Praca oryginalna *Original paper*

Macrophage regulation of Wnt pathway in canine and murine mammary cancer cells*

BARTŁOMIEJ TACIAK[1], MACIEJ BIAŁASEK[1], KAROL PAWŁOWSKI[2], EVA HELLMÉN[3], EMILIA GÓRKA[1], MAGDALENA KRÓL[1]

[1]Department of Cancer Biology, Institute of Biology, Warsaw University of Life Sciences – WULS, Nowoursynowska 166, 02-767 Warsaw, Poland
[2]Department of Preclinical Sciences, Institute of Veterinary Medicine, Warsaw University of Life Sciences – WULS, Nowoursynowska 166, 02-767 Warsaw, Poland
[3]Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Box 7011, SE-750 07 Uppsala, Sweden

Received 07.10.2022 *Accepted 11.01.2023*

Taciak B., Białasek M., Pawłowski K., Hellmén E., Górka E., Król M.

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Summary

Interactions between various cell types in a tumor microenvironment (TME) are important for cancer progression and metastasis. Tumor-associated macrophages (TAMs) play a key role in this intratumoral dialogue regulating Wnt signaling pathway in cancer cells. Co-culture of canine and murine tumor cells with TAMs lead to inhibition of the canonical Wnt pathway and activation of the non-canonical Wnt pathway in tumor cells resulting in change in tumor cell phenotype and subsequent higher potential to invasion and metastasis. These molecular changes in cancer cells that are induced by TAMs resemble the quercetin activity. This natural product is a plant flavonoid from the group of polyphenols. Its biological function is inhibition of the canonical Wnt pathway. As quercetin supplementation is broadly discussed in cancer patients, findings of this study show that this compound should be used with caution, as it may enhance cancer spread.

Keywords: TAMs, Wnt, mammary cancer, tumor microenvironment, canine, murine

Complex interactions between various cell types in a tumor microenvironment (TME) influence cancer progression and metastasis. Tumor-associated macrophages (TAMs) are important components of the TME and play a key role in this intratumoral dialogue (4, 12). Their involvement in cancer development has been widely shown in the literature and is now commonly accepted. Macrophages promote tumor malignant progression by stimulating angiogenesis, increasing cancer cell migration, invasion and intravasation. At the metastatic site they promote extravasation and tumor initiation. From a therapeutic point of view, TAMs became good targets for anticancer therapy through either their ablation or their re-differentiation and polarization from pro-tumoral towards antitumoral states (6, 20).

The TAMs promote tumor development by various ways. Most of the studies showed the importance of the TAM's secretion of various enzymes and metalloproteinases responsible for matrix remodeling in TME (23). Pukrop et al. suggested that TAMs can modulate Wnt signaling within TME. The authors observed Wnt-5a secretion by the TAMs, with subsequent induction of MMP-7 expression in cancer cells and their increased invasion (24). Another group observed that TAMs secrete Wnt-7b, which enhanced angiogenesis within the tumor facilitating cancer cell spread to other organs (23).

Wnt signaling is considered as one of the most important signaling pathways. Signaling network and the functional role of the Wnt pathway in growth and development is very complex. It is the most active pathway during embryogenesis playing a key role in the formation of new organisms, mediation of cell differentiation, polarization and migration. However, it is also activated during development of many types of tumors and various other diseases. Functionally, Wnt pathway runs along two ways: β-catenin dependent (canonical Wnt pathway) or β-catenin independent (non-canonical) (15). The canonical Wnt pathway plays a fundamental role during embryogenesis.

* This study was funded by UMO-2013/09/D/NZ5/02496 SONATA grant given to M. Król and by UMO-2014/15/N/NZ5/03649 PRELUDIUM grant given to B. Taciak from the National Science Centre.

Activation of the Wnt canonical pathway leads to the stabilization and translocation of β-catenin into the cell nucleus, where it promotes the transcription of genes (9). It is a crucial regulatory pathway that controls the developmental process and tissue morphogenesis (3, 7, 14). The main role of the canonical Wnt pathway is to provide a symmetry-breaking signal common to all metazoan organisms thus simultaneously deciding cell fate. The non-canonical Wnt signaling is dichotomized into two branches: planar cell polarity (PCP) and calcium-dependent. Non-canonical Wnt refers to a group of Wnt-dependent signaling pathways which do not lead to the cytoplasmic stabilization of β-catenin but is responsible for cell polarity.

The aim of this study was to evaluate the influence of macrophages on the Wnt signaling pathway in canine and murine cancer cells lines: CMT-U27, CMT-U309, P114, EMT6 and 4T1. The expression level of selected Wnt genes was examined and cellular location of selected Wnt proteins involved in cell motility and therefore, metastasis was shown. Moreover, the influence of quercetin on the Wnt signaling pathway in cancer cells was studied. Its biological function is inhibition of the canonical Wnt pathway. This is particularly important, as quercetin supplementation is one of the approaches in cancer patients.

Material and methods

Cell lines and culture conditions, co-culture experiments and cell sorting. Canine mammary cancer cells: CMT-U27 (simple carcinoma cell line), CMT-U309 (spindle-cell mammary tumor cell line) and P114 (anaplastic cancer cell line) were cultured as described in previous studies (19). They were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Life Technologies).

Murine macrophage-like cell lines RAW 264.7 and J7744 cell lines were purchased from American Type Culture Collection. Cells were cultured in DMEM high glucose medium supplemented with 10% FBS (all the experiments were performed using the same FBS batch) and 1% penicillin/streptomycin, in atmosphere of 5% CO_2 and 95% humidity at 37°C. Cells were passaged after reaching 90% confluence, detached with cell scraper and subcultivated in 1 : 6 ratio in T-75 flasks. All cell culture equipment (flasks, pipettes etc.) used in this study were from the same batch. Cells were regularly tested for mycoplasma contamination.

The EMT6 and 4T1 murine mammary carcinoma cell lines were purchased from American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 10% and 1% penicillin/streptomycin, in atmosphere of 5% CO_2 and 95% humidity at 37°C. Cells were passaged after reaching 90% confluence, detached with trypsin-EDTA and subcultivated in 1 : 6 ratio in T-75 flasks. Cells were regularly tested for mycoplasma contamination.

Monocyte-derived macrophages (MDM) were cultured as previously described (19). Blood leukocytes from dogs were obtained and CD64-positive cells were gated, sorted, and grown in co-culture with cancer cells or as a mono-culture supplemented with 25 μg/mLCSF-1 (5). Macrophage-conditioned medium was obtained from RAW 264.7 cells cultured in Dulbecco's modified Eagle's (DMEM) high glucose medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. The medium was collected, centrifuged 20 min at 3000 × g at 4°C, filtered and stored at 4°C until use.

For co-culture purposes neoplastic cell lines were grown in cell culture flasks, then stained with the Orange Cell Tracker fluorescent dye CMTMR (Invitrogen, USA) directly before co-culture. Sorted monocytes were seeded on top of the cancer cell monolayers at a ratio of 1 : 5, as previously described (19). After 72 h of incubation, co-cultured cells were harvested using trypsin, stained with anti-CD-64 antibody (BD Biosciences), and separated by cell-sorter FACS Aria II into two tubes: macrophages (CD-64-positive/CMTMR-negative) and neoplastic cells (CMTMR-positive/CD-64-negative).

Real-time PCR and a quercetin treatment. Reverse transcription of the isolated total RNA was performed using Universal cDNA synthesis kit II (Exiqon, USA). After cDNA was synthesized, quantitative RT-PCR was performed using SYBR Green Master Mix kit (Exiqon) on a aMx3005P Real Time PCR System (Agilent). The experiment was repeated three times and the data were analyzed by the comparative Ct method (27).

For quercetin study, cells were plated for 24 hrs in culture medium with 50 μM quercetin. After 24 h cells were collected and frozen. RNA isolation and quantitative RT PCR were performed as described above.

Confocal microscopy. Glass coverslips were washed with 70% ethanol, transferred into wells of 24-well plate and air-dried. Coverslips were then coated with 0.5% fish-skin gelatin (2% gelatin/PBS diluted in RPMI/FBS 5% : DMEM non-supplemented, 1 : 1) for 1 hr at 37°C. Gelatin was aspirated and coverslips were air-dried. EMT6 cells were seeded at low density (0.2×10^5 or 0.5×10^5 cells per well) in 500 μl media prepared by mixing equal volumes of DMEM (control) or conditioned media with 5% FBS/RPMI. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hrs, additional 500 μl of respective medium was added to the wells and cells were incubated for 24 hrs.

The cells were fixed for 10 min at RT with 4% paraformaldehyde/PBS, permeabilized for 10 min at RT with 0.1% Triton X-100/PBS and blocked for 15 min at RT with 2% BSA/PBS. The primary antibodies (Bioss Inc) used in immunostaining were: goat anti-Fzd6 (1 : 150), goat anti-Fzd7 (1 : 150), goat anti-Fzd10 (1 : 150), rabbit anti-Dvl1 (1 : 100), rabbit anti-Dlv3 (1 : 150), rabbit anti-Vangl1 (1 : 100) and rabbit anti-Vangl2 (P 1 : 200). The secondary antibodies used in immunostaining were: Alexa Fluor 488-donkey-anti-rabbit (1 : 500), Alexa Fluor 555-donkey-anti-goat (1 : 500). Samples were counterstained using Phalloidin-Atto 390 (3 μl per 100 μl of staining solution). Coverslips were mounted onto slides using Prolong Gold

Antifade mountant. Images were acquired using a 60 ×/1.35 NA (Olympus UPlanSApo) or 100 ×/1.40 NA (Olympus UPlanSApo) oil immersion objective lens, on an inverted laser scanning confocal microscope (IX70 FV 500, Olympus). Acquired images were edited and analyzed using ImageJ (https://imagej.nih.gov/ij/index.html) software.

Statistical analysis. Data is shown as mean ± standard deviation (SD). Statistical analysis was performed in GraphPad Prism software (GraphPad Software, San Diego, CA). The one way analysis of variance (ANOVA) and post-hoc HSD Tukey's Test were used for the comparison of means between cells from different passages. For all tests, p value < 0.05 was considered as significant, whereas p value < 0.01 was considered as highly significant.

Results and discussion

Macrophages induced expression of genes involved in non-canonical Wnt signaling. In order to test the hypothesis that macrophages activate the non-canonical Wnt pathway in cancer cells, expression of selected Wnt genes was analyzed using real-time PCR in three well characterized canine mammary cancer cell lines (18, 25) and two mouse mammary cancer cell lines grown in mono-culture and co-culture with macrophages.

All studied Wnt pathway genes belong to the non-canonical pathway and expression of all of them was increased in tumor cells due to co-culture with macrophages (Fig. 1). The most significant increase in expression was observed in the non-canonical Wnt pathway cell membrane receptors belonging to the Frizzled family: Fzd7 (CMT-U309, CMT-U27, P114) and Fzd10 (CMT-U309, P114). Increased expression was observed for Disheveled proteins and Daam1 protein, directly activated by stimulated Frizzled receptors in both canine and mouse models. Subsequently, the expression of RhoA, Rock1 and Rac1 kinases as well as Paxillin was increased in both canine and mouse tumor cells after contact with macrophages (Fig. 1). It is known that activation of these kinases increases cell adhesion and cytoskeleton remodeling (1, 16, 26, 29). In the present study, increased expression of genes belonging to the EphB receptor group was also observed in both models. EphB receptors are targeted by the Wnt signaling and they control cell compartmentalization along the crypt axis (10). Previous studies have shown that this group of receptors play a key role during colorectal cancer progression (8). Unaffected expression of sFRP5 gene, inhibitor of non-canonical Wnt pathway, and increased expression of Wif1, inhibitor of canonical Wnt pathway may indicate that macrophages induce 'switch' in Wnt pathway – from canonical to non-canonical – in cancer cells (11, 17).

The next important step of the study was to analyze Wnt protein location in the cancer cell. Mouse cancer cells from EMT6 cell line were grown in the presence

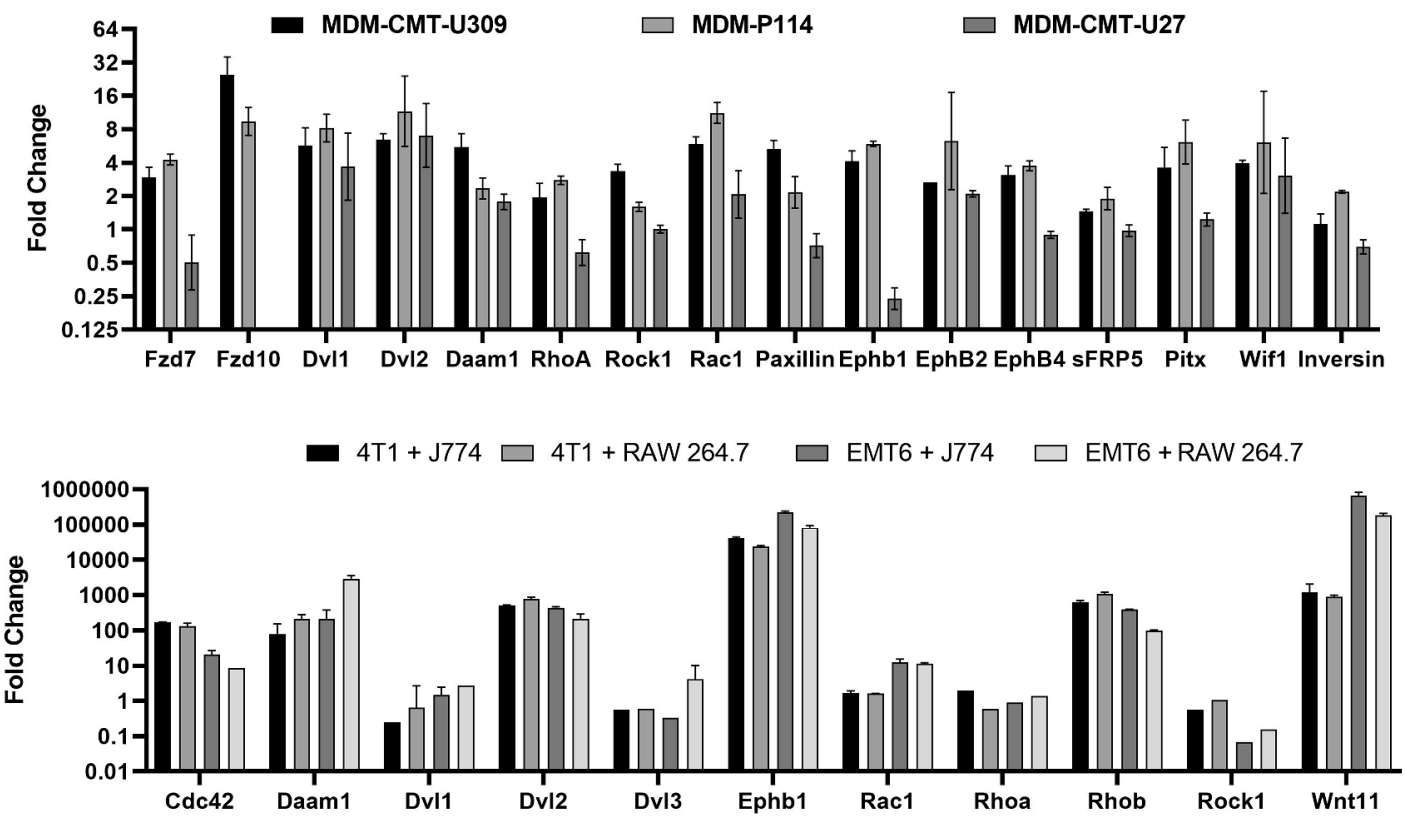
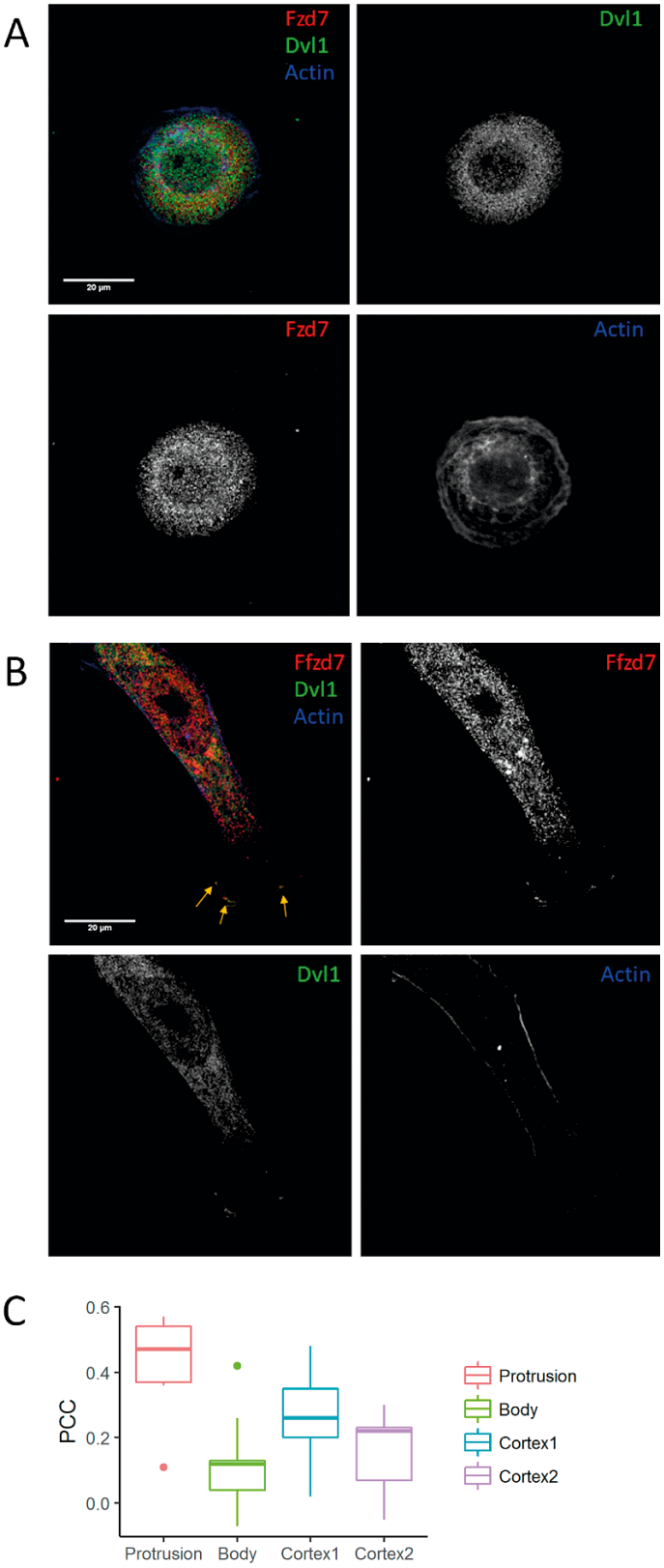


Fig. 1. Expression of selected Wnt genes in co-cultured canine mammary tumor cells and macrophages. Real-time RT-PCR analysis of Wnt genes in co-cultured and monocultured canine mammary neoplastic cells and macrophages. The results are expressed as the mean + range of expression based on standard deviation



of macrophage conditioned medium for 48 hrs. Next, the cells were observed under the confocal microscopy. Localization of Fzd7 receptor, Dvl1 and actin (F-actin) were examined in control cells and in cells treated with macrophage-conditioned medium. Due to lack of specific canine antibodies which are important for precise microscopic observations, the mouse model was used. After treatment with MCM, cancer cells changed morphology from epithelial cells to mesenchymal cells. The Fzd7 and Dvl1 co-localized at the leading front of protrusion in association with filamentous actin (Fig. 2). These results demonstrated that macrophage-conditioned medium stimulated mouse mammary cancer cells in a way that Fzd and Dvl were asymmetrically distributed with respect to cellular protrusions, in a manner analogous to planar-polarized epithelial cells.

In order to verify that the tested gene expression pattern is specific for inhibition of canonical Wnt signaling, gene expression in canine tumor cells was examined after treatment with the known inhibitor of canonical Wnt pathway – quercetin (2, 22). This natural product, which is a plant flavonoid from the group of polyphenols is known to inhibit cancer cell proliferation due to inhibition of canonical Wnt pathway. Indeed, quercetin activated most essential non-canonical Wnt pathway genes, especially in CMT-U309 and P114 cell lines (Fig. 3). Therefore, the quercetin mode of action in terms of Wnt signaling is consistent with the macrophage mode of action in cancer cells.

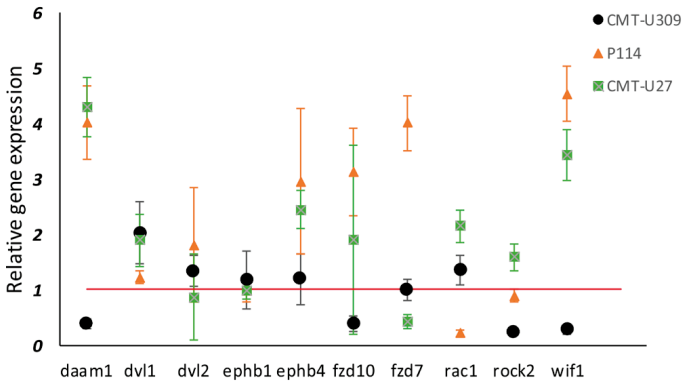


Fig. 3. Expression of selected Wnt genes in canine mammary tumor cells treated with quercetin. Real-time RT-PCR analysis of Wnt genes in canine mammary neoplastic cells treated with quercetin. The results are expressed as the mean + range of expression based on standard deviation

Fig. 2.A. Core planar cell polarity complexes localize symmetrically in untreated cancer cells from EMT6 cell line. F-actin structures were detected by phalloidin staining (blue, top left) and white (bottom right). The co-localization of Fzd7 and Dvl1 was not detected. Scale bars, 20 µm. B. Core planar cell polarity complexes localize asymmetrically in macrophage conditioned medium treated cancer cells from EMT6 cell line. F-actin-rich, cell protrusive structures were detected by phalloidin staining (blue, top left) and white (bottom right). The localization of Fzd7 at the leading edge of MCM-induced cell protrusions is indicated by yellow arrows (top left). The colocalization of Dvl1 with Fzd7 at MCM-induced cell protrusions is indicated (yellow arrows). Scale bars, 20 µm. C. Colocalization coefficient between Dvl1 voxels and Fzd7 voxels within ROIs defined in MCM-treated EMT6 cells were calculated and the results are plotted as box and whisker plots. n = 36 cells per group Actin staining was used to define the regions of interest (ROIs) including cell protrusions, the cell body and the cell cortex flanking the protrusion

As previously mentioned, canonical Wnt signaling pathway is frequently up-regulated in cancer cells. Its activation leads to uncontrolled cell divisions, tumor growth and increased inflammation (28). It has been shown that mouse macrophages secrete Wnt-5a, which activates NF-κB signaling that induces MMP7 expression in cancer cells and promotes invasion (13, 21, 24). Our group showed increased expression of Wnt-5b, Wnt-7a, and Wnt-7b in macrophages following co-culture with cancer cells (19). Moreover, our study showed that TAMs enhance pro-angiogenic properties of canine mammary cancer stem-like cells through the modulation of the Wnt/β-catenin signaling (25).

The results presented in the present study show that the presence of macrophages in the culture of cancer cells induced expression of key non-canonical Wnt genes for cell motility (Fzd7 and Dvl-2). This is in line with observed changes in cell morphology (to a more invasive one) and dislocation of these Wnt proteins within the cell body (in order to facilitate cell motility).

Interestingly, these molecular changes in cancer cells caused by the macrophages resemble those induced by the quercetin. Results of the present study showed that quercetin indeed induced non-canonical Wnt signaling in cancer cells. As quercetin supplementation is broadly discussed in cancer patients, findings of this study show that this compound should be used with caution, as it may enhance cancer spread. However these findings need to be further investigated in regard to details.

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Corresponding author: prof. dr hab. Magdalena Król, Department of Cancer Biology, Institute of Biology, Warsaw University of Life Sciences, Nowoursynowska 166, 02-767 Warsaw, Poland; e-mail: magdalena_krol@sggw.edu.pl