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Characterization of the canine *mda-7* gene, transcripts and expression patterns

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Abstract

Human melanoma differentiation associated gene-7/interleukin-24 (*mda-7/IL-24*) displays potent growth suppressing and cell killing activity against a wide variety of human and rodent cancer cells. In this study, we identified a canine ortholog of the human *mda-7/IL-24* gene located within a cluster of IL-10 family members on chromosome 7. The full-length mRNA sequence of canine *mda-7* was determined, which encodes a 186-amino acid protein that has 66% similarity to human MDA-7/IL-24. Canine MDA-7 is constitutively expressed in cultured normal canine epidermal keratinocytes (NCEKs), and its expression levels are increased after lipopolysaccharide stimulation. In cultured NCEKs, the canine *mda-7* pre-mRNA is differentially spliced, via exon skipping and alternate 5'-splice donor sites, to yield five splice variants (canine *mda-7sv1*, canine *mda-7sv2*, canine *mda-7sv3*, canine *mda-7sv4* and canine *mda-7sv5*) that encode four protein isoforms of the canine MDA-7 protein. These protein isoforms have a conserved N-terminus (signal peptide sequence) and are dissimilar in amino acid sequences at their C-terminus. Canine MDA-7 is not expressed in primary canine tumor samples, and most tumor derived cancer cell lines tested, like its human counterpart. Unlike human MDA-7/IL-24, canine *mda-7* mRNA is not expressed in unstimulated or lipopolysaccharide (LPS), concanavalin A (ConA) or phytohemagglutinin (PHA) stimulated canine peripheral blood mononuclear cells (PBMCs).

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Furthermore, *in-silico* analysis revealed that canonical canine MDA-7 has a potential 28 amino acid signal peptide sequence that can target it for active secretion. This data suggests that canine *mda-7* is indeed an ortholog of human *mda-7/IL-24*, its protein product has high amino acid similarity to human MDA-7/IL-24 protein and it may possess similar biological properties to human MDA-7/IL-24, but its expression pattern is more restricted than its human ortholog.

Introduction

Melanoma differentiation associated gene-7 (*mda-7*) was initially identified as a gene induced during induction of terminal differentiation in human melanoma cells (Jiang et al., 1995). Subsequent studies confirmed that *mda-7* had potent and selective growth suppressing and apoptosis-inducing properties in a broad spectrum of human cancers, without harming normal cells (Jiang et al., 1996; Dash et al., 2010a). Based on the structure and chromosome location, human *mda-7* is also referred to as interleukin-24 because it encodes a secreted protein with possible interleukin functions and a specific sequence common to members of the IL-10 gene family (Huang et al., 2001; Pestka et al., 2004). It is located in a cluster of IL-10 gene family members on human chromosome 1q31–1q32. A 2.0 kbps long mRNA is transcribed from the human *mda-7/IL-24* locus and encodes an evolutionarily conserved protein of 206 amino acids that belongs to the four-helix bundle family of cytokines (Jiang et al., 1996; Huang et al., 2001; Pestka et al., 2004; Zdanov, 2006). Human MDA-7/IL-24 protein has a 48 amino-acid long signal peptide, which is cleaved during its secretion (Jiang et al., 1996; Nielsen et al., 1997; Nakai, 2000). Normally, human MDA-7/IL-24 displays a highly restricted expression pattern. It is expressed at physiological levels by cells with immunological functions; including unstimulated or lipopolysaccharide (LPS) stimulated monocytes and anti-CD3 stimulated Th2 cells. Additionally, its expression is detected in melanocytes (Jiang et al., 1995; Huang et al., 2001; Allen et al., 2004). MDA-7/IL-24 expression can also be detected in ConA or LPS stimulated human peripheral blood mononuclear cells (PBMCs) (Huang et al., 2001; Caudell et al., 2002; Garn et al., 2002; Wolk et al., 2002; Poindexter et al., 2005). MDA-7/IL-24 expression is also observed in human monocytes during influenza A virus infection (Garn et al., 2002).

Human MDA-7/IL-24 is constitutively expressed at very high levels in normal human melanocytes (Jiang et al., 1995; Huang et al., 2001; Allen et al., 2004), and its expression decreases progressively during melanocytic transformation (Jiang et al., 1995; Madireddi et al., 2000; Ekmekcioglu et al., 2001; Ekmekcioglu et al., 2003; Allen et al., 2004). However, treatment with IFN- β and mezerein can induce its expression in metastatic human melanoma cells (Jiang et al., 1995; Dash et al., 2010a). Human *mda-7/IL-24* is not constitutively expressed in a wide variety of human tumor cells and promotes potent anti-cancer activity when reintroduced into primary and established human cancer cell lines of diverse lineages and in murine tumors (Jiang et al., 1995; Jiang et al., 1996; Fisher et al., 2007; Dash et al., 2010a). Despite much effort, little is known about the normal biological functions of MDA-7/IL-24. It may act as a Th1- inducing cytokine because it induces expression of Th1 cytokines such as IL-6, TNF- α and IFN- γ in human PBMCs (Poindexter et al., 2005). Human MDA-7/IL-24 may also function as an immunostimulatory cytokine with respect to

melanomas as it stimulates an immune response against melanoma associated antigens. The primary target tissue of MDA-7/IL-24, and its related cytokines, appears to be skin, where it controls the proliferation of dermal cells (Poindexter et al., 2010). Over the last decade, attention has been focused on its antitumor properties, which are observed when human MDA-7/IL-24 is expressed at high levels using either plasmid or replication deficient adenoviral vectors (Jiang et al., 1995; Jiang et al., 1996; Fisher et al., 2003; Fisher, 2005; Fisher et al., 2007; Dash et al., 2010a; Dent et al., 2010a; Dent et al., 2010b; Zhang et al., 2011). These studies led to a Phase I/II clinical trial in which administration of *mda-7/IL-24* using a replication incompetent adenovirus, Ad.*mda-7* (INGN 241), displayed significant clinical activity in patients with advanced cancers, including carcinomas and melanomas (Fisher et al., 2003; Cunningham et al., 2005; Tong et al., 2005; Fisher et al., 2007; Dash et al., 2010a).

Overexpression of human MDA-7/IL-24 causes growth arrest and induces apoptosis or toxic autophagy in a variety of tumor cells including malignant gliomas (Lebedeva et al., 2002; Su et al., 2003; Yacoub et al., 2008; Yacoub et al., 2010a; Hamed et al., 2013b), melanomas (Lebedeva et al., 2002; Chada et al., 2006; Sarkar et al., 2008) and carcinomas of breast (Su et al., 1998; Sarkar et al., 2005; Chada et al., 2006; Bhutia et al., 2013; Li et al., 2013), lung (Kawabe et al., 2002; Nishikawa et al., 2004; Inoue et al., 2007; Gupta et al., 2008), ovary (Leath et al., 2004; Gopalan et al., 2005; Gopalan et al., 2007; Yacoub et al., 2010b), kidney (Park et al., 2009; Eulitt et al., 2010; Park et al., 2011; Hamed et al., 2013a), colon (Emdad et al., 2007; Lebedeva et al., 2007; Azab B, In press) and prostate (Lebedeva et al., 2003; Sarkar et al., 2007; Dash et al., 2011; Azab B, In press). Importantly, none of the normal cells tested underwent apoptosis when exposed to MDA-7/IL-24, indicating that its effects are cancer-specific (Jiang et al., 1996; Fisher, 2005; Dash et al., 2010a; Dent et al., 2010a; Dent et al., 2010b). These growth suppressive and antitumor effects result from induction of apoptotic pathways and various other signaling pathways including down-regulation of anti-apoptotic genes, Bcl-2 and Bcl-xL, while causing up-regulation of pro-apoptotic genes such as Bax, Bad and Bak (Lebedeva et al., 2002; Sarkar et al., 2002; Fisher, 2005; Dash et al., 2010b). MDA-7/IL-24 inhibits angiogenesis, invasion and migration of tumor cells, thus making it a potent candidate for cancer treatment (Ramesh et al., 2003; Ramesh et al., 2004; Fisher, 2005; Ishikawa et al., 2005; Xie et al., 2008; Dash et al., 2010a; Wang et al., 2010).

Orthologs of human *mda-7/IL-24* have been identified in rats and mice (Soo et al., 1999; Schaefer et al., 2001). The murine ortholog of *mda-7/IL-24* is known as FISP (Interleukin 4 induced secreted protein) and is selectively expressed by Th2 cells. Recent studies suggest that unlike human *mda-7/IL-24*, FISP does not have antitumor properties (Nagakawa et al., 2012). The rat ortholog of *mda-7/IL-24* is known as c49a/Mob-5, and expression of this gene is increased during wound healing, primarily in fibroblast-like cells on wound edges and has also been suggested to be a ras-inducible gene with cancer growth promoting properties (Soo et al., 1999; Paoloni and Khanna, 2008). The genomic structure including exon/intron boundaries is conserved between these species and human *mda-7*. Given that dogs are considered to be useful intermediate animal models of many human cancers (Hansen and Khanna, 2004; Paoloni and Khanna, 2007; Paoloni and Khanna, 2008) knowledge of the canine *mda-7* gene and its functions would be extremely relevant.

However, no studies to either elucidate the biological properties or genomic structure of canine *mda-7* have been published to date.

In the present study, we sought to identify the canine ortholog of *mda-7* and examine its genomic structure and expression pattern. Our results demonstrate that the canine *mda-7* locus has a similar genomic structure when compared to human *mda-7*. However, canine *mda-7* has unique genomic elements, including novel exon sequences not identified in the human or murine genes, is restricted in its expression pattern and appears to undergo extensive splicing to generate five splice variants that encode four different protein isoforms with novel motifs, which could potentially have diverse biological properties.

Results

Genomic structure of canine *mda-7*

To identify the canine *mda-7* locus, we used the 552-bp long predicted canine mRNA sequence (XM_846427) from the NCBI database and BLASTed it against the canine genome. The putative canine *mda-7* was localized to canine chromosome 7 in a cluster of IL-10 related genes including canine IL-10, IL-19 and IL-20. To further characterize the canine *mda-7* locus, the exonic/intronic structures as well as promoter sequences were examined. *In-silico* analysis (Spidey-NCBI) revealed that the predicted canine *mda-7* mRNA sequence was composed of five exons. A TATA element was identified using FPROM software (<http://www.softberry.com/berry.phtml?topic=fprom&group=programs&subgroup=promoter>), which was located 1389-bps upstream from the first nucleotide of the predicted canine *mda-7* mRNA sequence. The sequences in and around the promoter were highly conserved when compared to the human *mda-7*/IL-24 locus. A transcription start site was also predicted at 1359-bps upstream of the first nucleotide of the predicted mRNA sequence, however *in silico* analysis failed to indicate the presence of exonic sequences in this region and showed very low sequence similarities with the identified human *mda-7*/IL-24 sequence.

To determine if the predicted promoter and transcription start site were used for transcription of canine *mda-7*, the 5'- and 3'-mRNA sequences were identified using rapid amplification of cDNA ends (RACE) from total RNA isolated from cultured normal canine epidermal keratinocytes (NCEKs). These amplifications were anchored in areas that were conserved between human *mda-7*/IL-24 and the predicted dog sequence. Initial experiments identified two mRNAs of differing lengths (963-bps and 1057-bps) that were named canine *mda-7*sv1 and *mda-7*sv2, respectively.

Alternative splicing is an important mechanism to increase the functional diversity of the eukaryotic transcriptome as it results in the expression of new protein isoforms. Splice variants have been characterized for both human *mda-7*/IL-24 as well as its murine ortholog FISP. To determine if there were any other splice variants derived from the canine *mda-7* gene, two PCR primer pairs were designed and used sequentially for nested PCR. The outer primer pair was complimentary to the sequences of the first and last exon. The inner primer pair was designed to amplify the open reading frame of the canine *mda-7* mRNA and was internal to the outer primer pair. These two primer pairs were used sequentially to perform a

nested PCR. The PCR products were cloned into a plasmid vector (pCDNA3.1+/Hygro) and sequenced, which resulted in the identification of the original two splice variants and three additional splice variants (Fig. 1).

Sequence data from the five splice variants was used to elucidate the genomic structure of canine *mda-7* (Fig. 2). 5' RACE indicated that the full-length mRNA of canine *mda-7* uses the predicted transcriptional start site for transcription initiation. When the canine *mda-7* mRNA sequences were aligned with the canine genome, the *mda-7* locus was determined to consist of eight exons that varied in size from 62- to 216-bps. The size of the introns varied from 47- to 1159-bps. All of the intron/exon boundaries had consensus-splicing signals (GT/AG) except the donor and acceptor sites in intron 7 (GC/TG) (Fig. 2). The first and fifth exons were not recognized by gene prediction software and have not been reported for the *mda-7* gene in any other species. The open reading frame started in the second exon and terminated in exon 7 (Fig. 2). Genomic analysis confirmed the presence of two polyadenylation signal motifs at the 3'-end of the mRNA.

A PCR primer set was designed to amplify the 6.0-kbps containing the canine *mda-7* locus. Genomic DNA isolated from canine (Beagle) muscle samples was used as template for PCR. In addition, genomic DNA from lymphocytes of an American grey wolf (*Canis lupus*) was also used as template for this PCR. A 6.0-kbp product from both templates was identified, gel-purified, cloned into a plasmid vector (pGEMT-easy) and partially sequenced. The sequences generated had 100 % similarity to each other as well as to the published canine genome (Boxer), thus confirming that this locus is conserved in different dog breeds as well as in American grey wolf (data not shown).

Canine *mda-7* pre-mRNA uses alternative splicing to generate five splice variants

Alternative splice acceptor sites (exons 2 and 3) and exon skipping (exons 4 and 5) are responsible for the five splice variants identified for canine *mda-7* (Fig. 3). Canine *mda-7*sv1 results from the use of the common downstream 5'-acceptor sites within the second and third exons and the skipping of exon 5. Canine *mda-7*sv2 is identical to *mda-7*sv1 with the exception that it uses the upstream splice acceptor site of exon 2, adding 93 bases to the mRNA. However, both canine *mda-7*sv1 and *mda-7*sv2 encode the same open reading frame (ORF), as the start codon is located in the 3'-end of the second exon. The translated product of this ORF has the highest level of similarity to human MDA-7/IL-24 protein. The third splice variant, canine *mda-7*sv3 results when exons 4 (63 bps) and 5 are skipped. Deletion of exon 4 does not cause a frameshift in the reading frame relative to *mda-7*sv1/sv2, but results in deletion of 21 amino acids when compared to the first two splice variants. The fourth and fifth splice variants, canine *mda-7*sv4 and *mda-7*sv5 contain a novel fifth exon that is only present in these two variants. The fifth splice variant, *mda-7*sv5, also utilizes a 5'-upstream alternate acceptor site for the third exon that adds 153-bps to this splice variant. This added sequence does not cause a frameshift, however, it results in the addition of 51 amino acids to the reading frame of canine *mda-7*sv5. Addition of the fifth exon (62 nucleotides) in *mda-7*sv4 and sv5 causes a frameshift, so the protein isoforms encoded by canine *mda-7*sv4 and *mda-7*sv5 are dissimilar at the C-terminus from the protein isoforms encoded by the

other three preceding splice variants. The *mda-7sv4* and *sv5* splice variants reading frames use a stop codon that is 29 bases upstream of the stop codon for *sv1-3* in exon 7.

Canine *mda-7* expression is restricted to normal canine epidermal keratinocytes (NCEKs)

A TaqMan based quantitative PCR assay was designed to detect and elucidate the expression pattern of canine *mda-7*. A canine *mda-7* specific primer pair and TaqMan probe were designed to amplify the 3'-region of the mRNA in order to detect all of the splice variants (Fig. 4). Canine *mda-7* mRNA was amplified by rt-PCR and cloned into a plasmid vector (pGEMT-easy). Recombinant plasmid was serially diluted (10-fold), amplified and used to construct a standard curve. The designed TaqMan assay had a high PCR efficiency (100%), correlation coefficient (99.9) and was successfully used to detect a minimum of 30 copies of the recombinant plasmid. This assay was then used to evaluate canine *mda-7* expression in different dog tissues (Table 1). Canine *mda-7* mRNA was expressed at very high levels in cultured normal canine epidermal keratinocytes (NCEKs) (225.72 ± 16 copies/ng of total RNA) and its expression increased significantly after LPS stimulation (329.54 ± 31.36 copies/ng RNA). Expression of canine *mda-7* mRNA was not detected in unstimulated canine PBMCs, PBMCs stimulated with PHA, ConA, LPS or anti-CD3 antibody, thymus, lymph node or spleen (Table 1). Stimulation of canine PBMCs by LPS, PHA and anti-CD3 was confirmed by quantifying expression levels of canine interleukin-6. The relative expression levels of canine IL-6 were statistically significantly increased (approximately 12 to 225 fold) in LPS, PHA and anti-CD3 antibody stimulated canine PBMCs, when compared to unstimulated PBMCs (data not shown). Cancer cell lines derived from various canine tumors were also evaluated. These cell lines included canine mammary tumors (CMT28, CMT27 and CMT12), melanoma (CML10) and lymphoma (OSW and 17-71). Five of the six cell lines, namely CMT28, CMT27, CML10, OSW and 17-71 did not express canine *mda-7*. However, CMT12 expressed canine *mda-7* endogenously at very high levels (378.23 ± 11.23 copies/ng RNA) (Table 1). In addition, RNAs isolated from primary canine tumor samples (splenic and hepatic hemangiosarcoma, mucinous adenocarcinoma, follicular thyroid carcinoma, hemangiopericytoma, squamous cell carcinoma, seven B cell lymphomas and two T cell lymphomas) were negative for canine *mda-7* expression (Table 1).

Canine *mda-7 sv1* is the predominant splice variant expressed by canine keratinocytes

The relative contribution of each of the five splice variants to the total amount of *mda-7* expression was evaluated with TaqMan PCR assays that were specific for each transcript. TaqMan probes were designed to be complementary to sequences that were either present only in specific transcripts (canine *mda-7sv2*, *sv4* and *sv5*) or to an exonic junction that was not present in the other transcripts (canine *mda-7sv3*) (Fig. 4). Copy number of canine *mda-7sv1* was obtained by subtracting the cumulative means of the remaining transcripts from the total copy number of canine *mda-7* mRNA, as a unique probe could not be identified for this variant. Likewise, because *mda-7sv4* contained no unique sequences when compared to *mda-7sv5*, but *mda-7sv5* does contain a unique sequence and could be quantitated, the amount of *mda-7sv4* was calculated from the total amount of *mda-7sv4* and *mda-7sv5* minus the amount of *mda-7sv5*. For absolute quantification, each transcript was cloned into a plasmid vector (pGEMT easy). These recombinant plasmids were used to

develop a standard curve for each TaqMan probe. All of the TaqMan assays had a high PCR efficiency in addition to having a correlation coefficient in the range of 0.965 to 0.999 (Table 2). When NCEK cells were assayed, *mda-7sv1* was the predominant splice variant expressed (128.74/ng RNA), sv2 and sv5 were expressed at intermediate levels (48.248 and 45.092 copies/ng RNA, respectively) and sv3 and sv4 were expressed at the lowest levels (1.104 and 2.528 copies/ng RNA respectively) (Table 2). When primary canine keratinocytes were stimulated with LPS, it resulted in a 50% increase in the copy number of *mda-7sv1* and a two-fold increase in the copy number of *mda-7sv2* with no alteration in the levels of the other splice variants ($p < 0.0001$, Table 2). CMT12 cells expressed *mda-7sv1* and sv2 at very high levels (258.22 copies/ng RNA and 72.33 copies/ng RNA, respectively), *mda-7sv5* at a relatively low level (22.42 copies/ng RNA), *mda-7sv3* at a very low level (4.78 copies/ng RNA) and the expression of *mda-7sv4* transcripts could not be detected (Table 2).

Predicted canonical canine MDA-7 protein has conserved elements

The five identified splice variants of canine *mda-7* encode four different protein isoforms. These protein isoforms differ in length as well as in amino acid sequences. The canine MDA-7 isoform encoded by canine *mda-7sv1* and *mda-7sv2* is a 183 amino acid protein with a predicted molecular weight of 18 KDa. Of the canine MDA-7 isoforms, this isoform has the highest amino acid similarity to the human MDA-7/IL-24 protein and was therefore designated as the canonical canine MDA-7 protein (Fig. 5). The isoforms encoded by *mda-7sv3*, sv4 and sv5 have predicted amino acid lengths of 162, 194 and 245 and molecular weights of 18.52 KDa, 22.0 KDa and 27.22 KDa, respectively. The canonical protein isoform has higher similarity to human MDA-7/IL-24 protein than it does to the rat (c49a) and murine (FISP) orthologs (Fig. 5, Table 3). *In-silico* analysis revealed that all of the canine MDA-7 protein isoforms have a putative 28 amino acid signal peptide and a predicted cleavage site that lies in between the 28th and 29th amino acid (Fig. 6). This is not conserved when compared to the cleavage site of human MDA-7/IL-24 (Fig. 7). All of the canine MDA-7 isoforms have conserved N-glycosylation sites and an IL-10 signature sequence (Fig. 7). However, the isoforms derived from splice variants sv4 and sv5 have a frame shifting insertion and use a different stop codon than the one used by the other splice variants, making these two protein isoforms dissimilar at their C-terminus from other isoforms (Fig. 7).

Discussion

MDA-7/IL-24 is an important gene that belongs to the IL-10 family of cytokines due to its chromosomal localization, sequence homology and functional properties (Huang et al., 2001; Pestka et al., 2004; Dash et al., 2010a). In the present study, we elucidated the genomic structure and expression profile of the canine ortholog of the human *mda-7/IL-24* gene. As expected, much of the structure and sequence is conserved, however, several novel features were identified for the canine *mda-7* locus. The canine *mda-7* gene contains eight exons compared to the seven identified for human *mda-7/IL-24* and consists of approximately 6.0-kbps, located on canine chromosome 7, within a cluster of IL-10 family members. The *mda-7* locus is conserved between different breeds of dogs as well as in their

close relative, the American grey wolf (*Canis lupus*). The canine *mda-7* promoter also contains a TATA element that has conserved sequences when compared to the promoter sequence of human *mda-7/IL-24*. Human *mda-7/IL-24* has a very long 3'-untranslated region (3'-UTR) that has three poly-A signal sequences that can be used for alternative polyadenylation. However, the third poly-A signal is used exclusively to add the poly-A tail in human *mda-7/IL-24* (Huang et al., 2001). Analysis of the genomic sequence at the canine *mda-7* locus identified two polyadenylation signals (Fig. 2). However, the canine *mda-7* mRNAs have a very short 3'-UTR, unlike human *mda-7/IL-24*. This is due to the use of the first poly-A signal sequence to add the poly-A tail rather than the third poly-A signal. The 3'-UTR of the human *mda-7/IL-24* mRNA also has three destabilization domains or AU rich elements (ARE) (3'-UTR-AUUUA). These AU rich elements interact with ARE binding proteins and target human *mda-7/IL-24* mRNA for degradation. This is one of the mechanisms used to regulate the expression levels of human MDA-7/IL-24 (Otkjaer et al., 2010). Although, canine *mda-7* mRNA is considerably shorter than human *mda-7/IL-24*, it still contains two of these AU rich elements, which suggests that the expression level of canine *mda-7* may also be regulated by a similar mechanism.

Canine *mda-7* is constitutively expressed by cultured normal canine epidermal keratinocytes (NCEKs), with an increase in expression after LPS stimulation. However, its expression is not detectable in RNA isolated from whole dog skin samples. Similarly, human *mda-7/IL-24* is expressed in cultured normal human epidermal keratinocytes, and its expression is undetectable in whole skin samples (Poindexter et al., 2010). This data suggest that the culturing of normal epidermal keratinocytes (both NHEKs and NCEKs) may induce MDA-7 expression. The rat ortholog of *mda-7/IL-24*, *c49a*, is expressed in fibroblast-like cells during wound repair. Human MDA-7/IL-24 is also expressed by epidermal keratinocytes during wound healing (Soo et al., 1999), and is hypothesized to play important role in maintaining the normal architecture of skin (He and Liang, 2010; Poindexter et al., 2010). It inhibits the proliferation of epidermal keratinocytes, inducing them to return to their differentiated, non-proliferative state after completion of wound healing (Poindexter et al., 2010). While it has yet to be demonstrated, canine MDA-7 may also play a similar role in wound repair in canine skin.

Human MDA-7/IL-24 protein is expressed by monocytes, subsets of T cells and in LPS and PHA stimulated PBMCs. However, canine *mda-7* expression was undetectable in unstimulated PBMCs as well as in LPS, PHA, ConA or Anti-CD3 stimulated PBMCs. The lack of expression in these canine cells is a significant difference between canine *mda-7* and human *mda-7/IL-24* and may have broad implications with respect to the impact, or lack thereof, of canine *mda-7* on conditions such as malignant cell growth, where the human ortholog has well-defined activity. Canine *mda-7* mRNA was not detected in primary canine tumor samples and in most of the tumor-derived canine cancer cell lines tested (OSW, 17-71, CML10, CMT28 and CMT27). However, CMT12 cells express canine *mda-7* mRNA at a very high level with no apparent effect on their growth. The mechanism that results in expression of canine *mda-7* in these cells is currently unknown.

Alternative splicing of pre-mRNA is an important mechanism to increase protein complexity in eukaryotes. In this study, we identified five different splice variants (sv1-5) encoding four

protein isoforms of canine MDA-7 that result either due to the skipping of exons (exon 4 and 5) or from the use of alternate splice acceptor sites (exon 2 and 3). All of the splice variants are constitutively expressed in cultured NCEKs with sv1 (57.03%), sv2 (21.37%) and sv5 (19.97%) being the predominant transcripts. LPS stimulation increases the expression of canine *mda-7*sv1 and sv2 in cultured NCEKs. However, it does not affect the expression levels of other transcripts. High-level expression of canine *mda-7*sv1 and sv2, and their change in expression due to LPS stimulation suggests that these two splice variants are the primary transcripts. Human *mda-7*/IL-24 pre-mRNA has also been reported to undergo alternative splicing to yield various splice variants (Allen et al., 2004; Allen et al., 2005). These splice variants either lack the third exon alone or third and fifth exon (canine exon 6) (Allen et al., 2004). The splice variant lacking the third and fifth exon was renamed as *mda-7s*, and its expression was found to inversely correlate to the stage of melanoma (expression decreases as the melanoma progresses). Human *mda-7s* encodes a truncated protein that only has 14 amino acids similar to human MDA-7/IL-24. However, this truncated protein can still co-precipitate and prevent secretion of human MDA-7/IL-24 protein (Allen et al., 2004; Allen et al., 2005). Similarly, a splice variant (FISP-sp) has also been reported for the murine ortholog of *mda-7*, FISP. FISP-sp lacks 29 amino acids from the 5'-end of the fourth exon. It can heterodimerize with FISP and blocks its secretion, inhibiting the apoptotic effects of FISP (Allen et al., 2004; Allen et al., 2005; Sahoo et al., 2008). Thus, it is possible that the protein isoforms encoded by different splice variants of canine *mda-7* may interact with each other to control the expression, activity and secretion of the primary splice variants, canine *mda-7*sv1 and sv2. This possibility requires experimental validation.

The protein isoforms encoded by splice variants sv1/2 and sv3 have high identity (75%) to human MDA-7/IL-24 at the amino acid level. All of the canine MDA-7 protein isoforms have a conserved IL-10 signature sequence (Fig. 7). Human MDA-7/IL-24 is a heavily glycosylated protein with three consensus N-linked glycosylation sites (Sauane et al., 2006; Fuson et al., 2009). Glycosylation at these sites is necessary for human MDA-7/IL-24 solubility and bioavailability, but is not necessary for anti-cancer apoptosis-inducing ability (Sauane et al., 2006). The canine MDA-7 isoforms have only one of these consensus N-linked glycosylation sites (Asn-85). The two other N-linked glycosylation sites (Asn-99 and Asn-126) are not present in any canine MDA-7 isoform (Fig. 7). Human MDA-7/IL-24 also possesses disulfide bond between the 59th and 106th cysteines (Fig. 7). These disulfide bonds are required for its secretion and activity (Fuson et al., 2009). These two cysteine residues are also conserved among the different isoforms of the canine MDA-7. Human MDA-7/IL-24 has a 49 amino acid signal peptide, which can target it for secretion. In this study, we have shown that canine MDA-7 has a predicted 28 amino acid signal peptide. Although the signal peptide sequence of canine MDA-7 is shorter than the human MDA-7 signal sequence, it appears to be large enough to still direct the canine MDA-7 protein for secretion. This possibility is being tested experimentally.

Thus, we conclude that the *mda-7* locus is evolutionarily conserved in dogs, but that it has a more limited range of tissue expression than its human counterpart. Canine *mda-7* pre-mRNA undergoes extensive alternative splicing to generate at least five splice variants,

which encode four protein isoforms. Canine MDA-7 has a potential signal peptide and conserved IL-10 signature sequence, thus confirming it to be a member of the IL-10 family of cytokines. Due to its high amino acid sequence similarity with human MDA-7/IL-24, we predict that one or more of the splice variants of canine MDA-7 may also have antitumor properties, which might prove useful in the design new cancer therapeutics. This hypothesis is currently being tested.

Material and methods

Isolation and culturing of normal canine epidermal keratinocytes (NCEKs)

Canine skin samples were collected in CnT-09 media (CELLnTEC advance cell system) supplemented with 10% supplement A (CELLnTEC), 400 nM L-glutamine, penicillin (500 I.U./ml) and streptomycin (500 µg/ml, complete CnT-09). Excess dermal tissue from the skin samples was removed under sterile conditions. The skin was cut into 0.5-cm square pieces. Cut skin samples were kept at 4° C in complete CnT-09 media supplemented with 10 mg/ml Dispase II (Roche applied science) for 21 hours. After that, the epidermis was removed from the dermis, washed in PBS (Mediatech, Inc.) and placed in TrypLE express (Invitrogen) for 30 min, at room temperature. The epidermis was rubbed with forceps repeatedly during this time to release the cells. After incubation, the cell suspension was filtered through a 0.70 µm filter (Beckson Dickinson Labware, France). An equal amount of complete CnT-09 media was added to the cell suspension. Cells were centrifuged at 300 x g for 5 min. The cell pellet was resuspended in complete CnT-09 media, 1.5×10^6 cells were seeded into a 75 cm² flask and maintained at 37°C and 5% CO₂. NCEKs were stimulated with lipopolysaccharides (100 ng/ml) for 24 hours.

RNA ligase mediated - rapid amplification of cDNA ends (RLM-RACE)

5'-RNA ligase mediated RACE (RLM-RACE) and 3'-RACE were performed according to the manufacturer's directions (First choice[®] RLM-RACE, Ambion, Inc.). Briefly, total RNA was isolated from normal canine epidermal keratinocytes (NCEKs) with TRI Reagent (MRC, Inc.). 1 µg of this RNA was treated with Calf Intestine Alkaline Phosphatase (CIP) to remove the 5'-phosphate group from rRNAs, tRNAs and broken mRNAs. CIP treated RNA was purified and by phenol:chloroform extraction and isopropanol precipitation. The RNA pellet was then dissolved in nuclease free water. After this, RNA was treated with Tobacco Acid Pyrophosphatase (TAP) to remove the 5'-cap from full-length mRNAs, leaving a 5'-monophosphate. CIP/TAP treated RNA was then ligated to a 45-bp long RNA 5'-adapter using T4 RNA ligase. Adapter-ligated RNA was reverse transcribed into first-strand cDNA using random-primers for 5'-RACE. An outer 5'-RLM-RACE PCR reaction was carried out under standard procedures using 5'-RACE outer and outer *mda-7*-specific antisense primers. 2 µl of the outer PCR reaction was used as template for inner PCR reaction using 5'-RACE inner and inner *mda-7*-specific antisense primers (supplemental Table S1). For 3'-RACE, RNA was reverse transcribed with the 3'-RACE adapter acting as a primer. An outer 3'-RACE PCR reaction was run using the 3'-RACE outer and outer canine *mda-7* specific sense primers under optimal PCR conditions. 2 µl of outer PCR reaction was used as template for inner PCR reaction using the 3'-RACE inner and inner *mda-7*- specific sense

primers under optimal PCR conditions (supplemental Table 1). Amplified products were cloned into pGEMT-easy vector and sequenced using T7 and SP6 primers.

Isolation of canine PBMCs

Canine PBMCs were purified from healthy dog blood by gradient density using Histopaque™ (1.077 g/mL, Sigma-Aldrich, Inc.) collected in EDTA coated blood collection tubes. Isolated canine PBMCs (1×10^6 cells/ml) were cultured in Roswell Park Memorial Institute medium-1640 (RPMI-1640, Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS), penicillin (100 I.U./ml) and streptomycin (100 µg/ml) and maintained at 37°C and 5% CO₂. Canine PBMCs were stimulated *in-vitro* with 25 ng, 100 ng or 200 ng/ml LPS. Canine PBMCs were also stimulated *in-vitro* with phytohemagglutinin (5 µg/ml) and Concanavalin A (25 µg/ml) for 24, 48 and 72 hours.

Culture of canine cancer cell lines

Canine cancer cell lines including CMT28, CMT12, CMT27, OSW, 17-71 and CML-10 were cultured in Dulbecco's Modified Eagle Media (DMEM, Corning, Inc.) supplemented with 10% fetal bovine serum (FBS), penicillin (100 I.U./ml) and streptomycin (100 µg/ml) and maintained at 37°C and 5% CO₂ (Wolfe et al., 1986). When the cells reached 80% confluence, total RNA was isolated using TRI REAGENT RNA isolation kit (Molecular Research Center, Inc.) as per manufacturer's instructions.

Amplification of canine MDA-7 locus

Genomic DNA was isolated from the cultured normal canine epidermal keratinocytes (NCEKs) using Genomic DNA mini kit (IBI scientific) as per manufacturer's instructions. Genomic DNA was also purified from the PBMCs isolated from whole blood of American Grey wolf. 100 ng of genomic DNA was used in 50 µl reaction to amplify canine *mda-7* locus by PCR using LA *Taq* (TAKARA, Inc.) under optimal PCR conditions that included preheating to 94°C for 1 min followed by 30 cycles of 98°C for 5 sec and 68°C for 7 min and final extension at 72°C for 10 min. A 5.5 kbp PCR product was successfully amplified, purified, cloned into pGEMT easy vector system (Promega, Inc.) and sequenced using multiple primer sets (Table 3).

Quantitative PCR

Different splice variants were amplified using nested PCR and cloned into pGEMT easy and pCDNA3.1+/Hygro (Invitrogen, Inc.) vectors. Absolute copy numbers of all the splice variants were calculated using quantitative polymerase chain reaction employing TaqMan® Probes. Primers and TaqMan® probes (Supplemental Table S2) were designed to amplify and differentiate among splice variants, respectively. Recombinant plasmids containing various splice variants were diluted according to the copy number to generate a standard curve. Total RNA was isolated from NCEKs, canine PBMCs, LPS stimulated canine PMBCs and NECKs. One microgram of total RNA was reverse transcribed using qScript cDNA synthesis kit (Quanta Biosciences). 4 µl of this reaction was used to quantify the copy number of splice variants using TaqMan Fast Universal PCR Master Mix (Applied

Biosystem) under optimal PCR conditions that included preheating to 95° C for 5 min followed by 40 cycles of 95° C for 10 sec and 62° C for 20 sec.

Relative quantification of canine IL-6 mRNA

Primers were also designed to amplify canine IL-6, L37 and eukaryotic elongation factor 2 (eEF2) (housekeeping genes) mRNA (Table 2). Total RNA was isolated from stimulated (lipopolysaccharides (LPS), phytohaemagglutinin (PHA) and anti-CD3 antibody) and unstimulated canine PBMCs as previously described (Ref?). The isolated RNA was reverse transcribed using qScript cDNA synthesis kit (Quanta Biosciences). 2 µl of the 5-fold diluted cDNA (20 µl of rt-PCR reaction + 80 µl of nuclease free water) was used for relative quantification of canine interleukin-6 (IL-6) using Sso EvaGreen superfast supermix (Biorad, Inc.) under optimal PCR conditions that included preheating to 95° C for 30 sec followed by 40 cycles of 95° C for 5 sec and 62° C for 3 sec and melt curve analysis. The amplified PCR products were also purified and sequenced to confirm the specificity of the primer sets. The expression levels of canine IL-6 were normalized to eEF2, L37, and to the expression levels of canine IL-6 in the unstimulated canine PBMCs.

In-silico analysis

The predicted canine *mda-7* gene sequence was obtained from the National Center for Biotechnology Information (NCBI) sequence database (Accession: XM_846427). This predicted sequence was BLASTed against the canine genome, and genomic sequence representing the canine *mda-7* locus (NW_876323.1). The canine *mda-7* gene locus was analyzed *in-silico* with different software packages including Vector NTI advance 10 (Invitrogen), Spidey (NCBI), Genescan (MIT) and FGANESH (Softberry, Inc.). FPROM (Softberry, Inc.) software was used to predict the promoter for canine *mda-7*. SignalP (3.0) software was used to predict the signal peptide in canine MDA-7 protein (Nielsen et al., 1997).

Statistical considerations and analysis

Equal amounts of cDNA were used to compare the expression levels of splice variants. All of the TaqMan PCRs were run in triplicate. A two sample t-test was used to compare the expression levels of canine *mda-7* between stimulated and unstimulated NCEKs at 95% confidence level, and a p-value of <0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MDA-7/IL-24	Melanoma differentiation associated gene-7/Interleukin-24
NCEKs	Normal canine epidermal keratinocytes
NHEKs	Normal human epidermal keratinocytes
Canine <i>mda-7</i>sv1, sv2, sv3, sv4 and sv5	Canine <i>mda-7</i> splice variant1, splice variant 2, splice variant 3, splice variant 4 and splice variant 5, respectively
PBMC	peripheral blood mononuclear cells
PHA	Phytohemagglutinin
ConA	Concanavalin A
LPS	Lipopolysaccharide

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Highlights

1. The canine *mda-7* locus contains 8 exons and 7 introns. Exons 1 and 2 differ significantly from other published *mda-7* sequences while exons 3–8 are highly conserved.
2. Canine *mda-7* mRNA undergoes extensive alternative splicing to produce 5 splice variants that encode four protein isoforms.
3. Canonical MDA-7 protein is constitutively expressed in normal canine epidermal keratinocytes (NCEKs). It has significant amino-acid similarity to human MDA-7, and possesses a 28 amino acid predicted signal peptide.

Figure 1.

Identification of splice variants of canine *mda-7*. Canine *mda-7* splice variants were amplified by nested PCR and cloned into the pCDNA3.1+/Hygro plasmid vector. Recombinant clones were digested with *HindIII* and *XhoI* restriction enzymes to release the canine *mda-7* inserts for *mda-7sv3* (lane 2), *mda-7sv1* (lane 3), *mda-7sv2* (lane 4), *mda-7sv4* (lane 5) and *mda-7sv5* (lane 6). Empty vector (pCDNA3.1+/Hygro) was also digested with *HindIII* and *XhoI* as a control (lane 1). The samples were visualized by agarose gel electrophoresis. Lane M1 and M2 are 100 bp and 1 kbp DNA ladders respectively.

Figure 2.

Genomic structure of canine *mda-7*. The genomic structure of canine *mda-7* was determined by comparing mRNA sequence with genomic sequences. The canine *mda-7* locus is composed of eight exons and seven introns. The TATA box and transcription start site are labeled. Splice donor and acceptor sites are shown in bold. Alternative splice donor sites are located within exons 2 and 3. The start codon, stop codons and Poly-A signals are shown in boxes. The size of each intron is given in base pairs. Exon numbers are based on relative position and not on the corresponding human exon numbering. AU-rich elements (ARE) are underlined.

Figure 3. Alternative splicing at the canine *mda-7* locus results in production of five splice variants. Splice variants range in size from 901- to 1179-bps. 5' alternate acceptor sites for the second and third exon are used by sv2 and sv5. The fourth exon is skipped in the third splice variant, while exon five is only present in the sv4 and sv5 transcripts. Lines below the introns denote commonly used splices, while the lines above the introns denote unique splices. Curved arrow indicates the position of start codon.

Figure 4.

Quantitative RT-PCR probe locations. TaqMan probes were designed against transcript specific sequences to detect sv2, sv3, sv4 and sv5. The copy number of canine *mda-7*sv1 was deduced by subtracting the sum of the copy number of sv2, sv3, sv4 and sv5 from the total copy number obtained from all transcripts PCR. Similarly, the copy number of sv4 was determined by subtracting copy number of sv5 transcript from copy number of sv4 transcript. Arrows indicate TaqMan PCR primers and bars indicate TaqMan probe positions.

Figure 5.

Protein similarities between in human, canine and murine MDA-7. The *in-silico* predicted canine MDA-7 amino acid sequence was aligned with the amino acid sequences of c49a (rat), FISP (mouse) and human MDA-7/IL-24 using vector NTI 10.0. A maximum likelihood phylogenetic tree was drawn showing that canonical canine MDA-7 has high sequence similarity to human MDA-7/IL-24 protein.

Figure 6.

Prediction of a signal peptide sequence for canine MDA-7 protein. Full-length mRNA sequence of canine *mda-7sv1* was translated *in-silico* using vector NTI 10.0 software. The *in-silico* translated protein sequence representing the canonical canine MDA-7 protein was then analyzed by signal peptide predicting software (signalP3.0 NN). The canonical MDA-7 protein has a 28 amino acid long signal peptide with a predicted cleavage site between the 28th and 29th amino acids.

Figure 7.

Alignment of canine MDA-7 protein isoforms with Human MDA-7/IL-24. Canine MDA-7 isoforms have a 28 amino-acid long predicted signal peptide sequence. The interleukin-10 signature motif is shown within the box. (*Conserved N-glycosylation site, ^Conserved cysteine amino acid).

Table 1

Quantification of mRNA levels of canine *mda-7* by TaqMan assay in various canine tissues.

Canine tissue type	Canine <i>mda-7</i> expression profile copies/ng of total RNA
Normal tissues	
Canine PBMCs	
Unstimulated PBMCs	Undetectable
ConA stimulated PBMCs	Undetectable
PHA stimulated PBMCs	Undetectable
LPS stimulated PBMCs (100ng/ml of media)	Undetectable
Anti-CD3 stimulated PBMCs	Undetectable
Spleen	Undetectable
Skin	Undetectable
Lymphnode	Undetectable
Thymus	Undetectable
Cultured normal canine epidermal keratinocytes	
Unstimulated NCEKs	225.72 $\pm$ 5.58
Lipopolysaccharides stimulated NCEKs	318.5 $\pm$ 3.35
Primary canine tumor samples and tumor derived cell lines	
Canine mammary tumor cell lines	
CMT-28	Undetectable
CMT-27	Undetectable
CMT-12	378.33 $\pm$ 9.12
Canine lymphoma cell lines	
OSW	Undetectable
17-71	Undetectable
Canine melanoma cell line (CML-10)	Undetectable
Primary canine tumor samples	Undetectable
Spleen hemangiosarcoma	Undetectable
Liver hemangiosarcoma	Undetectable
Squamous cell carcinoma	Undetectable
Mucinous adenocarcinoma	Undetectable
Follicular thyroid carcinoma	Undetectable
Hemangiopericytoma	Undetectable
B cell lymphoma (seven individual samples)	
T Cell lymphoma (two individual samples)	Undetectable

Quantification of mRNA levels of canine *mda-7* by TaqMan assay in various canine tissues. A primer set was designed to amplify sequences representing the sixth, seventh and eighth exons of canine *mda-7* mRNA. Amplified products were cloned into pGEMT easy vector and sequenced.

This recombinant plasmid was serially diluted to generate a 10-fold dilution series based on its copy number. This serially diluted recombinant plasmid was used as template from TaqMan PCR to generate a standard curve. Total RNA was isolated from the normal canine tissues and tumor samples reverse transcribed, which was then used for quantifying the canine *mda-7* mRNA copy number.

Table 2
Quantitative analysis of relative expression levels of different splice variants of canine *mda-7*

Transcript name	Number of amino acids in encoded protein	TaqMan Assay Efficiency/Correlation coefficient	Copy Number/ng of Total RNA in NCEK	Copy Number/ng of total RNA after LPS Stimulation (NCEK)	Copy number/ng of total RNA in CMT12 cells
Canine <i>mda-7</i> sv1	183	NA	128.74 ± 10.309	189.202 ± 9.08	258.22 ± 6.15
Canine <i>mda-7</i> sv2	183	100.0/0.998	48.248 ± 9.489	94.668 ± 6.39	72.33 ± 7.89
Canine <i>mda-7</i> sv3	162	96.2/0.967	1.104	Undetectable	4.78 ± 0.79
Canine <i>mda-7</i> sv4	194	89.0/0.965	2.528	Undetectable	Undetectable
Canine <i>mda-7</i> sv5	245	96.5/0.982	45.092 ± 0.82	45.13 ± 2.69	22.42 ± 2.48

Quantitative analysis of relative expression levels of different splice variants of canine *mda-7* in CMT12 cells and normal canine epidermal keratinocytes before and after LPS treatment.

Table 3

Protein similarities between in human, canine and murine MDA-7.

	C49a	FISP	Human MDA- 7
Canonical canine MDA- 7	65	64	66
C49a	-	82	63
FISP	-	-	60

The *in-silico* predicted canine MDA-7 amino acid sequence was aligned with the amino acid sequences of c49a (rat), FISP (mouse) and human MDA-7/IL-24 using vector NTI 10.0. Protein similarities are provided as percent identity between c49a, FISP, canine and human MDA-7 proteins.