Ann. Anim. Sci., Vol. 16, No. 1 (2016) 79–85 *DOI: 10.1515/aoas-2015-0068*

DE GRUYTER
OPEN

IDENTIFICATION OF UNBALANCED ABERRATIONS IN THE GENOME OF EQUINE SARCOID CELLS USING CGH TECHNIQUE*

Monika Bugno-Poniewierska[1♦], Beata Staroń[2], Leszek Potocki[2], Artur Gurgul[1], Maciej Wnuk[2]

[1]Department of Animal Genomics and Molecular Biology, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland
[2]Centre of Applied Biotechnology and Basic Sciences, University of Rzeszow, Werynia 502, 36-100 Kolbuszowa, Poland
♦Corresponding author: monika.bugno@izoo.krakow.pl

Abstract

Sarcoid is the most common skin cancer in horses. The etiology of the tumor is associated with BPV infection (BPV-1, -2, -13), which is an inducer of malignant transformation. The comparative genomic hybridization (CGH) technique identifying the unbalanced chromosome aberrations was used to analyze the genome of equine sarcoid cells and to diagnose the chromosome rearrangements involving large deletions or amplification. The results were based on the analysis of 100 metaphases and their karyograms as well as the diagram showing the average ratio of the intensity of the green to red fluorescence, using MetaSystems software (Isis). Based on a comparison of the fluorescence intensity ratios we found duplication in the subtelomeric regions of chromosome pairs 1, 4, 7, 8 and 23. Duplicated region of chromosome pair 1 also included the coding region of the rDNA. In the chromosome 23 next to the duplication occurring in the centromeric region of q arm (23q11) we also found the presence of deletions involving 23q18-23q19 region. For the chromosome pairs 25 to 31 and the X chromosome the software failed to generate CGH diagram, but on the individual karyograms we were able to observe fluorescence signals characteristic of duplication (red), in rDNA regions of chromosome pairs 28 and 31. The study showed that duplications of DNA present in the sarcoid cells are found mainly in the telomeric and rDNA regions. The presence of the duplication of telomeric regions is associated with increased activity of the telomerase enzyme, which is a hallmark of cancer cells, affecting the immortality of these cells. Accordingly, duplications of rDNA coding regions increase activity of nucleolar organizer region which is a tumor marker.

Key words: horse, sarcoid, comparative genomic hybridization

Equine sarcoid is the most common neoplasm in horses. A locally aggressive tumor with varying degrees of invasion, it is characterized by excessive proliferation of dermal fibroblasts, forming warts and ulcerations. Although not a fatal disease, dermal sarcoid is a significant problem causing economic losses to horse breeders

*The work financed from the research activities of the National Research Institute of Animal Production, project no. 07-4.06.7.

who have to remove the growth, administer treatment, and in some cases euthanize the animal. The equine sarcoid is associated with skin lesions, most commonly occurring on the head, neck and legs (Cotugno et al., 2013; Szczerba-Turek et al., 2009).

Because sarcoids have not been adequately studied, it is essential to identify changes that occur in this type of neoplasm at both chromosomal and molecular levels. Cancer cells are characterized by chromosomal instability (CIN) with accumulation of structural aberrations (deletions, duplications, translocations) and abnormal chromosome number (poliploidy, aneuploidy). Analysis of changes in the genome of cancer cells reveals the critical regions, namely genes or whole groups of genes important for the origin and development of cancer. In principle, karyotype determination may be based on classical cytogenetic methods (GTG, CBG, RBG and Ag-NOR banding), but these techniques are laborious, associated with errors, and above all require the use of cell culture, which in the case of cancers is often hard and time-consuming to optimize, and sometimes the preparations obtained are of poor quality. These problems can be circumvented by using comparative genomic hybridization (CGH), which is one of the basic techniques for analysis of chromosome aberrations in cancers, mainly solid tumors (Łaczmańska and Kozłowska, 2009) and sarcomas (Nishio et al., 2010), but also for identification and characterization of constitutive aberrations (e.g. marker chromosomes), and allows identifying genes responsible for the neoplastic lesions. CGH produces a map of DNA sequence copy number and location on all chromosomes in the genome.

This technique consists in hybridization of DNA isolated from test and reference cell populations, which are differently labeled and hybridized in a 1:1 ratio to a normal metaphase spread. The hybridization is detected with two different fluorochromes. Regions of gain or loss of DNA sequences, such as deletions, duplications, or amplifications, are seen as changes in the ratio of the intensities of the two fluorochromes along the target chromosomes. In the next stage, the karyograms are arranged and analyzed with specialized software. Ten to 16 chromosomes are usually analyzed for each chromosome pair to generate a sum karyogram of fluorescence intensity profiles in both colors along whole chromosomes. The intensity profiles are considered normal if they are within the range 0.75 to 1.25; values higher than 1.25 are considered as duplication (amplification) of the genetic material, and those below 0.75 as deletion of a given fragment (Kallioniemi et al., 1992; Kozłowska and Łaczmańska, 2010).

In the present study, comparative genomic hybridization technique was used to identify unbalanced aberrations in the genome of sarcoid cancer cells.

Material and methods

Metaphase slide preparation

Metaphase chromosomes are prepared according to standard protocols using pokeweed mitogen stimulated peripheral blood lymphocytes from a karyotypically

normal mare. High quality metaphase preparations for CGH should ideally have little cytoplasm (too much cytoplasm causes high background levels and may prevent optimal denaturation), minimal overlapping of the chromosomes (overlapping chromosomes need to be excluded from CGH analysis), and low cell density paired with a high mitotic index. In addition, the chromosomes should be of adequate length (400–550 bands) and not contain separated chromatids.

DNA isolation from tumor tissue

Biopsy of sarcoid tissue was taken from the abdominal area of a mare at the age of 3 years. Tissue samples came from the area of sarcoid and skin that was not covered by histopathological changes. Each sample was examined histologically. The tested sarcoid was classified as a fibroblast type. DNA was extracted from sarcoid tissue and healthy tissue of a horse. DNA isolation from tissue samples was performed using the commercially available Gentra Puregene kit (Qiagen, Germany) according to the manufacturer's protocol. Prior to labeling the isolated DNA, DNA fragmentation was performed to obtain fragments between 100 bp and 600 bp. Electrophoresis in 1% agarose gel was carried out to examine fragmentation results.

Isolated DNA was labeled according to FISH Bright Nucleic Acid Labeling Kit procedure (Kreatech, The Netherlands). 1 μg of reference DNA (from healthy tissue) was ULS-labeled in green, whereas 1 μg of sarcoid DNA was Cy3-ULS labeled in red. The normal labeled and tumor genomic DNA (1 μg each) was precipitated at –20°C for 16 hours in the presence of 50x horse Cot DNA and 5 μg ssDNA. The labeled probes were suspended in hybridization mixture (10% DS, 10% 20xSSC, 50% Formamid, 10% Tween 20, 20% water).

Hybridization

The probe and the normal metaphase slides were denatured separately: the slides in 70% formamide/2´ SSC at 72°C for 2–3 minutes (depending on the metaphase slide batch), and the probes in a water bath at 80°C for 10 minutes. The probes were mixed in a 1:1 ratio and applied to the denatured metaphase slides. The hybridization was conducted under a coverslip in a humid incubator at 37°C for 24 hours. After hybridization, the slides were washed and counterstained with DAPI (0.35 μg/ml in an antifade solution). Stained slides were analyzed under a fluorescence microscope (Olympus BX61 with Olympus DP72 camera). Isis software (version 5.3, MetaSystems) was used to construct the ideogram with the pattern of fluorescence ratios.

Results

The slides were analyzed under a fluorescence microscope equipped with a set of filters (DAPI, FITC, Spectrum Orange) and a CCD camera. For each chromosome, 12–20 copies from different metaphases were analyzed. The use of CGH technique and the analysis of 100 photographs in a dedicated program (Isis version 5.3 MetaSystems) produced complete karyograms of 7 metaphases (Figure 1) and a sum karyogram with the green to red fluorescence ratio (Figure 2).

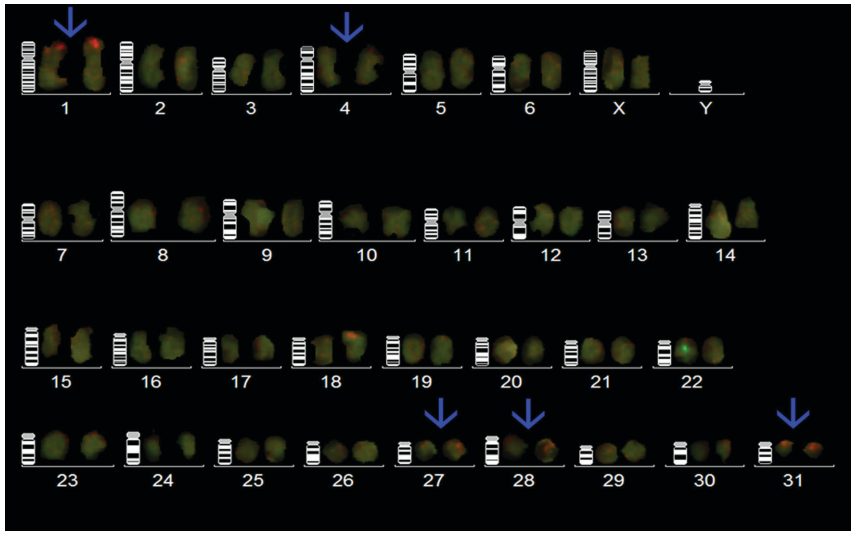

Figure 1. Sample karyograms of a mare (2n=64). Sarcoid DNA is stained red and reference DNA is green. Blue arrows indicate clear red fluorescence signals

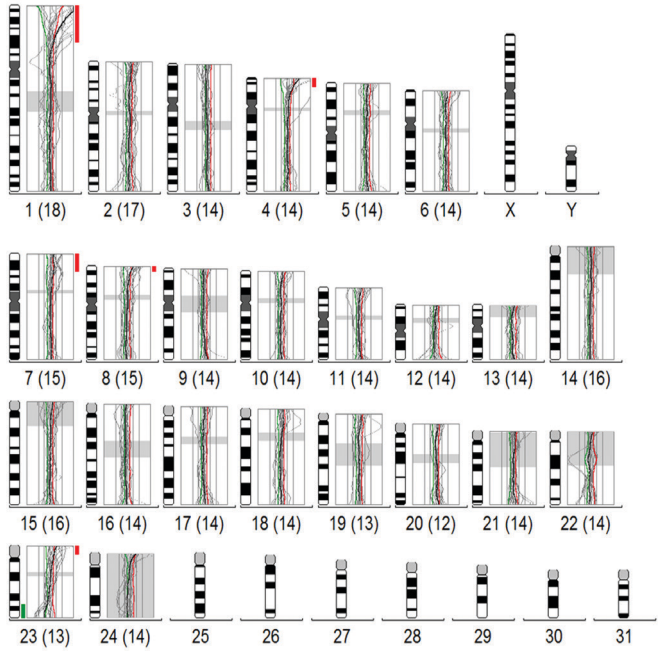

Figure 2. CGH profiles, comparison of fluorescence intensity ratios. Duplications were found in subtelomeric regions and in rDNA coding regions (chromosome pairs 1, 4, 7, 8, 23) – red markings on the right side of the diagrams, in addition to a deletion (pair 23) – green marking on the left side of the diagram. Numbers in brackets are the amount of chromosomes from which the CGH sum karyogram was created

Clear red signals (sarcoid DNA) were observed on chromosome pairs 1, 4, 27, 28 and 31 already at the stage of karyotype arrangement. The preparation of seven karyograms and selective analysis of chromosomes from successive metaphases helped to avoid random results and produced diagrams of averaged fluorescence ratios.

Comparison of fluorescence intensity ratios revealed duplications (pairs 1, 4, 7, 8, 23) in subtelomeric regions and in rDNA coding regions. A deletion was also found in pair 23. For chromosome pairs 25 to 31, the program failed to generate CGH diagrams, which was probably due to poor metaphase quality. A similar situation occurred when X chromosome was analyzed.

Discussion

The chromosomal instability characteristic of cancer cells is most often in the form of chromosomal rearrangements, namely deletions or amplifications. These regions can be detected using comparative genomic hybridization, which identifies unbalanced chromosomal rearrangements. This method is used to identify chromosomal aberrations also in farm animals (Hornak et al., 2012).

The comparative genomic hybridization produced results indicative of genetic material duplication in rDNA gene coding regions. Also the findings of Potocki et al. (2012) confirm that equine sarcoid fibroblasts show increased transcriptional activity of the rDNA coding regions (NORs). In addition, they are characterized by decreased DNA methylation levels, while DNA methylation is one of the main mechanisms that inactivate transcription. The amount and size of NORs in horses is associated with the amount of rDNA gene copies, the degree of their methylation, and the location of rDNA loci in telomeric regions (Słota et al., 2007). The presence of oxidative stress and the decreased expression of antioxidant enzymes has also been observed in sarcoid cells, resulting in an increased amount of reactive oxygen species (ROS) and DNA damage, as well as the formation of micronuclei (Potocki et al., 2012). Furthermore, sarcoid cells are characterized by prolonged S phase of the cell cycle, the presence of polyploidy, DNA damage in the form of double strand breaks (DSBs) and single strand breaks (SSBs). Sarcoid fibroblasts represent the heterogeneous fraction of cells that varies in some metabolic aspects. Infection with BPV1 virus induces the process of neoplastic transformation but also causes premature cellular senescence (Potocki et al., 2014).

The results obtained in the present study are indicative of clear DNA addition in telomeric and subtelomeric regions. However, Argyle et al. (2003) demonstrated that telomere length on horse chromosomes is strictly associated with the cellular aging process, whereas telomerase activity in equine sarcoid cells is not increased. Low telomerase activity may be due to oxidative stress (Bugno-Poniewierska et al., 2012; Potocki et al., 2014).

Our study also showed that DNA duplications in sarcoid cells mainly occur in the telomeric regions and in rDNA. The increased number of gene copies in telomeric regions is associated with increased telomerase enzyme activity, which is

a hallmark of cancer cells, affecting the immortality of these cells. Ribosomal DNA forms nucleolar organizer regions (NORs), whereas increased NOR activity is a tumor marker. The increased amount of rDNA copies will lead to an increase in the amount of rRNA, which is the precursor of ribosomes. By this means, a cancer cell satisfies the increased demand for production of proteins.

In summary it may be stated that comparative genomic hybridization is useful for finding unbalanced aberrations also in animals. This technique allowed identifying chromosome fragments, the deletion or amplification of which plays an important role in tumorigenesis.

References

Argyle D., Ellsmore V., Gault E.A., Munro A.F., Nasir L. (2003). Equine telomeres and telomerase in cellular immortalisation and ageing. Mech. Ageing Dev., 124: 759–764.

Bugno-Poniewierska M., Potocki L., Klukowska-Rötzler J., Lewińska A., Koch C., Mählmann K., Wnuk M. (2012). Cytogenetic analysis of horse sarcoid cells. Proc. 20th International Colloquium on Animal Cytogenetics and Gene Mapping. Chromosome Res., 20: 779–814.

Cotugno R., Gallotta D., d'Avenia M., Corteggio A., Altamura G., Roperto F., Belisario M.A., Borzacchiello G. (2013). BAG3 protects Bovine Papillomavirus type 1-transformed equine fibroblasts against pro-death signals. Vet. Res., 44: 61.

Hornak M., Oracova E., Hulinska P., Urbankova L., Rubes J. (2012). Aneuploidy detection in pigs using comparative genomic hybridization: from the oocytes to blastocysts. PLOS ONE, 7: e30335.

Kallioniemi A., Kallioniemi O.P., Sudar D., Rutovitz D., Gray J.W., Waldman F., Pinkel D. (1992). Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science, 258: 818–821.

Kozłowska J., Łaczmańska I. (2010). Genetic instability – its significance in cancer development and laboratory diagnostics (in Polish). J. Onc., 60: 548–553.

Łaczmańska I., Kozłowska J. (2009). Chromosomal instability in sporadic colorectal cancer (in Polish). Contemp. Onc., 13: 177–180.

Nishio J., Iwasaki H., Nabeshima K., Ishiguro M., Isayama T., Naito M. (2010). Establishment of a new human pleomorphic malignant fibrous histiocytoma cell line, FU-MFH-2: molecular cytogenetic characterization by multicolor fluorescence in situ hybridization and comparative genomic hybridization. J. Exp. Clin. Cancer Res., 29: 153. doi: 10.1186/1756-9966-29-153.

Pawlina K., Bugno-Poniewierska M. (2012). The application of Zoo-FISH technique for analysis of chromosomal rearrangements in the Equidae family. Ann. Anim. Sci., 12: 5–13.

Potocki L., Lewińska A., Klukowska-Rötzler J., Bugno-Poniewierska M., Koch C., Mählmann K., Janda J., Wnuk M. (2012). DNA hypomethylation and oxidative stress-mediated increase in genomic instability in equine sarcoid-derived fibroblasts. Biochemie, 94: 2013–2024.

Potocki L., Lewinska A., Klukowska-Rötzler J., Bielak-Zmijewska A., Grabowska W., Rzeszutek I., Kaminska P., Roga E., Bugno-Poniewierska M., Słota E., Mählmann K., Koch C., Wnuk M. (2014). Sarcoid-derived fibroblasts: links between genomic instability, energy metabolism and senescence. Biochemie, 97: 163–172.

Słota E., Wnuk M., Bugno M., Pienkowska-Schelling A., Schelling C., Bratus A., Kotylak Z. (2007). The mechanisms determining the nucleolar-organizing regions inactivation of domestic horse chromosomes. J. Anim. Breed. Genet., 124: 163–171.

Szczerba-Turek A., Siemionek J., Raś A., Platt-Samoraj A., Mikulska-Skupień E., Bancerz-Kisiel A., Szweda W. (2009). Clinical types of equine sarcoids (in Polish). Med. Weter., 65: 827–829.

W n u k i e w i c z J. (2008). Evaluation of argophylic nuclear organizer region in the oral cavity leukoplakia (in Polish). Dent. Med. Probl., 45: 136–140.

Received: 11 IX 2015
Accepted: 2 X 2015