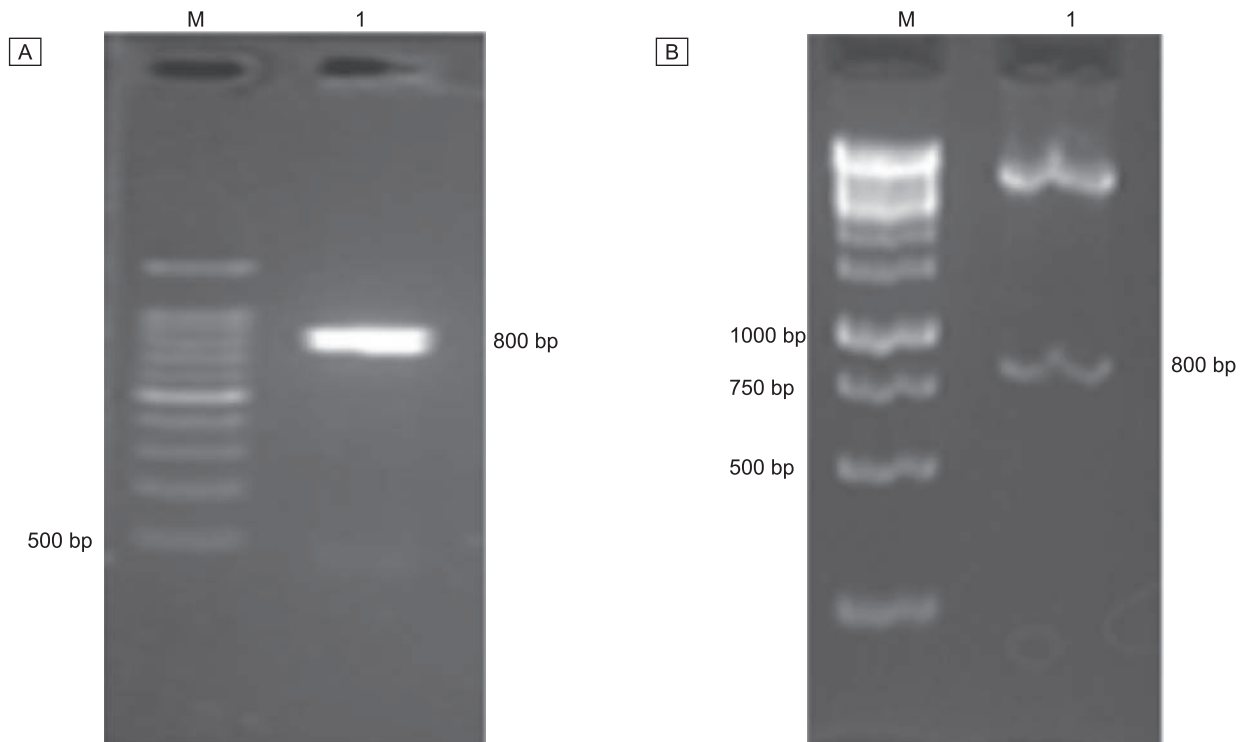


Fig. 2. Amplification and cloning of TPI gene. A. Amplification of TPI gene. Lane 1, amplified TPI PCR product; lane M, 100 bp DNA ladder. B. Confirmation of recombinant plasmid by RE digestion. Lane 1, *XbaI* and *ApaI* digested recombinant plasmid showing release of 800 bp insert; lane M, 1 kb DNA ladder.

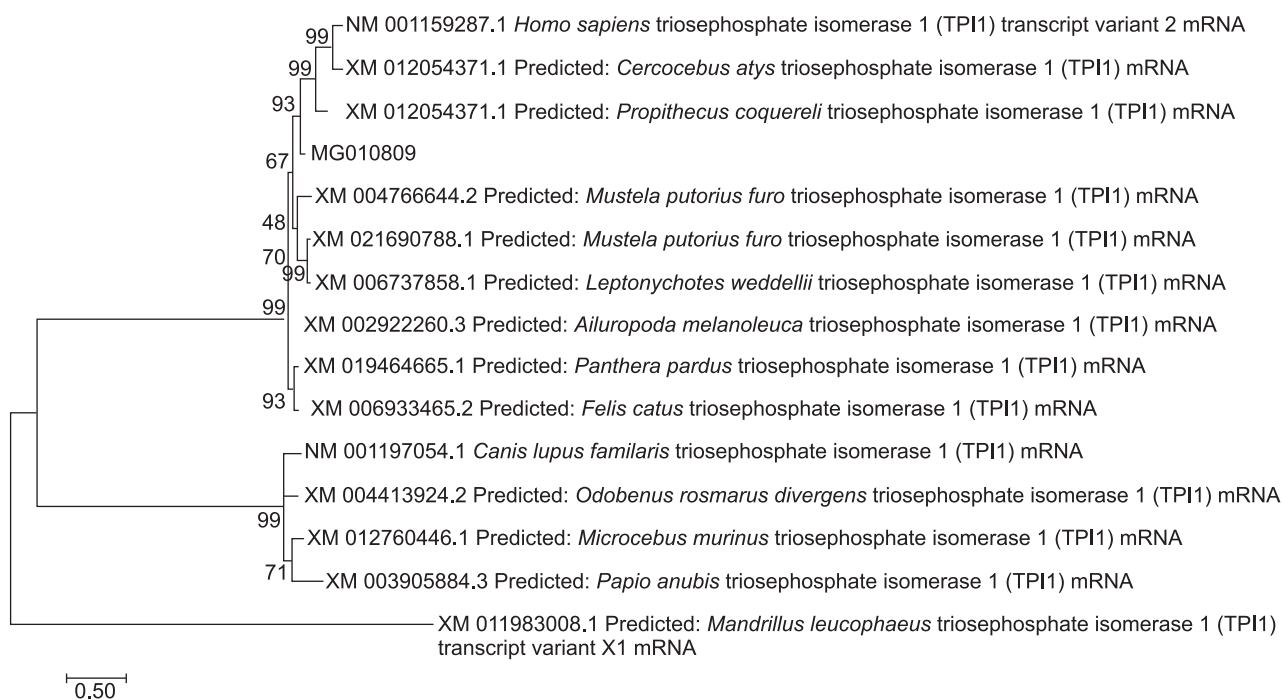


Fig. 3. Phylogenetic analysis of *Canis lupus familiaris* TPI gene coding region. Phylogram illustrating the evolutionary relationship of the TPI gene of dog (*Canis lupus familiaris*) with other species. The phylogenetic tree was constructed using Maximum-Likelihood method. Numbers represent bootstrap values (given as percentages) for a particular node. Thousand replicates were used in bootstrap analysis for a better statistical support. The branch lengths are scaled to represent the relative number of substitutions occurring along each branch.

in pH6HTN His-6 HaloTag[®] T7 vector and restriction digestion of the recombinant plasmid pH6HTN His 6 HaloTagTPI with *Xba*I and *Apa*I showed release of 800 bp fragment corresponding to the insert size (Fig. 2B).

The recombinant plasmid was confirmed by plasmid DNA sequencing and the sequence was submitted to GenBank (Accession No MG010809). The alignment of the TPI gene sequence showed 100% similarity with healthy dog (*Canis lupus familiaris*) TPI gene sequence (NM_001197054.1) available in the GenBank database (Fig. 3). These findings reveal absence of any mutation in the TPI gene coding region from canine mammary tumour case. Upon BLAST and phylogenetic analysis, TPI mRNA sequence showed greater than 90% similarity with sequence from other species available in the GenBank database showing conserved nature of the protein.

SUMMARY

Although TPI is a glycolytic enzyme, expressed by all the cells, still, autoantibodies against TPI were present in high frequency in sera of dogs suffering from CMT in comparison to normal healthy dogs. Thus, it was analysed whether these autoantibodies were against any mutated TPI protein or not. However, no mutation was found in the TPI gene coding region in the case of CMT. The sera sample from the same animal showed presence of high titre anti-TPI antibodies, which indicates that mechanisms other than gene alteration are responsible for production of autoantibodies. The same sample also showed high

expression of TPI by IHC. Thus, autoantibodies to TPI may not be due to abnormal structure or composition of mutated protein but possibly due to increase in antigenic load owing to increased gene expression level and reduced degradation.

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