NOTE *Internal Medicine*

Molecular Cloning of Canine *nm23* cDNAs and Their Expression in Normal and Tumor Tissues

Masashi TAKAHASHI[1)], Ryoma UNE[1)], Kenjiro FUKUSHIMA[1)], Makoto FUJIKI[2)], Kazuhiro MISUMI[2)], Noriaki MIYOSHI[3)], Yasuyuki ENDO[1)]*, Akihiro OHISHI[4)] and Masao AKUZAWA[1)]

[1)]*Laboratories of Veterinary Internal Medicine,* [2)]*Veterinary Surgery,* [3)]*Veterinary Pathology and* [4)]*Veterinary Animal Hospital, Faculty of Agriculture, Kagoshima University, Kagoshima 890–0065, Japan*

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ABSTRACT. Two canine *nm-23* cDNAs, designated as *nm23-C1* and *-C2*, were isolated and characterized. Both have a putative open reading frame consisting of 459-bp encoding 152 amino acids and are highly similar to human, mouse and rat homologues. To understand the potential role of *nm23-C1* and *-C2* in the development of mammary gland tumors (MGT), we analyzed the mRNA expression in 14 MGT samples by RT-PCR. The samples were divided into categories according to their histopathology (benign/malignant) and metastasis. No significant difference in the mRNA expression levels of either *nm23-C1* or *-C2* were observed between the benign and malignant groups or the metastatic and non-metastatic groups. These results suggest that *nm23-C1* and *-C2* are not related to the establishment of malignancy and metastatic lesions in canine MGT cases.

KEY WORDS: canine, NM23, tumor.

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Tumors are characterized by abnormal cell proliferation and tissue invasion. Since the discovery of oncogenes and tumor suppressor genes in the1980s, it has been recognized that a major cause of tumor growth is genetic abnormalities [4]. However, it has been shown that a single oncogene abnormality may not be enough to cause a tumor, and that the accumulation of multiple oncogene abnormalities, termed multi-step tumorigenesis, is required [2]. Recently, Steeg reported that metastasis might also develop because of abnormal expression of specific proteins or genes in tumor cells [18]. One metastasis inhibitory gene, designated as non-metastatic clone #23 (*nm23*), was isolated from a mouse melanoma cell K1735 [16]. Expression of *nm23* mRNA was high in the K1735 subclone with non-metastatic potential, and its expression was lower in the metastatic subclone [1, 9]. This finding indicated that *nm23* genes might play an important role in the development of metastatic lesions. Additionally, recent reports have shown that the incidence of metastatic lesions in patients with breast cancer, hepatoma, gastric cancer, and ovarian tumors was related to the expression level of *nm23* mRNA [7, 12, 16, 19]. This finding also suggested that determination of the *nm23* expression level would be a useful factor in predicting the prognosis for tumor bearing patients.

These days, more and more cases of cats and dogs with tumors are seen in small animal veterinary practices. Mammary gland tumor (MGT) is the most frequently occurring solid tumor in dogs. Most canine MGTs can be excised surgically, and the prognosis for these animals is good. Some of them, however, have metastasized, and, therefore, their prognosis is poor. It has been demonstrated that the cause of MGT is related to the hormone values [13], however, the molecular process for development of metastasis is still unclear. In studies examining the relationship between the development of metastasis and *nm23* expression levels in human breast cancer, it has been suggested that *nm23* genes may be one of the metastatic suppressor genes [3, 17]. In the present study, we focused on the function of *nm23* genes in the metastasis of canine MGT. Molecular cloning of canine *nm23* cDNAs was performed and their expression levels in normal and MGT tissues evaluated.

We first performed molecular cloning of *nm23-C1* and *nm23-C2* cDNAs using the RT-PCR method and DNA sequencing. Whole blood was collected from a clinically healthy dog to obtain peripheral blood mononuclear cells (PBMC). Total RNA was extracted from the PBMC with a commercially available kit (RNeasy mini, QIAGEN, Hilden, Germany), and then cDNA was synthesized from 1 μg of total RNA using a First strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ). Oligonucleotide primers, 5'-ATGGCCAACAGTGAACGCAC-3' (C1S) and 5'-TCACTCATAAATCCAGTTC -3' (C1R) for nm23-C1 and 5'-ATGGCCCACCAGGAGCGCAC-3' (C2S) and 5'-TTATTCATAGATCCAGTCA -3' (C2R) for *nm23-C2*, were designed based on the sequences of conserved human [6] and mouse [2] *nm23* cDNAs. The PCR amplifications were performed under the following conditions: 1 cycle of pre-denature (3 min, 94°C); 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and polymerization (1 min at 72°C); and 1 cycle of complete elongation (10 min at 72°C). The amplified DNA fragments were inserted into a pCR2.1 vector (Invitrogen, Calsbad, CA) and the nucleotide sequence of the inserted DNA fragments was determined by the dideoxy chain termination method (ABI Prism BigDye Primer Cycle Sequencing Ready Reaction kit, Applied Bio-

* CORRESPONDENCE TO: ENDO, Y., Laboratory of Veterinary Internal Medicine, Faculty of Agriculture, Kagoshima University, 1–21–24 Korimoto, Kagoshima 890–0065, Japan.

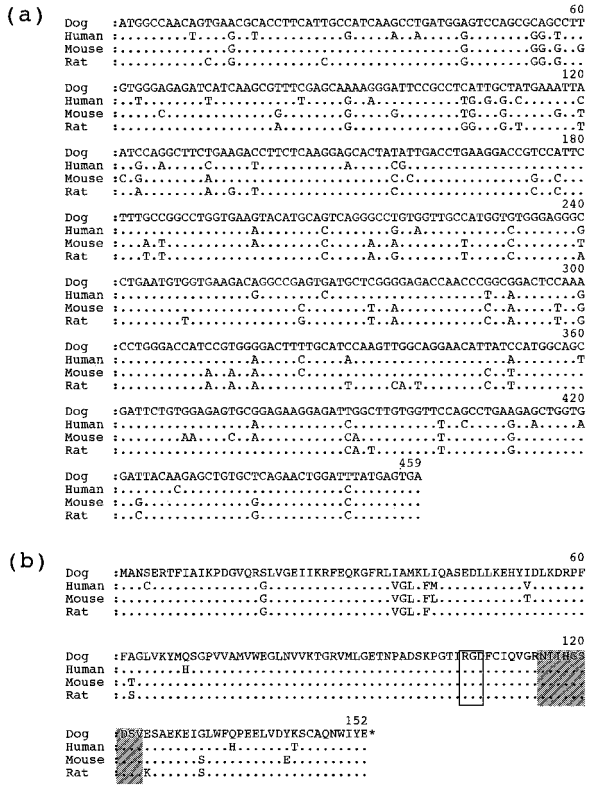

Fig. 1. Nucleotide and deduced amino acid sequences of *nm23-C1* cDNA. (a) Comparison of nucleotide sequences of *nm23-C1* cDNA with human, mouse, and rat counterparts. (b) Comparison of amino acid sequences of *nm23-C1* with human, mouse, and rat counterparts. Identical nucleotide and amino acid residues among species are shown as dots. The open box and shaded area indicate the RGD domain and NDPK domain, respectively.

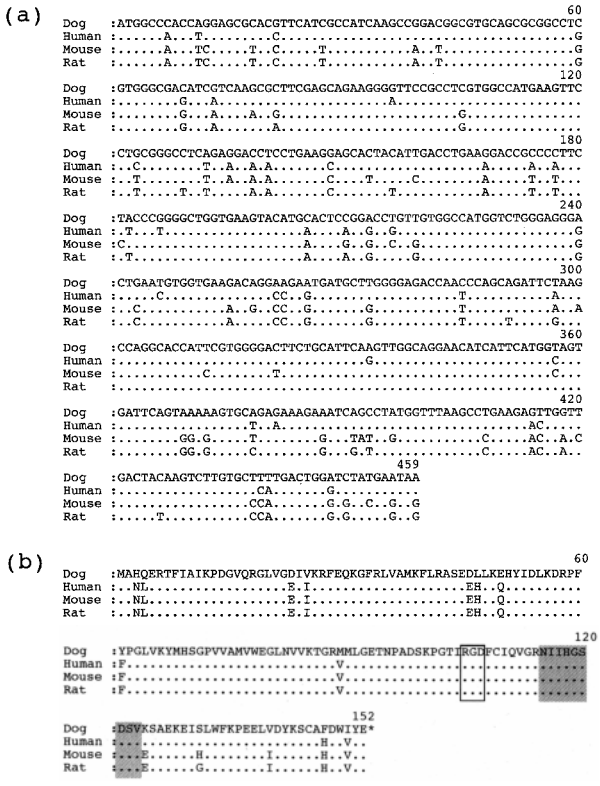

Fig. 2. Nucleotide and deduced amino acid sequences of *nm23-C2* cDNA. (a) Comparison of nucleotide sequences of *nm23-C2* cDNA with human, mouse, and rat counterparts. (b) Comparison of amino acid sequences of *nm23-C2* with human, mouse, and rat counterparts. Identical nucleotide and amino acid residues among species are shown as dots. The open box and shaded area indicate the RGD domain and NDPK domain, respectively.

systems, Foster City, CA). A DNA fragment of 495-bp long was obtained by RT-PCR using primers C1S and C1R. Based on the sequencing analysis, this DNA fragment contained a putative coding region that was 459-bp long and encoded 152 amino acid residues that showed high similarities to those of human (*nm23-H1*, 88.2%), mouse (*nm23-M1*, 87.6%), and rat (*nm23-R1*, 88.7%) homologues at the nucleotide level (Fig. 1). We analyzed this DNA fragment using FASTA system (DNA data bank of Japan), knowing that the *nm23* family has eight isoforms. This analysis also showed that the obtained DNA fragment resembled *nm23-H1, nm23-M1* and *nm23-R1* cDNAs. Therefore, we concluded that this DNA fragment is their homologue, and designated it as *nm23-C1* accordingly. Figure 1b shows the alignment of the deduced amino acid sequence of NM23-C1 with human, mouse, and rat homologues. At the amino acid level, NM23-C1 was shown to be between 92.8 and 95.4% similar to its human, mouse and rat counterparts. It has been reported that the NM23 family has an RGD domain (cell attachment sequence) that consists of three amino acid residues (Arg-Gly-Asp); this domain was also encoded in NM23-C1. Another important domain, the NDPK domain (NIIHGSDSV, aa 115–123), was also conserved in NM23-C1. The nucleotide and deduced amino acid sequences of *nm23-C2* were also determined using these same procedures. A 541-bp long DNA fragment was obtained by the RT-PCR method using primers C2S and C2R. This DNA fragment contained a coding region that was 459-bp long and encoded 152 amino acid residues. Analysis using the FASTA system showed that the nucleotide sequence of this DNA fragment was very similar to that of the human (*nm23-H2*, 91.7%), mouse (*nm23-M2*, 85.4%), and rat (*nm23-R2*, 87.1%) (Fig. 2). At the amino acid level, NM23-C2 was shown to be between 90.8 and 92.8% similar to its human, mouse and rat counterparts. RGD and NDPK domains were also conserved in NM23-C2 at positions 105–107 and 115–123, respectively. These findings suggested that the biological properties of *nm23* gene products are conserved among species.

Next, we analyzed the expression levels of *nm23-C1* and *-C2* mRNAs in normal tissue. Total RNA was extracted from the brain, lung, heart, liver, pancreas, small intestine,

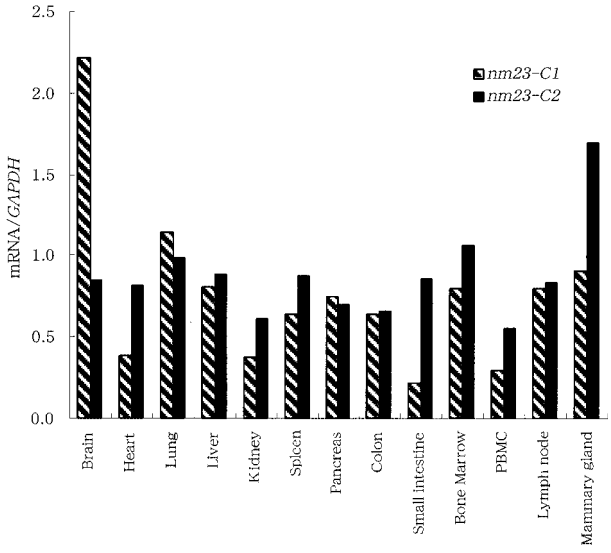

Fig. 3. Comparison of expression of *nm23-C1* and *nm23-C2* mRNAs in normal tissue. All data are depicted relative to the index against *GAPDH* mRNA level.

colon, spleen, kidney, lymph nodes, mammary gland, bone marrow, and PBMC of a healthy dog. cDNA was synthesized as previously described. Primer pairs 5'-AGGACCGTCCATTCTTTGCC-3' (nt 167–186 in *nm23-C1* obtained in this study) and 5'-GGACTCCAAACCTGGGACCA-3' (nt 310–291 in *nm23-C1*), and 5'-AAGGACCGCCCCTTCTACCC-3' (nt 166–185 in *nm23-C*2) and 5'-GCCTGGCTTAGAATCTGCTG-3' (nt 306–287 in *nm23-C2*) were used to amplify *nm23-C1* and *nm23-C2* mRNA, respectively. Sequencing analysis confirmed proper amplification of the derived *nm23-C1* and *nm23-C2* DNA fragments. As an internal control, canine GAPDH cDNA was amplified using the primer pair 5'-CTCATGACCACAGTCCATGC-3' (nt 515–533 in canine GAPDH cDNA, GenBank/EMBL/DDBJ accession number AB038240), forward, and primer 5'-TGAGCTTGACAAAGTGGTCA-3' (nt 925–906), reverse. The PCR products were electrophoresed through 2.5% agarose gel and stained with ethidium bromide for visualization. The expression levels of *nm23-C1* and *-C2* mRNA were evaluated by band density and compared to that of *GAPDH* mRNA. Graphic data from the stained gel was analyzed by computer using the NIH image 1.62 software (NIH, Bethesda, MD). As shown in Fig. 3, expressions of both *nm23-C*1 and *-C2* mRNAs were observed in a broad range of tissues. Expression of *nm23-C1* was high in brain and lung tissues, but was relatively low in the heart, kidney, PBMC, and small intestine. A high expression level for *nm23-C2* was detected in mammary gland tissue, however, no significant difference was observed in other tissues. Canine *nm23-C1* and *nm23-C2* mRNAs were confirmed to be expressed in a broad range of tissues similar to those in the human and mouse [2, 8]. It was noted that the *nm23-C1* expression level in brain tissue was much higher than in other tissues. This finding was also reported in a similar study on mice [2]. It has been shown that murine NDPK A, a gene product of *nm23-M1*, is required in the development of the brain and nervous system. Therefore, it is plausible that the biological function of *nm23-C1* may be the same as that of *nm23-M1*. Expression of *nm23-C2* was uniform in the tissues we examined, which may be due to the characteristic function of NDPK B, the gene product derived from *nm23-H2* and *-M2*. NDPK B is widely distributed in several tissues and works as an accelerator of cell proliferation [2]. A previous study identified NDPK B and PuF (purine-binding transcription factor), which is one of the transcriptional factors for c-*myc*, as the same molecule [15]. Therefore, canine *nm23-C2* can be considered one of the house-keeping genes for cells.

The relationship between the *nm23* gene expression level and the malignant property of canine MGTs was also evaluated. MGT samples were collected from 14 dogs referred to the veterinary hospital at Kagoshima University for diagnosis and treatment. Following a radiographic metastatic check, all cases underwent surgical removal of the tumor, and the MGT was diagnosed by histopathological examination. Fourteen MGT samples were categorized in two ways based on the histological findings and metastasis (Table 1). Total RNA extraction and RT-PCR of tumor samples were carried out as previously described. The student *t*-test was applied for statistical analysis. The indices of *nm23-C1* expression levels were 1.69 ± 0.45 (mean ± SD) in the benign tumor group (simple adenoma and benign mixed tumor) and the indices in the malignant group (simple carcinoma and complex carcinoma) were 1.69 ± 1.10. There was no significant difference between the two groups (p=0.780) (Fig. 4a). In addition, the indices concerning *nm23-C2* were 1.71 ± 0.86 and 1.12 ± 0.51 in the benign and malignant tumors, respectively (Fig. 4b). A slight decrease in the *nm23-C2* index was observed in the malignant MGT compared to the benign tumors, however statistical analysis revealed no significant change (p=0.144). Subsequently, tumor samples were divided into two groups based on the existence of metastatic lesions, and each index was calculated again. Metastatic lesions in lymph nodes were seen in 3 dogs (cases 9, 10, and 11) and the mean expression indices of *nm23-C1* and *nm23-C2* in tumor samples derived from these 3 dogs were 1.16 ± 0.47 and 0.98 ± 0.57, respectively (Fig. 4c and 4d). In contrast, the indices in the 11 cases without metastatic lesions were 1.76 ± 0.81 and 1.54 ± 0.77, respectively. Although a decreasing tendency for each parameter was observed in the indices of cases with metastatic lesions, no significant differences were detected in the statistical analysis (*nm23-C1*, p=0.253; *nm23-C2*, p=0.268). In this analysis, we unexpectedly found no relationship between the mRNA expression levels of *nm23-C1* and *-C2* and the existence of histopathological malignancy and metastatic lesions. For *nm23* genes, which act as metastatic inhibitory genes, it has been reported that the *nm23-H1* gene, or protein expression of this gene, is inversely related to survival time, remission period, frequency of metastasis,

Table 1. Profile of the MGT samples used in this study

Case No.	Pathological diagnosis	Group	Metastatic lesion
1	Simple adenoma	Benign tumor	(–)
2	Simple adenoma	Benign tumor	(–)
3	Benign mixed tumor	Benign tumor	(–)
4	Benign mixed tumor	Benign tumor	(–)
5	Benign mixed tumor	Benign tumor	(–)
6	Benign mixed tumor	Benign tumor	(–)
7	Benign mixed tumor	Benign tumor	(–)
8	Simple carcinoma	Malignant tumor	(–)
9	Simple carcinoma	Malignant tumor	(+)
10	Complex carcinoma	Malignant tumor	(+)
11	Complex carcinoma	Malignant tumor	(+)
12	Complex carcinoma	Malignant tumor	(–)
13	Complex carcinoma	Malignant tumor	(–)
14	Complex carcinoma	Malignant tumor	(–)

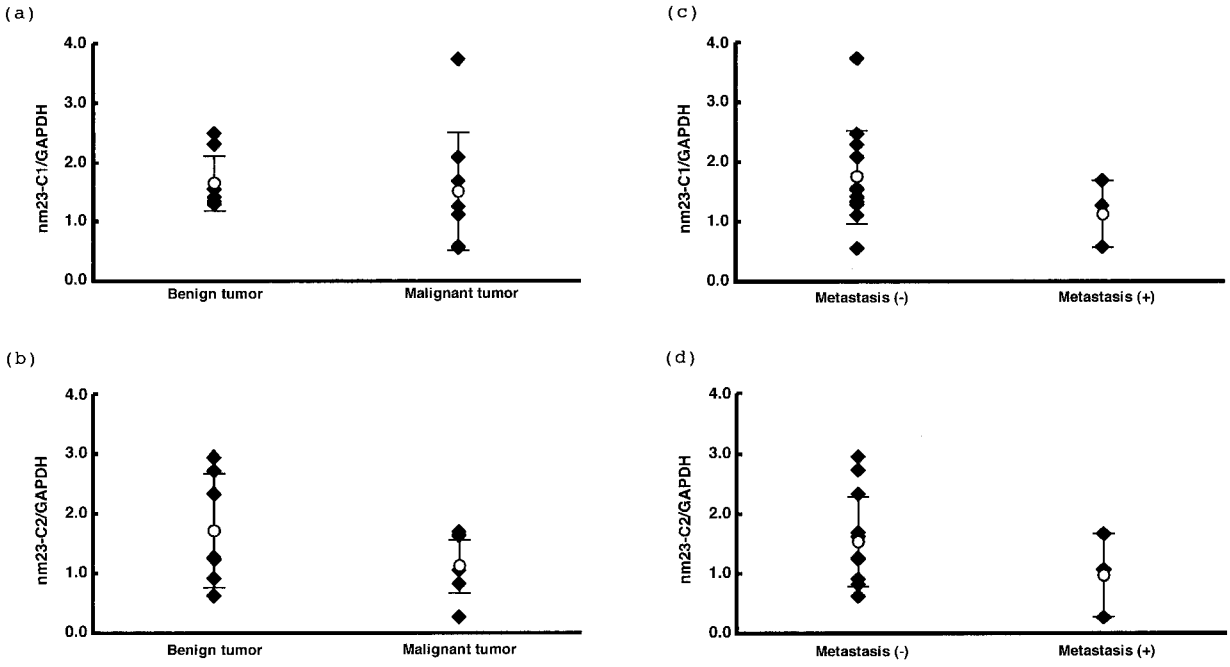

Fig. 4. Comparison of expression levels of *nm23* mRNAs in MGTs. Expression levels of *nm23-C1* and *nm23-C2* mRNA in malignant and benign MGTs are shown in (a) and (b), respectively. The correlation between the existence of metastatic lesions and expression of *nm23-C1* mRNA and *nm23-C2* mRNA is shown in (c) and (d). The index of each case is shown as a closed square. The average value and SD are shown as an open circle and error bar, respectively.

and histopathological malignancy [5]. Thus, our findings suggest a biological discrepancy between canine MGTs and human breast cancer. We analyzed the *nm23* expression levels in only a limited number of canine MGT tissues. If we had evaluated a larger number of MGTs, a significant difference between *nm23* expression levels and metastasis may have been observed. Additional studies with more samples are necessary to clarify this point. At the same time, the relationship between the *nm23* expression levels and the time frame of primary tumor development to metastatic lesion development should also be evaluated. It has been reported that the malignant characteristics (high metastatic rate and heteromorphism) would be observed in human breast cancer, hepatocarcinoma, gastric cancer, and ovarian tumors, if *nm-23* expression were low in these tumors [7, 12, 16, 19]. However, an inverse phenomenon has been shown in the cases of neuroblastomas and pancreatic tumors; it has been found that high *nm23* expression enhances the risk of metastatic lesions in these tumors [10, 11, 14]. These findings seem to suggest that the relationship between the malignant properties of tumors and the expression level of the *nm23* gene is dependent on the type of tumor. This point should also be clarified in the many different tumor types encountered in small animal practice.

In this study we determined the nucleotide and putative amino acid sequences of the canine homologues of the *nm23* genes, *nm23-C1* and *nm23-C2*, with the view of investigating their possible use as a predictive or prognostic factor for tumor bearing animals in veterinary medicine. Although we did not find any evidence of a relationship between future metastasis and *nm23* gene expression levels, our findings will be useful for the development of recombinant protein and polyclonal/monoclonal antibodies against gene products. These applications make it possible to evaluate tumors by viewing proteins and genes. Using these tools, canine tumors (not restricted to MGTs) can be analyzed more widely and provide useful information for increasing our understanding of the mechanisms of tumor establishment and metastasis.

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