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Differential expression of cell cycle regulators p21, p27 and p53 in metastasizing canine mammary adenocarcinomas versus normal mammary glands

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ABSTRACT

The cyclin dependent kinase inhibitors p21 and p27 are important regulators of cell cycle progression. To analyze their role in the malignant progression of canine mammary tumors expression levels of p27 and p21 and its major regulator p53 were compared in simple adenomas, adenocarcinomas of the mammary gland and lymph node metastases with normal mammary gland. Laser microdissection of tissue samples and real-time PCR were used for quantification of mRNA expression levels. p21 was overexpressed in adenocarcinomas, whereas adenomas and metastases expressed p21 more heterogeneously. Comparison of p21 expression in adenocarcinomas and their metastases revealed a significant decrease in expression in metastases. In contrast, p27 expression was reduced in the adenocarcinomas but heterogeneously expressed in adenomas and metastases. Taken together the results suggest that loss of p21 overexpression is associated with tumor metastasis while reduced cell cycle inhibition by p27 is associated with malignant progression.

1. Introduction

Little is known about the molecular mechanisms of malignant progression of canine mammary tumors despite their frequent incidence. Obviously, uncontrolled cell cycle progression is a major step in malignant transformation of this malignancy like in other tumor types. In normal cells, regulated progression of the cell cycle is governed by cyclins and cyclin-dependent kinases and their inhibitors (Sherr and McCormick, 2002). p21 and p27 are cyclin-dependent kinase inhibitors (CDKIN) and important regulators of the cell cycle. p21, also known as cyclin-dependent kinase-inhibitor 1 (CDKIN1A), is a nuclear protein and has been implicated in mechanisms of cell-cycle arrest that allow cell DNA repair, differentiation and apoptosis. These tasks are performed by the interaction with cyclinA-cyclin dependent kinase (CDK) 2 and cyclinD–CDK4 complexes (Gartel and Radhakrishnan, 2005). So far p21 expression and its interaction with p53 in canine mammary tumors are unclear. In human breast cancer the predictive value of p21 expression for clinical outcome has been discussed controversially (Colozza et al., 2005). Both loss of p21 expression and overexpression have been associated with shorter survival in human breast cancer patients (Abukhdeir and Park, 2008; Yang et al., 2003). Expression of p21 is mainly induced by the tumor suppressor protein p53 in response to several stress stimuli but several other factors can induce p21 expression and cell cycle arrest independent of p53 (Decesse et al., 2001; el-Deiry et al., 1994; Michieli et al., 1994; Somasundaram et al., 1997). Expression of p53 protein in canine mammary tumors has been investigated intensely. Here, immunohistochemical studies mostly reported p53 expression or loss in both benign and malignant tumors (Inoue and Shiramizu, 1999; Kumaraguruparan et al., 2006; Morris et al., 2008). Nevertheless, exact expression levels of p21 and p53 are not known due to limitations of immunohistochemistry to quantify protein expression. Direct interaction of p21 and p53 seems to be of importance at least in a portion of human breast cancers. Here, mutation and abnormal p53 expression is associated with loss of p21 expression (Ellis et al., 1997; Giannikaki et al., 1997). Similar studies are not available in the dog.

The cyclin-dependent kinase inhibitor p27 is regulated by extracellular stimuli like transforming growth factor beta (TGF-beta). Nevertheless, similar to p21 it regulates cell cycle progression by interacting with cyclins and CDK. Mutational loss of this gene may lead to uncontrolled cell cycle progression and cellular proliferation (Kiyokawa et al., 1996). However, reports on p27 expression levels in canine mammary tumors are not available so far. Decreased expression of p27 protein has been observed in up to 60% of human carcinomas (Slingerland and Pagano, 2000; Viglietto et al., 2002). Multivariate analyses of p27 expression along with other known clinical and pathologic prognostic markers have

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shown that loss of p27 protein is a negative prognostic factor for human breast cancer (Fredersdorf et al., 1997; Tan et al., 1997).

In the explorative study presented here we investigated the mRNA expression of p21, p27 and p53 in highly defined laser microdissected tissue samples of canine mammary adenomas, adenocarcinomas and their lymph node metastases. Expression data were normalized to three housekeeping genes and compared to non neoplastic mammary gland of the same dog. This approach allows exact quantification of gene expression and provides valuable evidence on molecular mechanisms of tumor development and progression that are not possible with semiquantitative immunohistochemistry. We show that p21 is overexpressed in primary adenocarcinomas but reduced in lymph node metastases independently of p53 expression. Furthermore, p27 is downregulated in primary and metastatic adenocarcinomas but regulated inconsistently in adenomas.

2. Materials and methods

2.1. Dogs and tissue processing

Hundred and ten canine patients with mammary tumors were screened for 10 simple adenomas and 10 simple mammary adenocarcinomas metastatic to the regional lymph node at the time of tumor resection (Table 1). All dogs had a comprehensive clinical examination including exclusion of pulmonary metastases by thoracic radiographs. Tissue specimens were sliced at 5 mm thickness and every other slice was immersion fixed in neutral-buffered 4% formaldehyde or snap frozen in liquid nitrogen at −80 °C within 15 min after resection and stored until further use.

Representative formaldehyde fixed tissues were routinely embedded in paraffin. Sections of 2 μm thickness were mounted on adhesive glass slides and stained with hematoxylin and eosin. Histologic evaluation of the tumors was performed independently by two board-certified pathologists, following the criteria of the WHO classification of canine mammary tumors (Misdorp et al., 1999) and the grading system of (Elston and Ellis, 1991).

2.2. Laser-capture microdissection and reverse transcription

Laser microdissection was used to obtain defined tissue samples of non neoplastic cells or neoplastic cells respectively without contamination by stromal and inflammatory cells. Five consecutive sections of 6–8 μm thickness from the frozen tissue samples were mounted on glass slides covered with a polyethylene naphthalate membrane (PALM Microlaser Technologies, Bernried, Germany) for laser microdissection. Sections were fixed for 2 min in 95% ethanol at −20 °C and stained with hematoxylin and eosin solubilized in diethylpyrocarbonate (DEPC) treated water. Subsequently, sections were dehydrated in ascending graded ethanol and air-dried at room temperature. More than 25×10^6 μm^2 of non-neoplastic epithelial cells, adenomas, adenocarcinomas and lymph node metastases were excised and laser pressure catapulted into caps of 0.5-ml reaction tubes containing 30 μl of lysis buffer (NucleoSpin RNA XS; Macherey & Nagel, Düren, Germany). Total RNA was extracted and purified using a commercial kit (NucleoSpin RNA XS; Macherey & Nagel, Düren, Germany) and reverse transcribed using iScript cDNA synthesis kit (Biorad, Germany).

2.3. Quantitative real-time polymerase chain reaction

Primer sequences for p21, p27, p53 and the housekeeper genes hypoxanthine-phosphoribosyl transferase (HPRT), ATP-synthase subunit 5B (A5B) and ribosomal protein L32 (RP32) are shown in Table 2. Real-time quantitative reverse-transcription PCR (RT-qPCR) and data analyses were performed using the MX 3000P Quantitative PCR System and MX Pro software (Stratagene, La Jolla, USA). The reactions were carried out in 96-well polypropylene plates covered with optical caps (Stratagene, La Jolla, USA). The plates contained triplicates of each cDNA sample and no-template controls with water instead of cDNA templates. During initial optimization runs, the exact primer concentrations and PCR time and temperature conditions were determined. The qRT-PCR efficiency of all assays was between 93% and 101% (data not shown) and all yielded products of the expected sequence. The 15-μl reaction mix contained 5 μl cDNA, 12.5 μl Brilliant SYBR Green QPCR Master Mix (Stratagene) with 300 nM of each primer. Cycling conditions were 10 min at 95 °C, followed by 40 cycles of 30 seconds at 95 °C, 1 min at 58 °C, and 30 s at 72 °C. The cDNA of all samples were amplified on the same plate for every primer pair to ensure equal amplification conditions. Specificity of amplification products was confirmed by melting curve analyses. For each sample, results were documented as cycle threshold (CT) set to 100 relative fluorescence units) values of background subtracted qPCR fluores-

Table 1
Animals and histologic grade of malignancy.

Dog	Breed	Age in years	Histologic diagnosis and grade	Affected lymph node
1	Cocker-mix	12	Simple adenoma, Grade I	–
2	Terrier-mix	11	Simple adenoma, Grade I	–
3	Miniature-pinscher	13	Simple adenoma, Grade I	–
4	WHW* terrier	12	Simple adenoma, Grade I	–
5	Yorkshire terrier	11	Simple adenoma, Grade I	–
6	Beagle	8	Simple adenoma, Grade I	–
7	WHW* terrier	11	Simple adenoma, Grade I	–
8	Golden retriever	9	Simple adenoma, Grade I	–
9	Labrador mix	11	Simple adenoma, Grade I	–
10	German spitz mix	7	Simple adenoma, Grade I	–
11	Bobtail	10	Simple adenocarcinoma, Grade III	Inguinal
12	Rottweiler	10	Simple adenocarcinoma, Grade III	Inguinal
13	Dachshund	13	Simple adenocarcinoma, Grade III	Inguinal
14	Bavarian mount. Hound	13	Simple adenocarcinoma, Grade III	Inguinal
15	Golden retriever	11	Simple adenocarcinoma, Grade III	Inguinal
16	WHW* terrier	12	Simple adenocarcinoma, Grade II	Axillary
17	WHW* terrier	16	Simple adenocarcinoma, Grade II	Inguinal
18	Spitz mix	16	Simple adenocarcinoma, Grade II	Inguinal
19	Beagle	11	Simple adenocarcinoma, Grade II	Inguinal
20	Mixed breed	9	Simple adenocarcinoma, Grade III	Inguinal

* WHW = West Highland White Terrier.