Abstract

The studies were aimed at immunohistochemical determination of telomerase expression in cells of primary adenocarcinomas in mammary glands of bitches. Moreover, it was aimed at comparing the obtained results with an extent of Ki-67 antigen expression, which reflects the proliferation rate of tumour cells. Material for the studies was sampled during surgery in 35 bitches of various breed, ranging in age from 5 to 14 years, which developed mammary tumour. Using histopathology, the tumours were verified as representing adenocarcinomas. This was followed by immunohistochemical reactions for the detection of telomerase and Ki-67 expression. The obtained preparations were photographed and the images were subjected to computer-assisted analysis using the MultiScaneBase V 14.02 software. The obtained mean positive correlation (r = 0.26) between the expression of Ki-67 proliferative antigen and that of telomerase in mammary adenocarcinoma of bitches points at the involvement of the enzyme in neoplastic processes in animals, in an analogy to breast cancers in women.

Key words: dog, mammary gland, telomerase, adenocarcinoma, Ki-67 antigen.

The name of telomere, representing a junction of two Greek words of telos (end) and meros (part), was presented for the first time at the beginning of the 20th century in studies of Muller and McClintock (20, 22). The authors were consistent in ascribing a protective role to the terminal fragments of chromosomes. Subsequent investigators, i.e. Olovnikow (27), in the second half of the 20th century, documented the relationship between the shortening of telomeres and cell survival, expressed by the number of mitotic divisions. The telomeres, localised at chromosome ends, consist of repeated hexanucleotides (TTAGGG) bound to the TBP (telomere binding protein) (31). Even if they contain no coding sequences, the structures are responsible for the normal course of several processes linked to cell division. The telomeres allow for a complete replication of cells, in the course of which no genetic information is lost and only the telomeres are shortened by 50-200 base pairs (1, 17). In addition, due to their structure, the telomeres protect chromosomes from the harmful action of endonucleases and from abnormal (non-homologous) joining of chromosome ends, preventing the development of the so-called chromosomal instabilities (2, 4, 19, 26). Moreover, the telomere is regarded as a specific clock, which informs the cell when a critical number of divisions is exceeded, which stimulates mechanisms leading to cell apoptosis i.e. a programmed cell death (9, 10, 29, 34). The telomerase represents an enzyme, which is strictly linked to the structure and function of the telomere. The enzyme posses reverse transcriptase activity and adds nucleotides to the terminal portions of chromosomes taking advantage of its own RNA template and in this way elongates the telomere (4, 8).

It should be stressed that the structure and biological behaviour of the telomere and telomerase are very similar in human and dog cells. This has been confirmed by the isolation of canine catalytic subunit of telomerase (dogTERT), representing a functional analogue of human transcriptase (hTERT) (18, 24, 25). In many studies of human or animal tissues, the activity of telomerase was demonstrated in embryonic cells and in male genital cells. Among somatic cells, the activity of the enzyme was demonstrated only in cells of regenerating tissues, i.e. in dermal stem cells, haematopoietic stem cells, activated lymphocytes, cells of proliferating endometrium, and in cells of intestinal crypts (3, 33). In contrast to the majority of somatic cells, neoplastic cells manifest augmented activity of telomerase in over 90% types of tumours (7, 33). It
should be mentioned that presence of active telomerase in a cell by itself is not equivalent to the acquisition by the cell of a neoplastic phenotype (5, 15). Nevertheless, constant presence of the telomerase and, thus, uncontrollable cell divisions promote mutation accumulation, which when their critical number (around 4 to 6) is exceeded, may induce neoplastic transformation of the cell. It should also be added that studies in recent years demonstrated the presence of a telomerase-independent alternative mechanism of telomere lengthening: ALT (alternative lengthening of telomeres) based probably on homologous recombination between sister chromatids (6, 23).

As mentioned above, in contrast to normal somatic cells, most of tumour cells manifest clearly shorter telomeres than those in normal cells as well as high activity of telomerase (12, 13, 16). Such a situation requires permanent activity of telomerase due to which neoplastically transformed cells may continue proliferation bypassing the control of the "mitotic clock" (11, 35). Function of "the clock" in the life of a cell consists of a few stages. At the first stage, when the cell has divided around 60-70 times, is accompanied by shortening of its telomeres to such an extent that their spatial structure is disturbed. This results in stopping of cell divisions or in a dormant (rest) phase, termed also the M1 phase. If the cell inherits or acquires defects in the key proteins required for detection of short telomeres (p53/pRb), it will continue proliferation until a time when it contains already very short telomeres (M2 phase). At the phase M2, the short telomeres switch on DNA reparative processes (among other, the HR or homologous recombination mechanism), which in most cases result in disordered joining of various chromosomes ends, leading to the so-called mitotic catastrophe, most frequently terminating by apoptosis. However, not all cells undergo apoptotic death following the M2 phase. It is estimated that probably due to inherited mutations in suppressor genes and/or oncogenes, approximately 1/10^7 of the cells activate proliferation rate. Formalin-fixed and paraffin-embedded tissues were freshly cut (4 µm). The sections were mounted on Superfrost slides (Menzel Gläser, Germany), de-waxed with xylene, and gradually rehydrated. The activity of endogenous peroxidase was blocked by 5 min exposure to 3% H_2O_2. The detection of telomerase and Ki-67 antigen expression was preceded by 15 min exposure of the sections to boiling Antigen Retrieval Solution (DakoCytomation, Denmark) in a microwave oven at 250 W. For the demonstration of telomerase and Ki-67 antigen expression in the paraffin sections, mouse monoclonal antibodies were used in the following concentrations: clone 44F12 (1:75) (Novocasta, UK), clone MIB-1 (1:100) (DakoCytomation, Denmark). The antibodies were diluted in the Antibody Diluent, Background Reducing (DakoCytomation, Denmark). The sections were incubated with the antibodies for 1 h at room temperature. Subsequently, incubations were performed with biotinylated antibodies (15 min, room temperature) and with streptavidin-biotinylated peroxidase complex (15 min, room temperature) (LSAB2, HRP, DakoCytomation, Denmark). DAB (DakoCytomation, Denmark) was used as a chromogen (7 min, room temperature). All the sections were counterstained with Mayer’s haematoxylin. In every case, controls were included in which specific antibody was substituted by the Primary Negative Control (DakoCytomation, Denmark).

Microphotographs of the obtained preparations were subjected to computer-assisted image analysis via a computer coupled to an Axioshot optical microscope (Carl Zeiss, Germany). The set had the potential to record images and to perform their digital analysis. The measurements took advantage of the MultiScanBase V 14.02 software (Computer Scanning Systems, Poland).

The expression of telomerase was appraised using the modified semiquantitative IRS scale, according to Remmle (Table 1) (30).

The method takes into account both the proportion of positive (stained) cells and the intensity of the reaction colour, while its final result represents the product of the parameters, with values ranging from 0 to 12 points (no reaction = 0 points (--); weak reaction = 1-2 points (+), moderate reaction = 3-4 points (++), intense reaction = 6-12 points (+++)). The expression of Ki-67 was evaluated quantitatively by the estimation of the percentage of positive cells (0-5% = no reaction (--), 6-25% = weak reaction (+), 26-50% = moderate reaction (++), above 50% = intense reaction (+++)). The results were subjected to statistical analysis using the Statistica PL 7.1 software (STATSoft, Krakow, Poland) employing Spearman’s correlation analysis.

Material and Methods

Material for the studies was obtained in the course of surgery in 35 bitches of various breeds, aging 5 to 14 years, which developed a mammary tumour. The tumours were histopathologically verified as representing adenocarcinomas.

Results and Discussion

The expressions of both telomerase (Fig. 1) and of Ki-67 proliferation-associated antigen (Fig. 2) were demonstrated in mammary adenocarcinomas in bitches.
Table 1
Semiquantitative IRS scale taking into account both percentage of positive cells (A) and intensity of the reaction colour (B), with the final score representing product of the two variables (A x B)

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<th>A</th>
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<td></td>
<td>0 pts – no cells with positive reaction</td>
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<td>0 pts – no colour reaction</td>
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<td>1 pts – to 10% cells with positive reaction</td>
<td>1 pts – low intensity of colour reaction</td>
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<td>2 pts – 11-50% cells with positive reaction</td>
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<td>3 pts – 51-80% cells with positive reaction</td>
<td>3 pts – intense colour reaction</td>
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<td>4 pts – &gt; 80% cells with positive reaction</td>
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Moderate or high levels of telomerase expression were noted in over 36% of the examined tumours, while Ki-67 antigen was expressed in slightly less than 14% of tumours. Evident differences were observed in expression intensity of the studied markers (Fig. 3).

In over 40% of tumours, telomerase expression was quantitated at +, in over 27% it was valued at ++, and in over 9% of tumours at ++++. A similar level of marker expression (65-80% telomerase-positive tumours) was documented by Saito et al. (32) and by Umbricht et al. (36) in studies on breast carcinoma in women. Panarese et al. (28), in turn, examined telomerase activity in mammary cancers in bitches and detected telomerase expression in just 51% of studied tumours.

In the case of Ki-67 antigen, the expression of the protein at the + level was noted in 18% of the studied tumours and expression at the ++ level was observed in almost 18% of the tumours. It should be mentioned that over 68% of tumours failed to manifest expression of the protein and no tumour showed the expression at the +++ level. Over 57% of tumours with Ki-67 expression manifested in parallel a moderate (3-4 points) or intense (6-12 points) expression of telomerase. It should be added that among tumours with moderate or high expression of telomerase, 50% demonstrated Ki-67 expression at the + or higher level.

Statistical analysis performed on the entire group of studied tumours using the Spearman's test demonstrated positive correlation of a moderate extent between expression of telomerase and that of Ki-67 antigen ($r = 0.26$) (Fig. 4).

In analogous studies conducted by Ikeda et al. (14) and those performed by Mokbel et al. (21) on breast carcinomas in women, the positive correlation was also disclosed between expression intensities of the mentioned markers with such a difference that in female breast cancers, the correlation was even more accentuated.

The positive correlation between expression of Ki-67 proliferative antigen and that of telomerase in mammary adenocarcinoma in bitches detected in our studies, may point to the involvement of the enzyme in neoplastic processes in animals, in comparison to the situation in women. The finding may provide additional proof for the suitability of a canine body as an experimental model in studies on mechanism of carcinogenesis in humans.
Fig. 3. Distribution of expression intensities of telomerase and Ki-67 antigen in mammary adenocarcinomas in bitches.

Fig. 4. Correlation between telomerase and Ki-67 antigen expression in canine mammary adenocarcinomas, \( r = 0.26, \ P<0.05 \) (Spearman’s correlation).

References


