Reactivity of p53 protein in canine transmissible venereal tumor

[Reatividade da proteína P53 no tumor venéreo transmissível canino]


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ABSTRACT

The expression of p53 protein was evaluated in canine transmissible venereal tumor (CTVT), as following: natural occurrence (n=8); resistant to chemotherapy (n=4); and allogeneic transplanted in progression (n=8), stable (n=8), and regression (n=8) stages. The collected specimens were submitted to GM1 immunohistochemical reaction. Results showed a mean percentage of immunomarked cells around 18.6% in CTVT of natural occurrence, 23.8% in CTVT resistant to chemotherapy, 22.9% in allogeneic transplanted CTVT in both progression and stable stages, and 35.8% in transplanted CTVT in regression stage. The results suggest that there is a functional abnormality in p53 gene and its products in the studied tumors; although, it is not possible to correlate the percentage of cells marked by p53 and a prognosis.

Keywords: immunohistochemistry, p53, oncology, transmissible venereal tumor

RESUMO

A expressão da proteína p53 foi avaliada em espécimes de tumor venéreo transmissível canino (TVT) de ocorrência natural (n=8); resistente à quimioterapia (n=4) e transplantado em câo nas fases de progressão tumoral (n=8), de latência (n=8) e de regressão (n=8). Os espécimes foram submetidos à reação de imunoistoquímica. Os resultados mostraram porcentagem média de células imunomarcadas de 18,6% no TVT de ocorrência natural, 23,8% no TVT refratário, 22,9% nos TVTs transplantados nas fases de progressão e latência e de 35,8% na fase de regressão. Os resultados sugerem que há uma anormalidade funcional no gene P53 e seus produtos nos tumores estudados, apesar de não ser possível correlacionar a porcentagem de células marcadas pelo p53 ao prognóstico.

Palavras-chave: câo, imunoistoquímica, p53, oncologia, tumor venéreo transmissível

INTRODUCTION

Canine transmissible venereal tumor (CTVT) is contagious and it is naturally transmitted (Knapp et al., 2000) or by experimental transplants in dogs (Karson and Mann, 1952). In this case, it passes by consecutive phases of progression, latency, and regression within two to six months (Koike et al., 1979), with important participation of the immune system (Rogers et al., 1998). CTVT is mainly located on the external genital mucosa, but it has also been reported in other organs (Knapp et al., 2000; MacEwen, 2001).

Around 90% of the dogs treated with vincristine sulphate respond to treatment and a small percentage is resistant to chemotherapy (Rogers, 1997). The multiple drug resistance gene (RMD1), that confer resistance to cytotoxic drugs, is responsible by failure in chemotherapy in several types of cancer. The evidence that RMD1 expression is regulated by certain p53 mutant genes has suggested that the response to radiotherapy or chemotherapy may depend, in part, on the condition of p53 in the tumor before the treatment (Harris and Hollstein, 1993).
The p53 gene encodes a nuclear phosphoprotein with 393 amino acids which is able to bind to specific DNA sequences, acting as a transcription factor. In normal cells, damage to the DNA leads to increase in p53 protein and arrests the cell cycle at G1 stage, allowing the repair of this damage. If the DNA is not repaired, a programmed cell death (apoptosis) may occur. Thus, p53 is considered to maintain genomic stability. The deficiency of p53 in tumor cells is responsible for the genomic instability, manifested by aneuploidy and by the ability to produce gene amplification (Kelley and Johnson, 1994). Several forms of mutant p53 protein are oncogenic and stimulate cell division without repair mutations in DNA during replication (Levine et al., 1991).

The wild-type p53 protein is quickly eliminated due to its short half-life. In contrast, the mutant p53 protein has a half-life of several hours and may be detected by immunohistochemistry (Prokokimer and Rotter, 1994). Correlation between immunohistochemical detection of p53 protein and mutations in p53 gene has been described (Davidoff et al., 1991). These mutations often lead to production of an altered p53 protein that binds to and inactivates the normal, wild-type p53 protein, thereby promoting tumorigenesis. Thus, immunohistochemical detection of p53 protein is equated to the detection of the mutant p53 protein or otherwise stabilized abnormal p53 protein, rather than due to overexpression of wild-type p53 (Ginn et al., 2000).

Changes in p53 expression have been the aim of several studies in canine tumor of natural occurrence (Sagartz et al., 1996; Gamblin et al., 1997; Wolf et al., 1997; Ginn et al., 2000; Jaffe et al., 2000). Jaffe et al. (2000) found that canine cutaneous grade III mast cell tumors had greater p53 content than those of grade I or II. On the other hand, Ginn et al. (2000) did not correlate the expression of p53 to the histological grade of canine mast cell tumors. In canine osteogenic tumors, there was a significant association between highly aggressive tumor behavior and p53 expression in osteosarcomas versus multilobular tumors of bone (Sagartz et al., 1996).

The polyclonal antibody CM1 is able to bind both wild and mutant types of p53 protein in tumor tissues of dogs. Successful immunohistochemical analysis for mutant p53 protein has been performed and may be useful as prognostic factor, although it may vary according to the type of tumor and the species (Sagartz et al., 1996; Gamblin et al., 1997; Ginn et al., 2000; Jaffe et al., 2000).

The aim of this study was to verify the expression of p53 protein in CTVT to (1) determine and compare the percentage of immunomarked cells by p53 protein in CTVT of natural occurrence, allogeneic transplanted, and those resistant to conventional chemotherapy with vincristine sulphate; and (2) evaluate if p53 can be employed as a prognostic factor in these experimental conditions.

**MATERIAL AND METHODS**

The following specimens of CTVT were used: (1) natural occurrence (n=8), with no previous treatment; (2) transplanted from one dog to another by infecting them with tumor cells (allogeneic CTVT): in progression (n=8), stable (n=8), regression stages (n=8), and (3) resistant (n=4), after four administration of vincristine sulphate (0.5 - 1.0mg/m²). Specimens were fixed in 10% buffered formalin and embedded in paraffin wax. Four-micrometer thick tissue sections were mounted on Poly-L-lysine (code P4832 – Sigma Chemical Co., St. Louis, USA) treated slides and submitted to immunohistochemical reaction, according to ABC method (Hsu et al., 1981).

The human polyclonal antibody CM1 (code NCL-p53-CM1 – Novocastra Laboratories Ltd., UK), diluted at 1:100, served as the primary antibody for the detection of p53 protein. Vectastain Elite ABC kit (Kit Rabbit IgG - code PK-6101 – Vector Laboratories, Burlingame, USA) was used as biotinylated secondary antibody.

Briefly, formalin-fixed sections were dewaxed, rehydrated, and then rinsed in phosphate buffer saline (PBS). Endogenous peroxidase activity was quenched by immersion of the slides in 8% hydrogen peroxide diluted in methanol p.a. for 20 minutes. Sections were then subject to heat for antigen retrieval in a pressure cooker containing TRIS-EDTA buffer (pH 9.0). Unspecific protein was blocked by incubating...
sections in 1.0mL PBS supplemented with 0.06g commercial skim milk powder (Molico - Nestle, Brazil) and one drop of normal goat serum for 30 minutes. Sections were then incubated with primary antibody p53-CM1 overnight at 4°C in a moist chamber. The sections were incubated with biotinylated secondary antibody prepared according to the instructions of the manufacturer for 45 minutes at room temperature and were incubated for 45 minutes in the avidin-biotin complex.

Slides were made in a solution of 3,3-diaminobenzidine (DAB), counterstained with Harris Hematoxylin. The sections were evaluated by three distinct observers at an optical microscope. Six fields were chosen at random and the percentage of marked cells was extracted from the total of a hundred cells counted. The results were established based on the percentage of marked cells.

To compare the frequency of p53 expression among the histological specimens, the F test and Tukey test (P<0.05) were performed for comparison of the means. The collected data were analyzed using SAS GLM Software.

RESULTS

All specimens showed reactivity to p53, with diverse immunomarked cells. Results are shown in Table 1.

### Table 1. Significance and percentage of immunoreactive cells to p53 (mean±standard deviation) in specimens of canine transmissible venereal tumor (CTVT) immunomarked with p53-CM1 antibody

<table>
<thead>
<tr>
<th>Tumor</th>
<th>% of marked cells</th>
</tr>
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<tbody>
<tr>
<td>Transplanted CTVT – regression stage</td>
<td>35.8±5.7a</td>
</tr>
<tr>
<td>Refractory CTVT</td>
<td>23.8±4.9b</td>
</tr>
<tr>
<td>Transplanted CTVT – stable stage</td>
<td>22.9±6.2b</td>
</tr>
<tr>
<td>Transplanted CTVT – progression stage</td>
<td>22.9±6.3b</td>
</tr>
<tr>
<td>Natural occurrence CTVT</td>
<td>18.6±2.8b</td>
</tr>
</tbody>
</table>

Means followed by same letters do not differ by Tukey test at 5%. Coefficient of variation = 21.51%.

The specimens of CTVT allogeneic transplanted in progression stage (Figure 1C) showed the highest percentages of marked cells (35.8%), and a significant difference was observed when it was compared to the other groups.

The percentage of immunomarked cells by p53 in refractory (Figure 1E) CTVT (23.8%) did not differ from that transplanted CTVT either in stable (Figure 1B) (22.9%) or in progression (Figure 1A) stages (22.9%), as well as from that of CTVT of natural occurrence (18.6%). The percentage of marked cells in CTVT of natural occurrence (Figure 1D) only differed in transplanted CTVT in regression stage.

**DISCUSSION**

In humans, altered p53 proteins caused by mutations in the wild-type p53 protein enable detection by immunohistochemistry and have been considered a potential factor of prognosis for several tumors. CTVT of natural occurrence showed the smallest reactivity to p53 protein. This result is suitable with the less aggressive characteristic of CTVT, which may persist on the external genital structures of the host for undetermined period, without apparent systemic consequences (MacLachlan and Kennedy, 2002).

Among the CTVT specimens employed in this study, allogeneic transplanted CTVT in regression stage was the group with the greatest percentage of immunomarked cells by p53, in contrast to the initial expectation. Regression of the transplanted CTVT is a spontaneous event that demonstrates the immunological competence of the host (Ogilvie and Moore, 1995). For that reason, it was expected a greater percentage of immunomarked cells in the group of transplanted CTVT in progression stage, which did not occur. In CTVT resistant to chemotherapy, the percentage of immunomarked cells could be compared with that of CTVT of natural occurrence, suggesting that the p53 gene is not involved in this type of tumor, at least alone, with resistance to chemotherapy (Harris and Hollstein, 1993). This demonstrates that p53 gene may not be the only involved in oncogenesis of this tumor and, consequently, it is
not the only prognosis factor to be considered for CTVT. The evidence that RMD1 expression is regulated by certain p53 mutant genes has suggested that the response to radiotherapy or chemotherapy may depend, in part, on the condition of p53 in the tumor before the treatment (Harris and Hollstein, 1993) and not exclusively on p53 protein overexpression.

Figure 1. Specimens of canine transmissible venereal tumor (CTVT) immunomarked with p53-CM1 antibody. (A) transplanted CTVT in progression stage; (B) transplanted CTVT in stable stage; (C) transplanted CTVT in regression stage; (D) CTVT of natural occurrence; and (E) CTVT resistant to vincristine sulphate. ABC Method, Harris’ Hematoxylin counterstain. 200x.
The results obtained in the present study could be due to the use of a polyclonal antibody (CM1). Polyclonal antibodies are generally less specific than monoclonal; however, most of these antibodies used in human pathology may not react with canine tissue (Sueiro et al., 2004). Zhang (1999) verified in human colorectal adenocarcinomas that the polyclonal antibody CM1 marked 49% of the specimens, while monoclonal antibodies Pab1801 18%, DO1 44%, and DO7 30%, justifying that the polyclonal antibody CM1 recognizes the fully segment of p53 protein (amino acids 1-393), while others recognize only specific segments of this protein.

Wolf et al. (1997) detected in canine colorectal epithelial tumors, a greater p53 expression in benign lesions (48%) compared to malignant neoplasms (41%). Overexpression of p53 protein is a good, but not unique, indicator of mutation in p53 gene (Vogelstein and Kinzler, 1992). Authors of other investigation mentioned that p53 detected by immunohistochemical evaluation was not always mutant (Roels et al., 2001). However, it is possible that wild-type or mutant p53 expression in benign tumors may be associated with subsequent malignant transformation (Lee et al., 2004). In human tumors, positive immunohistochemical staining is often accepted as evidence of an underlying p53 genetic abnormality, and as there is limited DNA sequence data available to verify this conclusion in dogs, the potential for detection of the wild-type protein in this species merits consideration (Wolf et al., 1997).

In human tumors, experimental data showed that there is a direct correlation between damage in the function of p53 protein and reduction in long-term survival. However, this association may not occur in all types of tumors (Harris and Hollstein, 1993) and, regarding domestic animals, the results for the same type of cancer can be controversial (Ginn et al., 2000; Jaffe et al., 2000).

A positive correlation was verified between p53 overexpression and prognosis in canine osseous tumors (Sagartz et al., 1996), astrocytomas (Stoica et al., 2004), and mammary tumors (Lee et al., 2004), but not in gastrointestinal mastocitomas (Ozaki et al., 2002), colorectal epithelial tumors (Wolf et al., 1997), and canine and feline melanomas (Roels et al., 2001). In the present study, p53 expression could not be correlated to the degree of malignity and, therefore, p53 expression in CTVT is not a reliable prognostic indicator.

CONCLUSIONS

Allogeneic transplanted CTVT in regression stage was the group with the greatest percentage of immunomarked cells by p53, and significant difference was not observed among the other groups. CTVT of natural occurrence showed the smallest reactivity to p53 protein. It can be concluded that there is functional abnormality on p53 gene and p53 products in CTVT. However, it was not possible to establish a correlation between the percentage of marked cells by p53 and a prognosis in CTVT specimens.

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