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Characterization and *in vitro* expression studies of a potential xenogeneic DNA vaccine against canine mammary tumours

PAVAN KUMAR YADAV[1], SHISHIR KUMAR GUPTA[2], SAROJ KUMAR[3], MOHINI SAINI[4], SUMIT RANJAN MISHRA[5], P NANDAKUMAR[6] and MEENA KATARIA[7]

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

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ABSTRACT

Matrix metalloproteinases-7 (MMP-7) which is expressed in a wide variety of malignant cells has been seen to be extensively up-regulated in mammary carcinomas. MMP-7 can promote cancer invasion and angiogenesis through proteolytic cleavage of extracellular matrix and basement membrane proteins. This property of MMP-7 makes it a promising target in the context of immunotherapy. Further, to enhance DNA-based immunization, a cytokine gene can be employed as an adjuvant. Interleukin-18 (IL-18) is a Th1-type cytokine that has been demonstrated as a potential biological adjuvant in murine tumour models. The present study was undertaken to clone murine MMP-7 (mMMP-7) and IL-18 genes in pVIVO2.mcs eukaryotic expression vector and to characterize their expression by immunofluorescence and Western blotting. This double gene construct now may be used as a potential xenogeneic DNA vaccine against canine tumour model.

Key words: Cancer, Interleukin-18, Matrix metalloproteinase-7, Tumour, Xenogeneic DNA vaccine

Matrix metalloproteinases (MMPs) are the multigene family which share a similar structure and have the capacity to degrade virtually every component of the extracellular matrix (ECM). This activity has been implicated in a number of key normal and pathologic processes such as tumour growth, progression and metastasis as well as the dysregulated angiogenesis (Westermarck and Kahari 1999, Chang and Werb 2001). There are several evidences that MMPs have multiple roles, as they are necessary for the creation and maintenance of a microenvironment that facilitates growth and angiogenesis of tumours at primary and metastatic sites (Nelson *et al*. 2000). MMP-7 is one of the smallest MMPs and lack the C-terminal hemopexin domain present in the majority of its family members (Nagase *et al*. 2006). It additionally cleaves a wide range of ECM proteins including fibronectin, vitronectin, elastin, type IV collagen, aggrecan and proteoglycans as well as other molecules including β4 integrin, E-cadherin, Fas ligand, tumour necrosis factor–α and other proteases (Woessner and Taplin 1988, Miyazaki *et al*. 1990). MMP-7 has been shown to be over-expressed in several pathological conditions like cancer, inflammation and degenerative disorders such as atherosclerosis, multiple sclerosis and Alzheimer disease (Adachi *et al*. 2001, Li *et al*. 2002, Tan *et al*. 2005). MMP-7 expression has been described in cancers of different origin, particularly in malignant tumour and its presence is associated with tumour progression and poor prognosis (Ii *et al*. 2006, Wielockx *et al*. 2004). There is evidence that ovarian carcinomas over express these genes compared to breast cancer metastases (Davidson *et al*. 2011), and that lung adenocarcinomas similarly overexpress MMP-7 compared to breast carcinomas (Davidson *et al*. 2012).

A lot of xenogeneic DNA vaccines targeting various tumour-associated antigens have been found to elicit both humoral and/cytotoxic T-cell immune response. Besides, DNA vaccine targeting MMPs could induce anti-cancer effects in various cancer models (Su *et al*. 2003). Several studies have demonstrated that breaking immune tolerance against tumour growth may be a useful approach for cancer therapy (Wei *et al*. 2000). Sequence analysis indicated that mouse MMP-7 is 68% identical with that of dog at the amino-acid level. Thus, mice MMP-7 cDNA vaccine could be constructed to break immunity tolerance to MMP-7 in canines. Further, a common strategy to further enhance the efficacy of DNA-based immunization is by using cytokine genes as adjuvant. Interleukin-18 is a member of IL-1 family of pro-inflammatory cytokines, which was identified for its ability to induce high levels of IFN-γ secretion from natural killer (NK) and T-cells (Okamura *et al.* 1995). IL-18 is produced by dendritic cells and activated macrophages and plays a crucial role in driving Th1-dominated immune

Present address: [1,3]Assistant Professor (pavanivri2010@gmail.com, drsarojvet@gmail.com), [2]Scientist (shishirgupta.biotech@gmail.com), [4,7]Principal Scientist (mohini@ivri.res.in, mkataria97@rediffmail.com). [5,6]Ph D Scholar (smsumit5@gmail.com, vetnandha@gmail.com), Division of Animal Biochemistry.

responses (Micallef *et al*. 1997). Thus, a bivalent vaccine consisting of mouse MMP-7 and IL-18 may be a potential useful strategy to treat canine mammary tumours. Therefore, the present study was undertaken to clone mMMP-7 gene and molecular adjuvant IL-18 in eukaryotic expression vector pVIVO2–mcs and characterize the double constructs in mammalian cells (HEK–293).

MATERIALS AND METHODS

Cell line and culture media: HEK-293 cells were adapted to grow in DMEM (high glucose) 2 mM L-Glutamine, 1.5 mM $NaHCO_3$, 10 mM HEPES buffer (Himedia, India) supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin at 37°C under 5% CO_2 in 25 cm^2 flask (Nunc, Denmark).

Cloning and characterization of murine IL-18 gene in pVIVO2-mcs mammalian expression vector

Isolation of total RNA and cDNA synthesis: Spleen was dissected from a Swiss albino mouse and single cell suspension of splenocytes (5×10^6 cells/ml) was prepared in RPMI-1640 containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells in a 6-well plate were cultured in the presence of concanavalin A (5 µg/ml) for 16 h in humidified atmosphere at 37ºC and 5% CO_2. Following incubation, the cells were harvested from wells and washed with PBS and total RNA was extracted using total RNA SafeKit^{TM} (MP Biomedical, USA) as per the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from isolated RNA sample using Oligo-dT primer and RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA) following the manufacturer's protocol.

PCR amplification for cloning mice IL-18 gene: Messenger RNA (mRNA) coding region of *Mus musculus* IL-18 gene (Accession No: NM_008360.1) was used to amplify the mature sequence, using cDNA as template. The primer sequences (5'-GCACTCTAGAATGGCTGCCAT GTCAGAAGAC-3´ (Forward) and 5'-GCATCAGTATACC ACAAACCCTCCCCACCTAA-3' (Reverse)) were chosen to amplify 594 bp fragment encoding IL-18 coding sequence (CDS). Designed primers were checked for correct reading frame within their expression vectors by *in silico* translation of the target fragments using DNA Star programme of Lasergene suite 6.0 and online programme ExPASY (SwissProt).The amplification was carried out in 25 µl final volume using *Pfu* DNA polymerase (Thermo Scientific, USA). After a denaturation at 95°C for 5 min, 35 cycles of amplification were performed as follows: denaturation at 94°C for 45 sec, annealing at 61°C for 45 sec and extension at 72°C for 45 sec. A final extension step of 5 min at 72°C ended the reaction. The PCR product was recovered from low melting point agarose and purified. The purified IL-18 product and eukaryotic expression vector pVIVO2-mcs (InvivoGen, USA) were digested with restriction enzymes *Xba*I and *Bst*1107I, ligated and transformed into *E. coli* DH5α competent cells. The desired recombinant plasmid pVIVO2.IL18 was confirmed by colony PCR and restriction enzyme digestion with *Xba*I/*Bst*1107I.

In-vitro *expression analysis of recombinant pVIVO2.IL-18 plasmids in HEK-293 cells*

To check the functionally active recombinant plasmids, *in-vitro* expression studies were carried out in HEK-293 cells. For this, transfection grade pVIVO2.IL-18 prepared using Endo Free Plasmid Maxi kit (QIAGEN, USA) as per the manufacturer's protocols. Purified plasmids were used to transfect 70–80% confluent HEK-293 cells in a 12 well plate using lipofectamine 2000 (Invitrogen, USA) transfection reagent. For this, 2 µg plasmid DNA was mixed with 5 µl lipofectamine in 250 µl of opti MEM (Invitrogen, USA). The mixture was incubated for 30 min at room temperature for complex formation. Meanwhile, the cells were washed once with 1 ml of PBS and 1.5 ml of fresh cell growth medium without antibiotics (DMEM with 10% FBS) was added to each well. After incubation, DNA-lipofectamine complexes were added over the cells and plate was incubated at 37ºC for 4 h. After 4 h, media was replaced with fresh growth medium and plate was incubated again at 37ºC under 5% CO_2 for 48 h and expression was analyzed by following methods.

Immunofluorescence assay: HEK-293 cells cultured in 12 well plates were transfected with pVIVO.IL18 and incubated at 37°C and 5% CO_2 for 48 h. After 48 h of incubation, the transfected cells were washed twice with PBS for 5 min each. Then cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Following two washes with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. Then free sites were blocked with 3% BSA in PBS (pH 7.4) for 1 h. After discarding the blocking buffer, the cells were incubated with pre-diluted (1:100 in PBS) mice monoclonal IL-18 antibodies (Santa Cruz, USA) at 4ºC overnight. The cells were washed thrice with PBS and incubated with goat anti-mouse IgG-FITC conjugate (Bangalore Genei, India) at 1:2000 dilution at RT for 1 h. Further, after 2 washings with PBS, the cells were stained with DAPI followed by 2 washings with PBS and observed under UV florescence microscope (Nikon, USA).

Western blotting: Western blot analysis was done using the total proteins of HEK-293 cells transfected with pVIVO2.IL18 construct and the total protein of HEK-293 cells untransfected as control. For Western blot, transfection was carried on monolayer of HEK-293 cells (60–70% confluent) grown in 25 cm^2 tissue culture flasks. Dilution of primary antibodies and secondary rabbit anti-mouse HRP-conjugate used were 1:200 and1:5000, respectively. The rest of procedure was similar to our previous study (Yadav *et al*. 2014).

Cloning and characterization of mMMP-7 in MCS2 of pVIVO2-mcs eukaryotic expression vector

The primers for *Mus musculus* MMP-7 gene (forward 5'-GCCCATGGATCTGCCACTGTCCCAGGAAGCTG-3´ and reverse 5'-GCGCTAGCTCACAGCGTGTTCC

TCTTTCCAT-3´) were designed as described above. pMD-mMMP7 (Sino Biological Inc.) was used as a template for amplification of mMMP-7 gene by the above primers. PCR amplification was carried out as described above with annealing at 58ºC for 45 sec and extension at 72ºC for 60 sec. PCR product was cloned at *Nco*1 and *Nhe*1 MCS 2 site of pVIVO2.mcs. Presence of insert mMMP-7 was confirmed by colony PCR and restriction double digestion.

In-vitro *expression analysis of recombinant pVIVO.mMMP-7 plasmids in HEK-293 cells:* To check the functionally active recombinant plasmids, *in-vitro* expression studies were carried as described above. And expression was checked by Immunofluorescence assay and Western blotting using hyper-immune sera raised against canine MMP-7 (cMMP-7) in rabbit (available in lab.). TRITC labeled secondary goat anti-rabbit IgG (Sigma, USA) for Immunofluorescence assay and HRP conjugated labeled secondary mouse anti-rabbit IgG (Benglore Genei, India) was used for Western blotting.

Cloning and expression of mMMP-7 in pVIVO2.IL-18 backbone: mMMP-7 fragment was released from pVIVO2.mMMP-7 construct using *Nco*1 and *Nhe*1 restriction enzymes and further cloned in pVIVO2.IL-18 backbone at the same site. The plasmids were verified by restriction digestion with *Xba*1, *Bst*11071, *Nco*1 and *Nhe*1 followed by 1% agarose gel electrophoresis.

In-vitro *expression analysis of recombinant pVIVO.IL-18.mMMP-7 plasmids in HEK-293 cells:* To check the functionally active recombinant plasmids, *in-vitro* expression studies were carried out as described above and expression was checked by double Immunofluorescence (van der Loos 2008), and Western blotting using hyper-immune sera raised against cMMP-7 in rabbit (available in lab.). TRITC labeled secondary goat anti-rabbit IgG (Sigma, USA) for IF and HRP conjugated labeled secondary mouse anti-rabbit IgG (Benglore Genei, India) was used for Western blotting.

RESULTS AND DISCUSSION

Cloning and characterization of murine IL-18 gene at MCS-1 position of pVIVO-2mcs eukaryotic expression vector: The full-length cDNA of murine IL-18 gene was amplified from RNA isolated from ConA stimulated mice splenocytes and a 594 bp PCR amplicon was resolved in agarose gel (Fig.1a). The PCR amplicon was purified, digested with *Xba*1 and *Bst*11071 restriction enzymes and gel purified for cloning in to MCS-1 of pVIVO-2mcs. The positive colonies were screened by colony PCR (Fig.1b) and further confirmed by restriction digestion of the plasmids with *Xba*1 and *Bst*11071 enzymes which released the desired fragment of 594 bp (Fig.1c). The recombinant pVIVO.IL-18 was prepared in bulk and purity of DNA preparation was checked by measuring absorbance at 260/280. The functional expression of the pVIVO.IL-18 recombinant plasmid was verified by transfection into HEK-293 cells followed by immunofluorescence staining 48 h post transfection. Immunofluorescence staining showed

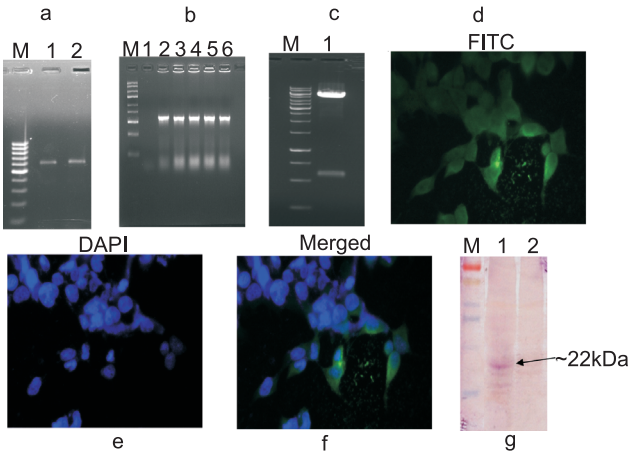

Fig. 1. Generation of pVIVO.IL-18 plasmid and it's functional assessment. (a) PCR amplification of murine IL-18 gene, Lane M- DNA ladder and lanes 1 and 2- amplified 594 bp product; (b) Colony PCR for screening of positive clones; (c) Double endonuclease digestion of the recombinant vector pVIVO.IL-18, Lane M - DNA maker; lane 1- release of insert after digestion with *Xba*1 and *Bst*1107; (d) Photomicrograph of HEK-293 cells showing IL-18 expression; (e) DAPI positive cells indicating nuclei of HEK-293 cells; (f) Merged image of fields shown in (d) and (e); (g) Western blot analysis of extracts from untransfected control cells and transfected cells expressing IL-18 protein (~22 kDa), using anti– IL-18 antibody. Lane M- protein ladder; lane 1- transfected lysate; lane 2- Non-transfected lysate.

optimum fluorescence in the test sample (Figs.1d-f). The cell lysate from transfected cells showed a expected band of ~22 kDa in Western blot using anti-human interleukin-18 antibodies (Fig.1g).

Cloning and expression of mMMP-7 gene at MCS-2 position of pVIVO-2mcs eukaryotic expression vector: The cDNA corresponding to the full-length mature peptide of mMMP-7 protein was amplified from the TA clone mentioned above and a 744 bp PCR amplicon was resolved on 1% agarose gel (Fig. 2a). The amplicons were purified and digested with *Nco*1 and *Nhe*1 and cloned at MCS2 of vector pVIVO-2mcs. The positive colonies screened by colony PCR revealed 691 bp products (Fig. 2b). The recombinant plasmids were confirmed by restriction digestion with *Nco*1 and *Nhe*1 enzymes which released the desired fragment of 744 bp (Fig. 2c). The *in vitro* expression analysis of purified recombinant pVIVO.mMMP-7 was performed by transfecting HEK-293 cells and the expression of cloned mMMP-7 gene at protein level was confirmed by IFAT using polyclonal serum raised in rabbit against r-cMMP-7 protein followed by TRITC labeled anti-rabbit secondary antibody. The nucleus of the cells was stained by nuclear stain DAPI and cells were visualized under fluorescent microscope. The expression of mMMP-7 protein was confirmed by the characteristic red fluorescence (Figs 2d-f). The cell lysate from transfected cells exhibited expected band of ~28 kDa in Western blot using anti r-cMMP-7 polyclonal serum raised in rabbit (Fig. 2g).

Cloning and expression of both mMMP-7 in pVIVO2.IL-18 backbone: For the cloning of mMMP-7 at MCS-2, both

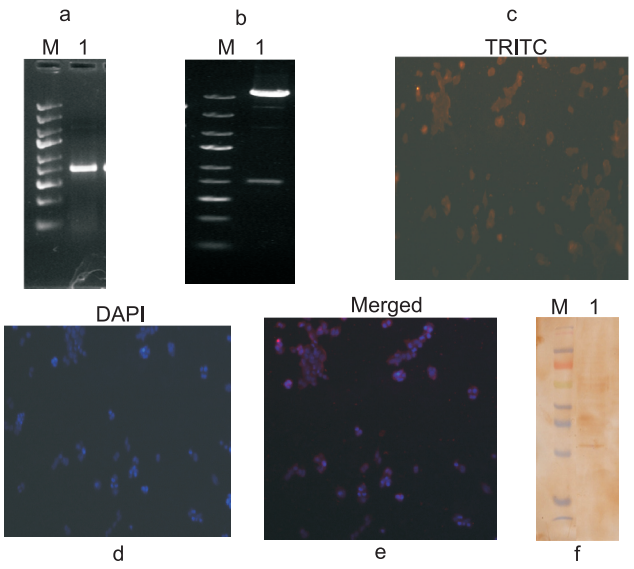

Fig. 2. Generation of pVIVO.mMMP-7 plasmid and its functional assessment. (a) PCR amplification of mMMP-7, Lane M- DNA ladder and lane 1-amplified 744 bp product; (b) Double endonuclease digestion of the recombinant vector pVIVO.mMMP-7, Lane M - DNA maker; lane 1-release of insert after digestion with *Nco*1 and *Nhe*1; (c) Photomicrograph of HEK-293 cells showing mMMP-7 expression; (d) DAPI positive cells indicating nuclei of HEK-293 cells; (e) Merged image of fields shown in (c) and (d); (f) Western blot analysis of extracts from untransfected control cells and transfected cells expressing mMMP-7 protein (~28 kDa), using anti– cMMP-7 antibody. Lane M- protein ladder, lane 1- transfected lysate.

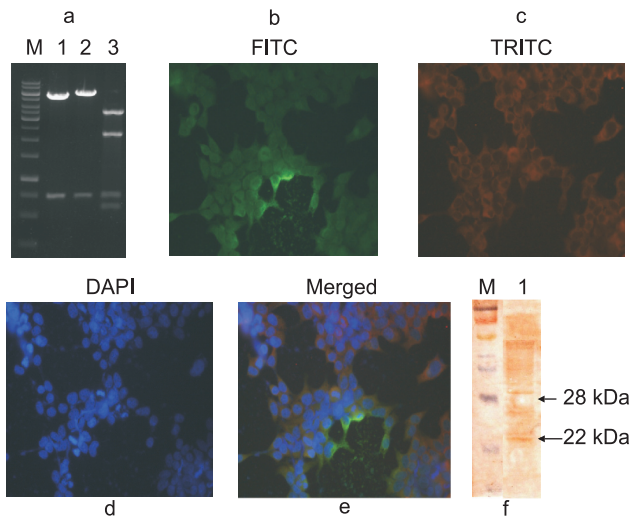

Fig. 3. pVIVO.IL-18.mMMP-7 plasmid characterization and functional assessment. (a) Confirmation of recombinant pVIVO.IL-18.mMMP-7 by RE, Lane M- DNA ladder, lane 1- pVIVO.mMMP-7 clones digested with *Nco*1 and *Nhe*1; lane 2, pVIVO.IL-18.mMMP-7 clones digested with *Nco*1 and *Nhe*1 and lane 3, pVIVO.IL-18.mMMP-7 clones digested with *Xba*1, *Bst*11071, *Nhe*I and *Nco*1 REs. Desired fragments (744 bp for mMMP-7 and 594 bp for IL-18) were released; (b) Photomicrograph of HEK-293 cells showing IL-18 expression; (c) Photomicrograph of HEK-293 cells showing mMMP-7 expression; (d) DAPI positive cells indicating nuclei of HEK-293 cells; (e) Merged image of fields shown in (b), (c) and (d); (f) Western blot analysis of extracts from transfected cells expressing mMMP-7 protein (~28 kDa), and IL-18 protein (~22 kDa), Lane M- protein ladder, lane 1- transfected lysate.

recombinant pVIVO.mMMP-7 and pVIVO.IL-18 plasmid were digested with *Nco*1 and *Nhe*1. The mMMP-7 insert released from pVIVO.mMMP-7 was cloned into pVIVO.IL-18 recombinant plasmid. The presence of the mMMP-7 and IL-18 genes in recombinant pVIVO2.IL-18.mMMP-7 was confirmed by restriction digestion using *Nco*1, *Nhe*1 (for mMMP-7 gene) and *Xba*1 and *Bst*11071 (for IL-18 gene), respectively. The release of desired fragments of IL-18 gene (594 bp) and the mMMP-7 gene (744 bp) were checked by agarose gel electrophoresis, which confirmed the respective inserts in the recombinant plasmids (Fig. 3a).The transfection grade recombinant pVIVO2.IL-18.mMMP-7 was prepared and after transfection the expression of mMMP-7 and IL-18 gene at protein level was confirmed by immune-fluorescence assay (IFAT) by using r-cMMP-7 anti-rabbit polyclonal serum and IL-18 mice monoclonal antibodies used simultaneously, followed by TRITC labeled anti-rabbit and FITC labeled anti-mice secondary antibody and simple fluorescence microscopy after nuclear staining with DAPI, respectively. The results depicted both bright red and green fluorescence in pVIVO2.IL-18.mMMP-7 transfected HEK-293 cells (Figs 3b-e). The cell lysate from transfected cells showed desired bands of ~22 kDa and ~28 kDa from pVIVO2.IL-18.mMMP-7 recombinant plasmid in Western blot using mice IL-18 monoclonal and anti r-cMMP-7 polyclonal serum raised in rabbit (Fig. 3f).

In conclusion, in the present study, the mMMP-7 gene was cloned along with the molecular adjuvant IL-18 in the pVIVO2.mcs eukaryotic expression vector, which have two independent multiple cloning sites regulated by ubiquitous ferritin promoter. The ferritin levels were elevated in malignant tumour (Fan *et al.* 2013), presumably due to hypoxic condition of highly proliferative malignant cells (Dunn 1997). The gene construct pVIVO2.IL 18.mMMP7 might expected to be expressing both MMP-7 protein as well as IL-18 protein *in vivo*. Further, the molecular adjuvant IL-18 has been demonstrated to have potential as a biological adjuvant in murine tumour models (Osaki *et al*. 1998, Marshall *et al*. 2006). IL-18 improves both humoral and cellular immune responses that protect against cancer. The gene constructs pVIVO2.IL-18.mMMP7 proven for its expression of mMMP-7 protein and IL-18 protein can now be used as a xenogeneic DNA vaccine as therapeutic as well as prophylactic regimen against canine mammary tumours.

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