Canine Scent Detection of Volatile Elements, Characteristic of Malignant Cells, in Cell Cultures

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ABSTRACT: **Background:** In recent years several reports have been published describing dogs' ability to detect, by scent, patients with cancer. This ability is based on the sniffing of volatile organic elements that are secreted by malignant cells or react to them.

Objectives: To evaluate the ability of trained dogs to detect breast cancer cell cultures (MCF7) compared to the control pseudo-normal keratinocyte cell line (HaCaT), and to detect melanoma (BG) and type 2 epithelial lung carcinoma (A549) malignant cell cultures to which they were not previously exposed in the course of their training.

Methods: Cell cultures were prepared in a standard manner. Two Belgian Shepherd dogs were trained and then tested in a single-blind test (for dogs and trainers) on their ability to detect the "target specimen," a MCF7 breast cancer cell culture. Following this, the ability of the dogs to detect cancer cell cultures that they were not previously exposed to (i.e., A549, BG) was tested. In each test round, four specimens placed in identical blocks were arranged in a line with one meter between them: one target specimen (MCF7, A549, BG), two control specimens (HaCaT), and a sample containing cell culture medium only.

Results: The two dogs picked out all the target specimens of MCF7 breast cancer cell cultures that they were trained to detect (10/10) as well as all the target specimens that they were not previously exposed to [A549 (5/5) and BG (5/5)], but did not pick out the control specimens or the cell culture medium. Thus, the sensitivity, specificity, and positive and negative predictive values for both dogs were 100%.

Conclusions: The results of this study support the assumption that cancer cells have a unique odor pattern, and that this odor pattern is common to different types of cancer.

IMAJ 2015; 17: 567–570

KEY WORDS: canine scent detection, malignant cells, cancer

"Does hot water have a smell?" asked the Doctor. "Certainly it has," said Jip. "Hot water smells quite different from cold water. It is warm water – or ice – that has the really difficult smell. Why, I once followed a man for ten miles on a dark night by the smell of the hot water he had used to shave with – for the poor fellow had no soap..."

A monolog by Jip the dog after his sniffing ability was challenged by Gab-Gab the pig, from *The Story of Doctor Dolittle* by Hugh Lofting, 1920, page 155

The effectiveness of early detection tests for colon and breast cancer or premalignant clinical conditions has been proven repeatedly through controlled studies [1,2]. Early detection testing for colorectal and breast cancer is in broad clinical use today, but there are other types of cancer for which there are no effective early detection tests. Therefore, a test that could identify biological markers that are secreted by cancer cells, or by healthy cells in reaction to the malignant process, could be of great value in increasing the sensitivity and specificity of existing early detection tests or could itself serve as an early detection test where none exists [3].

In the last decade few controlled trials evaluated the ability to detect specific organic volatile compounds by "electronic nose" or chromatography, using expired air from cancer patients as compared to that from healthy volunteers, or to define these compounds from different cell lines. The volatile compounds that differed between the expired air of patients and of healthy volunteers were alkanes, some aromatic compounds, and benzene derivates [4-6]. A recent study by Fu et al. [7] using mass spectrometry technology showed that the concentrations of 2-butanone, 2-hydroxyacetaldehyde, 3-hydroxy-2-butanone, and 4-hydroxyhexanal in the exhaled breath of lung cancer patients were significantly higher than in the exhaled breath of healthy smoker and non-smoker controls and patients with benign pulmonary nodules. The concentration of 2-butanone in exhaled breath of patients with stages II through IV non-small cell lung cancer was significantly higher than in exhaled breath of patients with stage I.

The idea of using a dog's olfactory sense for the early detection of cancer was first raised by Williams and Pembroke [8] and reported in *The Lancet* in 1989. These authors described the case of a patient who visited the clinic because her dog showed a particular interest in a skin nevus she had. Following its excision the pathological examination revealed malignant melanoma. Few studies have reported the results of a systematic assessment of the ability of dogs to detect different types of cancer by smelling [3,9-12].

The present study was designed to address two key issues arising from previous studies that dealt with the ability of dogs to detect volatile elements associated with cancer. The first is whether the source of the odor pattern is in the cancer cells themselves or is a reaction of healthy cells to the malignant process. The second is whether there are volatile elements that are common to different types of cancer.

MATERIALS AND METHODS

CELL CULTURES

The dogs were trained using cell cultures of breast cancer MCF7 (ATCC, HEB-22) cells (target specimens) compared to the pseudo-normal keratinocyte cell line HaCaT (CLS-HaCaT-DKFZ), which does not have malignant potential in laboratory animals (control specimens). The cell cultures were prepared in Prof. Gopas laboratory in the Faculty of Health Sciences of Ben-Gurion University of the Negev. The cell culture medium used was DMEM+10% heat-inactivated fetal calf serum + penicillin/streptomycin, purchased from Biological Industries, Beit HaEmek, Israel. The cell number in all cell cultures (target and control) was 10^6. The cell cultures were prepared in cell culture plates with a diameter of 5 cm. The volume of medium in all plates was 5 ml. The cell cultures were stored at 4ºC, up to 24 hours, until the training began. Cell cultures were used only for one day of training. Cell cultures from type 2 epithelial lung carcinoma (A549 ATCC CCL-185) and melanoma (BG) [13] that were used in the testing stage (see below) were prepared using the same techniques and conditions.

DOGS AND TRAINING PROCEDURES

Two female Belgian Shepherd dogs were trained by licensed dog trainers with experience in training dogs to detect explosives and to identify people who need to be singled out in a group. The trainers chose the dogs based on their proven success in previous missions. One was a 5 year old dog of the Groenendael type, and the other, of the Malinois type, was 2½ years old. Their training lasted for 6 months, January to June 2011, in two training phases with a rest interval of 3 months in between. During the training the dogs were kept under appropriate conditions with veterinarian surveillance, as required. Training took place in a kennel with a dirt surface. The target and control specimens were placed in identical concrete blocks,

which were laid out so that neither dog could get to the specimen. The blocks were arranged in a straight line at one meter intervals. During each search round for the target specimens the dogs were accompanied by a trainer who ordered them to search for the target specimens using the words "search" or "where." During the search the dogs were either tied to a leash or were free but kept next to the trainer at his discretion, based on the dog's behavior on any given training day. The guiding principal for training the dogs was continuous positive reward, i.e., the dogs were allowed to play with a tennis ball or were given food for a correct detection. In the first phase the dogs were trained to detect the smell of the target specimens that were presented to them and hidden in different places in the training site. In the second phase the dogs had to identify the target specimen when they had also been presented with a specimen that contained cell culture medium without cells (the correct of the two); in the third phase they had to differentiate between the target and control specimens in the same fashion. When the dogs reached the level where they no longer made incorrect decisions on the two choices, they progressed to the training phase in which the study tests were implemented. In this phase they had to detect the target specimen from among four specimens that included, in addition to the target specimen, two control specimens and one sample with cell culture medium without cells, to ensure that the dogs were not detecting the target specimens "by exclusion."

CLASSIFICATION OF THE DOGS' REACTIONS

During the training period and the test stage the dogs learned the course they were expected to follow when they tried to detect the target specimen from among the others. They were trained to sniff all the specimens before they marked the target one. If no specimen was detected at the end of one round of sniffing the dogs were allowed a second round. A correct detection was defined as: (i) identification of the target specimen by sitting or lying down in front of the block that contained the specimen, or (ii) sniffing while ignoring the control specimen. An incorrect detection was defined as: (i) identification of the control specimen as the target specimen, (ii) sniffing without sitting or lying down in front of the target specimen, or (iii) unclear behavior on the part of the dog.

THE TEST

When the dogs succeeded in detecting the target specimen consistently it became possible to test their ability to detect the target specimen in an objective manner. A test was conducted on one day at the training site. In every test round four specimens were arranged in a row of identical blocks placed one meter apart. The specimens included the target specimens (MCF7, A549, BG), two control specimens (HaCaT), and a specimen that contained cell culture medium only. At the end of each round the specimens and the placement within the row

were changed. Arrangement of the specimens before each test round took place beyond the field of view of both the dogs and the trainer. When allowed to do so the dog and trainer started out on the detection round. After detection of a specimen and confirmation from the study coordinator, who had arranged the specimens and stayed in a corner of the kennel, that the identification was correct the dog was rewarded as described above. Each dog's ability to detect correctly was tested separately. Breaks for rest and drink were taken, as deemed necessary by the trainer. During the course of the test each dog made the following detection rounds: (i) ten rounds to detect the target specimen they were trained to identify (cell cultures of MCF7 cancer cells), (ii) five rounds to detect each of the target specimen they were not exposed to during training (A549, BG), and (iii) a single round in which there were three control specimens, one sample with nutrient medium, and no target specimens. In all, there were 21 detection rounds for each dog.

RESULTS

The specimen arrangement for the test round was single blind, i.e., for the dog trainer and the dogs. Both dogs marked all target specimens as positive in all ten rounds in which there were MCF7 breast cancer cell cultures (10/10) and did not mark any of the control specimens or the cell culture medium as positive. The dogs did identify all target specimens in the rounds in which they were type 2 epithelial lung cancer (A549) (5/5) and all the rounds in which the target specimens were melanoma BG (5/5). In these cases they did not select any of the control specimens or the cell culture medium as positive. The two dogs also did not select any specimen as positive in the lone round in which only control specimens and nutrient medium without any target specimens were presented.

Thus, the sensitivity, specificity, and positive and negative predictive values for both dogs for the detection of positive and negative specimens was 100%. Both dogs were particularly hesitant in making their selections in the round in which the target specimens were cells that they had not been trained to detect (A549, BG). Although the level of accuracy in detecting these specimens was also 100%, there was a clear difference in the dogs' decisiveness in these rounds compared to the round with MCF7 as the target specimen.

DISCUSSION

In the present study, we tested the ability of dogs to detect cancer cell culture specimens in comparison with control specimens. The two trained dogs demonstrated a 100% ability to detect the target cell culture [MCF7 breast cancer cultures, type 2 epithelial lung carcinoma cells (A549) and melanoma (BG) cells] while the expected success rate would be 0.25^n (n = number of search rounds). Their success rate did not differ for

the cell culture for which they were trained and for cell cultures that were presented to them without any previous training.

This study has several limitations. First, the presence of the study coordinator, who arranged the cell specimens, in the kennel during the test could have influenced the detection of the target specimens. However, there was no eye contact with the study coordinator until the dogs had detected the specimens. Second, we assessed only one concentration of cell cultures. Accordingly, it is impossible to propose a certain "smell threshold" for the dogs' detection ability. The most important limitation is the question of the generalizability of this study, which was conducted on cell cultures only, to human studies based on the olfactory ability of dogs for the early detection of cancer.

Studies to date that evaluated the ability of dogs to detect, by sniffing, volatile elements secreted from malignant tumors have raised four main issues:

- Do dogs actually have the ability to detect malignant tumors by smelling expired air, stool, or urine?
- Do different types of cancer have unique odor patterns so that dogs trained to detect a specific type of cancer would not be able to detect the smell of another?
- Are volatile elements that the dogs detect secreted by the malignant cells themselves or as a reaction to them?
- The issue of generalizability, i.e., under the assumption that the dogs do, indeed, have the ability to detect volatile organic elements, can this ability be exploited as a complementary or unique screening method for early cancer detection?

Three of the controlled trials that were published to date demonstrated success in detecting specimens taken from cancer patients in comparison to controls. McCullen et al. [3], in a study of four trained dogs, reported sensitivity and specificity rates of 99% for the detection of samples of expired air from lung cancer patients, and sensitivity and specificity rates of 88% and 98%, respectively, for the detection of breast cancer patients. Furthermore, there was no difference in the dogs' ability to detect target specimens from patients with early-stage cancer compared to advanced cancer [3]. Similarly, Horvath and team [10], in a study of the ability of a single dog trained for one year to detect ovarian cancer in pathology specimens, reported sensitivity and specificity rates of 100% and 97.5%, respectively [10]. Sonoda and co-authors [11] who studied dogs' ability to detect colon cancer from expired air and stool specimens reported a sensitivity rate above 90% and a specificity rate of 99% for both types of specimens. These controlled studies, taken together with the results of the present study, provide definitive proof for the contention that dogs can detect different types of cancer by sniffing the volatile elements secreted by the tumor cells or in reaction to them. In studies that found a lower detection rate, such as the study by Willis et

al. [12], the reason seems to lie in the choice of dogs or in the less than optimal training process.

There is a significant discrepancy between the results of the two studies that investigated whether there is a unique odor pattern for different types of cancer. While the dog that was trained by Horvath et al. [10] did not detect specimens from other types of malignant tumors than ovarian cancer, the dog in the study by Sonoda and colleagues [11] which was trained to detect colon cancer did mark as positive expired air and stool specimens from patients with other types of cancer (prostate, breast, stomach). It should be emphasized that the question of unique odor pattern was not the central study question in these investigations and was not tested directly.

In the present study the sensitivity and specificity for the detection of malignant cells that the dogs were not exposed to over the course of their training was 100%. We purposely chose lung carcinoma cells (A549), which are close in origin to the breast cancer cell cultures (MCF7) that the dogs were trained to detect, as well as melanoma cell cultures (BG) that have an ectodermal origin rather than an endodermal origin like breast and lung cancer. The dogs' detection ability was related to the presence of volatile elements that are characteristic of different types of cancer, even types that are very distant from each other in terms of their origin.

In addition, one of us (J.G.) recently published an article where urine of mice inoculated with B16 melanoma was compared with urine of tumor-free animals [14]. Specific volatile tumor-specific molecules were described. Increased concentration of these molecules was detected in correlation with tumor mass. Others also detected some of the same molecules in additional melanomas or in other tumor types. Thus, it is expected that increased tumor mass will result in higher concentrations of tumor-related molecules. These molecules may be both specific and restricted to a defined tumor type or of wider expression among different tumors. In principle, dogs may be trained to detect single defined compounds or combinations of various molecules and concentrations.

In conclusion, the results of the present study support the assumption that malignant cells have unique odor patterns that can be distinguished from cells that do not have malignant potential and that a common odor pattern exists for different types of cancer. The question of the generalizability of the results presented here to early detection of cancer pro-

grams remains unresolved. To date, no study has checked the feasibility of using this ability of dogs for screening purposes. This should be tested in more comprehensive and larger studies, because the potential appears to be promising.

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"It is a curious thought, but it is only when you see people looking ridiculous that you realize just how much you love them"

Agatha Christie (1890-1976), English crime novelist, short story writer and playwright

"Be not too hasty to trust or admire the teachers of morality; they discourse like angels but they live like men"

Samuel Johnson (1709-1784), British poet, essayist, moralist, literary critic, biographer, editor and lexicographer