Program: Human resources

Type of the project: Research projects for young research teams – TE type

Title: Effects of polyphenols in modulating the relationship between ErbB proteins and cell cycle progression in breast and epidermoid adenocarcinoma

Project number: PN-II-RU-TE-2011-3-0204

Contract: 111/10.10.2011

Scientific Report Summary

on project implementation in the period October 2011 - October 2014

1. Phase I: October 2011 - December 2011

Introduction

Involvement of ErbB proteins in cancer is known since the early 1980s, when it was observed that avian erythroblastosis virus encodes a protein similar to the epidermal growth factor receptor known as ErbB1 (Yarden Y. & al. [1]). Epidermal growth factor family is a family of tyrosine-kinase receptors consisting of four members: ErbB1 (EGFR, HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) (Y. Yarden & al. [1], JW Park & al. [2]). ErbB proteins are involved in many cellular events which include cell division, migration (associated with tumorigenesis), cell adhesion, differentiation and apoptosis (Yarden Y & al. [1]). After coupling of ligands to receptors, it occurs their association in form of homo- and heterodimers, and out of these, ErbB2 protein heterodimers are those which have the most power in triggering cell division processes and tumorigenesis (Yarden Y. & al. [1]). In the case of tumor cells which overexpress ErbB2 oncoprotein, their activation occurs independent of ligand (Park JW & al. [2]). Also, ErbB2/ErB3 heterodimer is considered one of the most mitogenic and transforming combinations of ErbB protein (Y. Yarden & al. [1] Park JW & al. [2]). Oncoprotein ErbB2 activation is leading to cell proliferation and inhibition of apoptosis by disruption of proteins involved in cell cycle progression (T Gelardi & al. [3]).

Green tea made from *Camellia sinensis* leaves contain a number of phenolic compounds (Fig. 1), such as (-) - epigallocatechin-3-gallate (EGCG), (-) - epigallocatechin (EGC), (-) - epicatechin 3-gallate (ECG) and (-) - epicatechin (EC) (M. Shimizu & al. [4]). Anti-tumor effect of EGCG is partially attributed to the antioxidant properties or ability to remove reactive oxygen species (ROS) (Fujiki H. & al. [5]). EGCG has an effect at the level of survival and cell proliferation, inducing apoptosis and cell cycle arrest in tumor cells (Tachibana H. [6]), and Kuzuhara et al have been proposed DNA and RNA as new targets for the EGCG (T. Kuzuhara & al . [7]). Recent studies have indicated tyrosine kinase receptors, respectively ErbB family

proteins, as targets of EGCG. It has been demonstrated that EGCG inhibits the activation of ErbB3 protein in tumor cells of colon cancer (M. Shimizu & al. [8]). In addition, EGCG interfere with intracellular signaling of the epidermal growth factor receptor (EGFR) in head and neck carcinomas (M. Masuda & al. [9]).

In an attempt to better understand the mechanisms of action of EGCG on tumor cells that overexpress ErbB family proteins it was assessed their ability to act *in vitro* on cell cycle progression.

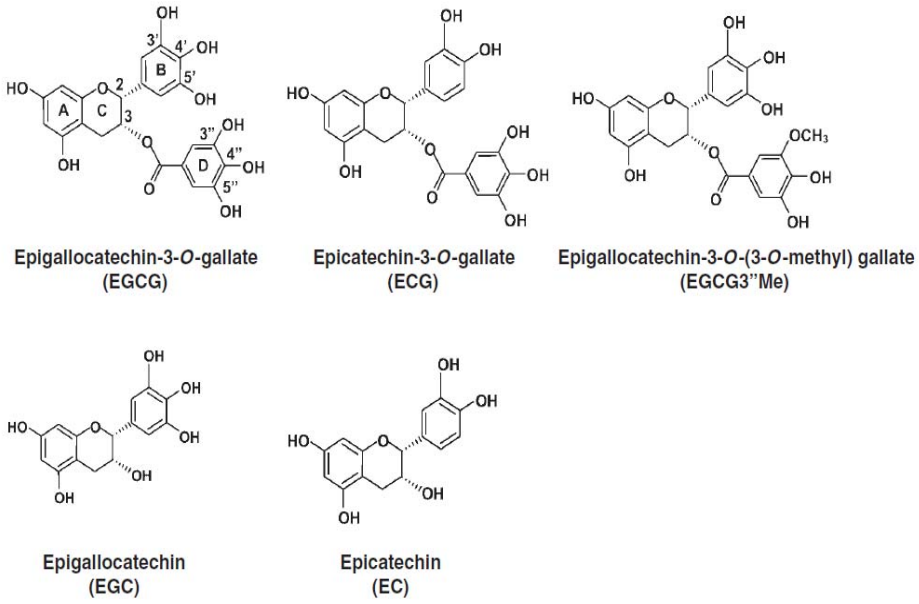


Figure 1: Structure of the polyphenols from the leaves of *Camellia sinensis* (green tea).

Adaptation after Tachibana, H. Proc. Jpn. Acad., Ser. B 87 (2011)

In these preliminary experiments it was observed the increase in the number of breast tumor cells SK-BR-3 in the S phase and the reduction in the number of cells in phase G0/G1. Experiments in the breast tumor cell lines SK-BR-3 is a continuation of previous experiments conducted at the Medical University of Debrecen, Hungary where gene expression of c-erbB2 in breast tumor tissue has been investigated.

Materials and methods

Cell cultures and treatment with EGCG. Breast tumor cells SK-BR-3 were grown to 60-70% confluence in DMEM with L-glutamine (Lonza) supplemented with 10% fetal bovine serum, 100 units penicillin/ml and 0.01 mg streptomycin/ml. Cell cultures were maintained at 37°C in an incubator with a humidified atmosphere and CO2 (5%). Medium was renewed in 2-3 days, and subculturing was in the proportion of 1:2. Adherent sub-confluent cells were treated with different concentrations of EGCG: 5, 10, 50 and 75 µM (Sigma) dissolved in DMSO (up to 0.1%) for 36 hours.

DNA staining. After trypsinization, cells were washed in PBS and ready for cell cycle analysis. Briefly, 10^6 cells were re-suspended in 0.5 ml cold PBS, transferred to 4.5 ml cold 70% ethanol (ice cold, -20°C) and incubated overnight at 4°C. After centrifugation, the pellet was washed with 5 mL PBS. After another centrifugation, the pellet was

suspended in 0.5 ml propidium iodide / RNAse (BD Pharmingen) and incubated 15 minutes in the dark at room temperature.

Flow cytometry. Cell cycle phase distribution was performed using flow cytometer: Becton Dickinson FACSCalibur (FACS, fluorescence-activated cell sorting) equipped with CellQuest data acquisition program. After the binding of propidium iodide to DNA, the absorption maximum is at 535 nm and emission maximum is at 617 nm. For recording fluorescent signal of propidium iodide Argon ion laser emitting at 488 nm was used and fluorescence was collected using a 585 nm bandpass filter (FL2 channel of the cytometer). For each measurement were analyzed 10,000 events. Data analysis was performed with the program MODFIT 3.3 and WinMDI 2.8 software.

Results and discussion

Objective 1: Study of the effects of polyphenols on the cell cycle, apoptosis and cell viability

Preliminary measurements of the cell cycle progression in the presence of polyphenols

Previous studies have shown ECGC to have antimitotic effect (H. Tachibana [6], CS Yang & al. [10]). To investigate whether the activity was associated with blocking the progression of the cell cycle, were performed by flow cytometry measurements, in which breast tumor cells SK-BR-3, which overexpress ErbB2 oncoprotein (MM Mocanu & al. [11]) were treated with various concentrations of EGCG (5, 10, 50 and 75 µM) for 36 hours.

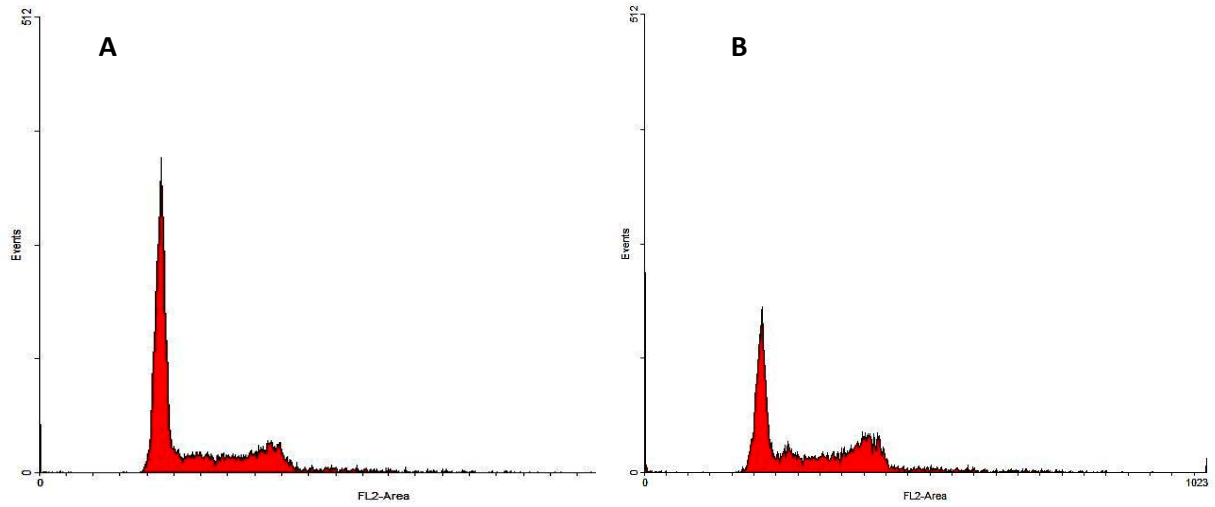


Figure 2: The effect of EGCG on cell cycle progression. DNA content and cell cycle progression was analyzed by flow cytometry. For DNA staining using propidium iodide. The two peaks of the histogram indicate the G0/G1 phases of G2/M cell cycle, and between the two peaks is the phase S. The abscissa represents the fluorescence intensity and the ordinate the relative number of cells (10,000 events were investigated). **A.** Cell cycle progression line SK-BR-3 without treatment with EGCG. **B.** G0/G1 phase cells reduction after incubation with 75 µM EGCG for 36 hours

Fig. 2A shows the phases of the cell cycle G0/G1, S and G2/M for the untreated tumor cells. Treatment of breast cancer cells SK-BR-3 with 75 µM EGCG for 36 hours (Fig. 2B) induces cell cycle arrest in the S phase. These results are consistent with previously published in vitro studies conducted in the lung carcinoma cells (Sadava D. & al. [12]). Fig. 3 shows the quantification of results of flow cytometric analysis performed by the 10000 event. There was a decrease in the number of tumor cells SK-BR-3 in the G0/G1 from 53% (control) to 38% (cells treated with 75 µM EGCG) and an increase in the number of cells in S phase from 17 % to 26% (34.61%), suggesting the blocking of the progression in the cell cycle of S phase. It

was also observed a slight increase in the number of cells in the G2/M fraction or sub-fraction G0/G1 after treatment with 75 µM of EGCG.

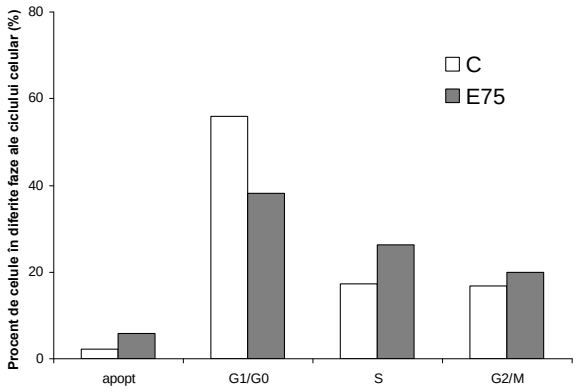


Figure 3: Percentage of SK-BR-3 cells in the different phases of the cell cycle. White columns represents the results obtained from the analysis of cell cycle progression of the SK-BR-3 in the absence of EGCG (control, C), and the gray columns represent the SK-BR-3 cells in the presence of EGCG (E75). Breast tumor cell line SK-BR-3 was treated with 75 µM of EGCG for 36 hours. Quantification of flow cytometry data indicates the G1/G0 stage reduction and increasing of the number of cells in the phase S, G2/M. Also, there is a slight increase in the number of apoptotic cells in the case of cells treated with EGCG.

The effect of EGCG on cell cycle progression was investigated in the presence of low doses of EGCG (5 and 10 µM) or relatively high doses of EGCG (50 and 75 µM). Fig. 4 shows little influence 5 şi 10 µM EGCG at phase G0/G1 relative to the control sample. Reducing the number of cells in the G0/G1 is evident from relatively increased doses of EGCG, 50 and 75 µM, respectively. Preliminary data obtained indicate an increase in the number of cells in S-phase after incubation of tumor cells with 10, 50 and 75 µM EGCG for 36 hours, the concentration of EGCG 5 µM inducing no change on the number of cells in the S phase as compared to the control sample.

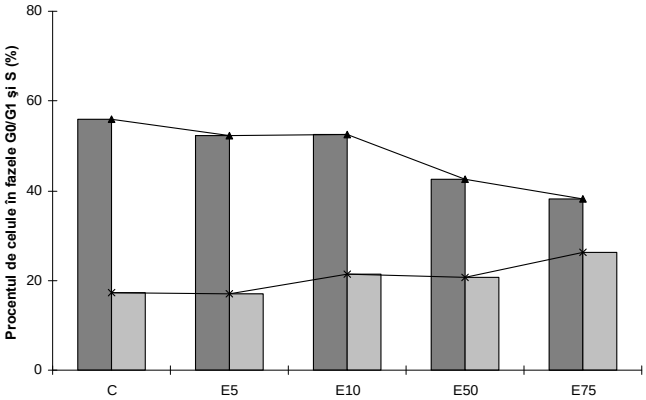


Figure 4: The effect of EGCG on phase G0/G1 and S. Breast tumor cell line SK-BR-3 was treated with different concentrations of EGCG: 5, 10, 50 and 75 µM EGCG for 36 hours. It is observed the decrease in the number of cells in the G0/G1 cell cycle (dark gray columns), at the same time increasing the number of cells in the S phase (gray columns).

Conclusions

In these preliminary experiments there was presented the effect of different doses of EGCG on cell cycle progression of breast adenocarcinoma cell line SK-BR-3. It has been found that relatively high doses of EGCG, 50 and 75 µM cause the reduction of the number of cells in the G0/G1 and increasing the number of

cells in the S phase of the cell cycle in a similar way to the data from the literature (D. Sadava & al. [12]). It was also observed that the effect of EGCG in the tumor cell cycle progression is dependent on the dosage used.

2. Phase II: January 2012 – December 2012

Introduction

At this stage we continued to study the effect of polyphenols, respectively epigallocatechin gallate (EGCG) extracted from green tea (*Camellia sinensis*) in the breast (SK-BR-3) and epidermal (A431) tumor cell lines that overexpress ErbB proteins.

Materials and methods

Cell cultures and treatment with EGCG. Breast tumor cells SK-BR-3 were grown to 60-70% confluence in DMEM with L-glutamine (Sigma) supplemented with 10% fetal bovine serum, 100 units penicillin/ml and 0.01 mg streptomycin/ml. Cell cultures were maintained at 37°C in an incubator with a humidified atmosphere and CO2 (5%). Medium was renewed in 2-3 days, and sub-culturing was in the proportion of 1:2 for SK-BR-3 and 1:5 for A431. Adherent sub-confluent cells were treated with different concentrations of EGCG: 5, 10, 50 and 75 µM (Sigma) dissolved in DMSO (up to 0.1%) for different periods of time.

DNA staining. After trypsinization, cells were washed in PBS and prepared for cell cycle analysis. 10^6 cells were re-suspended in 0.5 ml cold PBS, transferred to 4.5 ml cold 70% ethanol (ice cold, -20°C) and incubated overnight at 4°C. After centrifugation, the pellet was washed with 5 mL PBS. After another centrifugation, the residue was suspended in 0.5 ml propidium iodide / RNAse (BD Pharmingen) and incubated 15 minutes in the dark at room temperature. Cell cycle phase distribution was analyzed using a flow cytometer: Becton Dickinson FACSCalibur (FACS, fluorescence-activated cell sorting) equipped with CellQuest data acquisition program. For recording fluorescent signal of propidium iodide Argon ion laser emitting at 488 nm was used and fluorescence was collected using a 585 nm bandpass filter (FL2 channel of the cytometer). For each measurement were analyzed 10,000 events. Quantitative analysis of the data was carried out with the MODFIT 3.3 software, WinMDI 2.9.

Apoptosis. After the incubation of breast (SK-BR-3) and epidermal (A431) tumor cells with various concentrations of ECGG for 24 hours and 72 hours, their staining was carried out for the measurement of apoptosis. The cells were harvested by trypsinization (all cell fractions were collected), and for each sample 10^6 cells were prepared. After washing twice in PBS, the supernatant was removed and the residue was taken over in 100 µl AVBB (Annexin V binding buffer) according to the manufacturer's instructions (Beckman Coulter). Each sample was incubated with 1 µl Annexin V-FITC and 7-AAD 2.5 µl for 15 minutes in the dark at room temperature, after which the sample was completed with another 400 µl AVBB. Samples were analyzed by flow cytometry in less than one hour with intrument using a FACSCalibur (Beckton Dickinson). Wavelength fluorophores were excited was 488 nm (argon laser, 15 mW), and data were recorded using bandpass filters type 530 nm for FITC and >670 nm for 7-AAD.

Assessment of cell viability - WST-1 assay. Cell viability was assessed by measuring the oxidation of water soluble tetrazolium salts using the reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). The adherent cells were cultured in 96-well plates at a concentration of 7 x 10^3 cells/well 24 hours prior to the treatments. Adherent tumor cells were incubated at 37°C in CO2 atmosphere with various concentrations of EGCG for 24 and 48 h in triplicate. The absorbance of WST-1 reagent was measured using a spectrophotometer for 96-well plates (Synergy HT Multi-Mode Microplate Reader, BioTek) at wavelengths of 450 and 620 nm. The concentration of polyphenols that leads to

the death of 50% of tumor cells (IC50) was calculated using MATLAB (Mathworks Inc., Natick, MA) [2011 Varadi et al., Eur J Pharmacol]:

$$A_{\min} + \frac{A_{\max} - A_{\min}}{1+10^{n(\log(c)-\log(IC50))}} \quad (1)$$

where, A_{min} and A_{max} are the absorbance for the lowest and highest values of absorbance, ***n*** is Hill's coefficient, ***c*** is the concentration of polyphenol.

Rezults

Objective 1: The study the effects of polyphenols on the cell cycle, apoptosis and cell viability

The effect of EGCG on the viability of breast SK-BR-3 and epidermal A431 tumor cell lines, was studied by incubating cells in the presence of several concentrations of polyphenols.

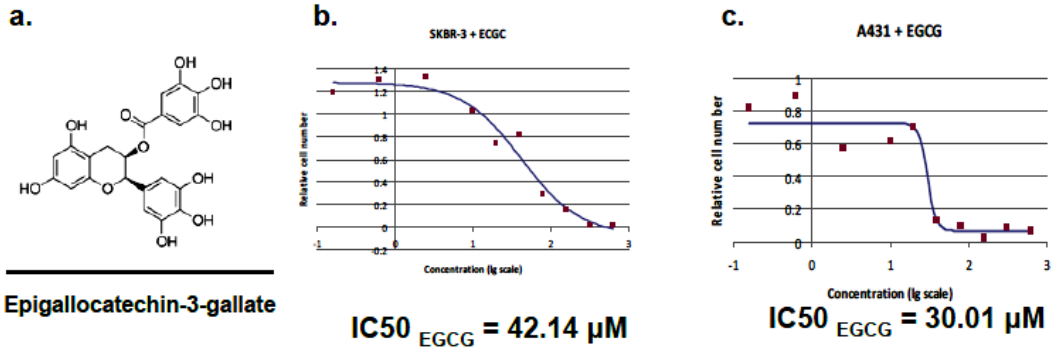
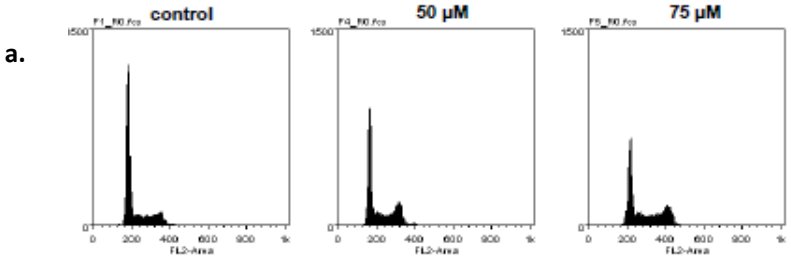


Figure 1: Viability curves for the incubation of tumor cells which overexpress ErbB protein with EGCG. a. The structure of EGCG; b. Cell viability curve for the SK-BR-3 following incubation with different concentrations of EGCG; c. The cell viability curve for A431 after incubation with EGCG.

Breast cell line overexpress ErbB2 oncoprotein and epidermal cell line A431 overexpress the protein ErbB1 (epidermal growth factor receptor). Cell viability was evaluated 24 and 48 hours. Figure 1 shows the viability curve for breast cancer cells SK-BR-3 (Figure 1b) and epidermal cancer cells A431 (Figure 1c). Also, the IC50 value was calculated and it was obtained 42.14 ± 1.29 µM for the breast tumor cell line SK-BR-3 and 30.01 ± 0.72 µM for the A431 epidermal tumor cell line after incubation with EGCG for 24 h.

Figure 2a shows the influence of EGCG on cell cycle phase for mammary tumor line SK-BR-3. There were investigated more polyphenol concentrations: 5, 10, 50, 75 µM EGCG. Low concentration of polyphenol did not significantly influence cell cycle progression and the concentrations of 50 and 75 µM EGCG resulted in a decrease in the number of cells in the G0/G1 phase and its growth in the S and G2/M phases (Figure 2b).



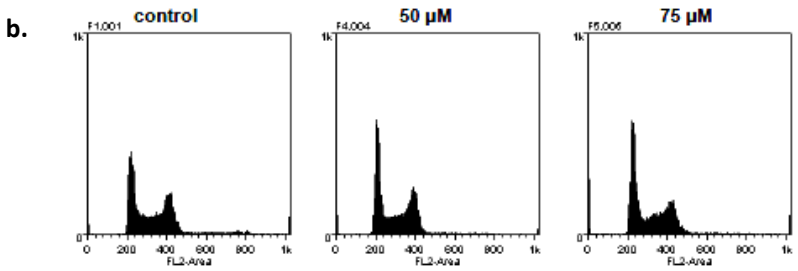


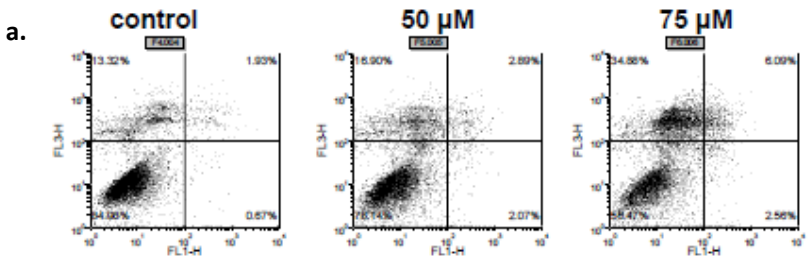
Figure 2: The effect of EGCG on cell cycle progression in breast tumor cell line SK-BR-3. a. Comparative images of cell cycle progression in case of control sample and samples incubated for 24 h with 50 and 75 µM ECGC. b. Cell cycle phases for epidermal tumor cells A431 incubated for 24 h in the presence of 50 and 75 µM EGCG.

The quantitative analysis of cell cycle phases in the case of epidermal tumor line A431 and breast tumor line SK-BR-3 for concentrations of 50 and 75 µM EGCG showed changes in the percentages of each phase of cell cycle (Table 1):

Cell line	Sample	G0/G1 (%)	S (%)	G2/M (%)
SK-BR-3	Control	62.25	32.09	5.67
	50µM EGCG	49.81	36.76	13.44
	75 µM EGCG	41.31	43.50	15.19
A431	Control	27.75	47.27	24.98
	50µM EGCG	31.26	49.58	19.16
	75 µM EGCG	27.97	58.88	13.16

Table 1: Percentage of cell cycle phases after incubating with epigallocatechin gallate

In the next stage we were interested to investigate the effect of EGCG on the apoptotic process. Tumor cells were treated with different concentrations of polyphenols, 5, 10, 50, 75 µM EGCG for 72 hours. After incubation, the tumor cells were stained with Annexin V-FITC and 7-AAD to identify viable cells (lower left quadrant), early apoptotic cells (bottom right quadrant), and late apoptotic or necrotic cells (the two upper quadrants of the graphs the dots). Figure 3 shows the effect of EGCG in the apoptotic processes. It was found that EGCG at concentrations of 50 and 75 µM induces late apoptotic/necrotic cells both in the breast tumor line SK-BR-3 (Figure 3a) and in the epidermal tumor line A431 (Figure 3b). The observed increase in the percentage of late apoptotic/necrotic cells with increasing concentrations of EGCG, shows the dependency of the apoptotic process on the used dose of EGCG. Flow cytometric measurements were performed in the Laboratory of Immunology, National Institute Victor Babes.



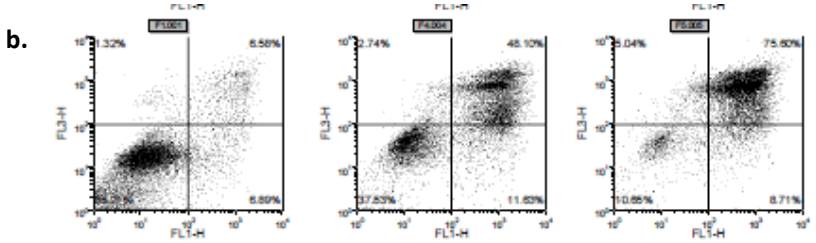


Figure 3: Effect of treatment with EGCG on the apoptosis assessed by staining of cells with Annexin V - FITC and 7-AAD.

a. mammary tumor cell line SK-BR-3; b. epidermal tumor cell line A431

Objective 2: Investigation of the effect of polyphenols on the expression of ErbB proteins

Since in the literature it was mentioned the influence of EGCG on the ErbB family and on β1-integrin expression level, as well as on the 67kDa laminin receptor (67LR) which is considered the receptor for EGCG, these membrane proteins were investigated by flow cytometry.

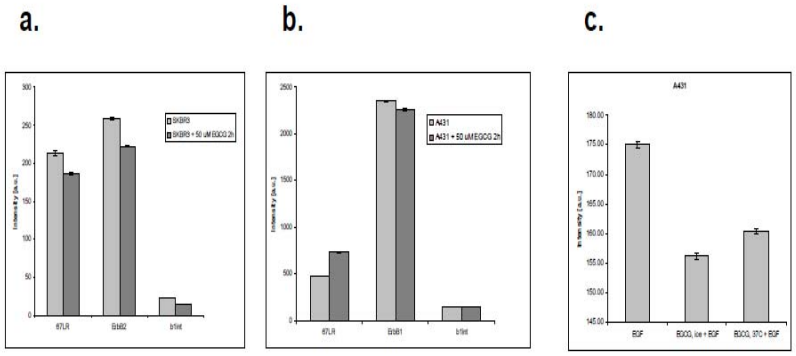


Figure 4: The effect of EGCG to the expression of membrane proteins. a. The level of expression of membrane proteins: ErbB2, 67LR, β1-integrin was reduced after incubation of SK-BR breast tumor cells with 50 μM EGCG-3 for 2 hours; b. Incubation of epidermal cancer cells A431 with 50 μM EGCG for 2 hours.

Breast SK-BR-3 and epidermal A431 tumor cells were incubated in the presence of 50 μM of EGCG for 2 h. It was investigated the effect of EGCG on the expression of ErbB2 protein, 67LR, β1-integrin in SK- BR-3 cell line and ErbB1 protein level, 67LR, β1-integrin in the case of tumor cell line A431 (Figure 4a,b). In SK-BR-3 cell line it was noticed a moderate decrease of 67LR and ErbB2 proteins, while not significant changes in β1-integrin were observed. At the same time the expression level of ErbB1 was moderate decreased, while no significant changes were observed in case of β1-integrin on A-431 cell line. The experiments regarding this objective were conducted in the laboratories of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Hungary.

Conclusions

In conclusion, the measurements support the involvement of polyphenols, EGCG, respectively, in blocking cell cycle progression, promoting late apoptosis/necrosis, the reduction of the ErbB protein expression in the investigated epidermal and breast tumor cell lines. It was also found that EGCG effect is dose-dependent. Further experiments are needed to evaluate the effect of other polyphenols, and combinations thereof, in order recommendation polyphenols as possible chemotherapeutic agents of cancer.

3. Phase III: January 2012 – October 2013

Introduction

Quercetin (3,3 ', 4', 5,7-pentahidroxi-flavone) is a secondary metabolite of plants that can be found in the onion, apples, grapes, green tea etc. (G. S. Kelly [13] Dajas F. [14]). Numerous studies have shown chemopreventive and chemotherapeutic effects of quercetin based on its two core activities, namely its anti-oxidant and pro-oxidant properties. Anti-oxidant properties of the flavonoids quercetin are related to the ability to donate electrons to reactive oxygen species (ROS) that will be less harmful to the DNA macromolecule (AJ & Vargas al. [15]). Anti-oxidant properties of quercetin were observed at lower concentrations, of up to 40 μM (AJ & Vargas al. [15]) and have been associated with both anti-inflammatory activity, and the neuroprotective and cardioprotective effects (F. Dajas [14]). Pro-oxidant properties of quercetin were linked with its ability to inhibit tumor cell proliferation and it has been appreciated that these antitumor properties are manifested in the concentration range 40-250 μM (AJ Vargas & al. [15]). Other reports indicate, however, an anti-tumor activity even at lower concentrations of quercetin (3-50 μM) (Gibellini L. & al. [16])

It should be noted that the anti-proliferative role of quercetin was only evident in the case of tumor cells (G. Wang & al. [17] Baran I. & al. [18] US Mertens-Talcott & al. [19]), while such effects were not observed in normal cells (JH Jeong & al. [20]). According to previous reports, quercetin induced cell cycle arrest in G2/M phase (K Bishayee & al. [21]), the G0 / G1 (M. Yoshida & al. [22]), or the S-phase (H. Zhang & al. [23]). Divergent data on concentrations that occur in case of anti-proliferative activity and variety of effects on cell cycle progression after incubation with quercetin led us to investigate these effects in epithelial tumor cells.

Induction of apoptosis in tumor cell lines by flavonoids has been reported by many studies. Flavonoids modulate the apoptotic pathways both extrinsic and intrinsic leading to the increased expression of caspase 3/7, and by inhibiting the anti-apoptotic proteins such as Bcl-2 (MH Lee & al. [24]). Recent studies demonstrate that the pro-apoptotic activity of EGCG is mediated by a 67kDa laminin receptor (67LR), and the blocking of this receptor with specific antibodies gave almost complete inhibition of apoptosis in multiple myeloma cell line (M. Kumazoe & al . [25]). It has also been reported the influence of cell cycle progression by flavonoids: while EGCG may cause cell cycle arrest in the G0/G1, genistein could result in blocking progression of the cell cycle in G2/M (HC Huang & al. [26]).

Multiple scientific results support the hypothesis that consumption of certain food constituents such as epigallocatechin gallate (EGCG) from green tea and soy genistein, is associated with both reduced risk of cancer (CS Yang & al. [27] SJ Park & al. [28]). The effect of flavonoids on apoptosis, cell cycle progression, and phosphorylation of the receptor tyrosine kinase (RTK) has been studied in various tumor cell lines (M. Masuda & al. [9], M. Shimizu & al. [29]). However, the molecular mechanism of action of the flavonoids in cell lines that overexpress ErbB protein remains to be elucidated. ErbB proteins, a family of RTKs have a central role in the development of various forms of cancer (Yarden Y. & al. [1] NE Hynes & al. [30]). ErbB protein family has four members, from the ErbB1 to ErbB4, and overexpression thereof was associated with invasiveness, metastasis, and inhibition of apoptosis in tumor cells (Yarden Y & al. [1]). ErbB1 receptor, epidermal growth factor (EGF), form homo- and heterodimers, triggering the activation of intracellular signaling pathways after ligand coupling (Hynes NE & al. [31])

Characterization of the association between the proteins or their modifications induced by the coupling of physiological ligands or other molecules with therapeutic effect, does not just allow the elucidation of the onset of intracellular signaling, but also provides a therapeutic modality which may be addressed in the future. A variety of methods are available for investigation of the interactions between proteins, including: molecular biology techniques (for example, immunoprecipitation, "yeast two-hybrid" (Williamson MP & al. [32]), techniques based on the different manifestations of the fluorescence transfer energy (FRET) (EA Jares-Erijman & al. [33]) or variations of fluorescence spectroscopic techniques (fluorescence correlation spectroscopy, FCS) (E. Haustein & al. [34]).

This study was designed to evaluate the ability of quercetin, EGCG and genistein to inhibit the proliferation of tumor cells in two cancer cell lines of epithelial or mammary tumor cells SK-BR-3 and epidermoid carcinoma cells A-431. We studied the anti-proliferative effects of quercetin, first by assessing the progression of the cell cycle and, secondly, by investigating its pro-apoptotic activity. Our data support both the ability of quercetin to interfere with the progression of the cell cycle and its ability to produce pro-apoptotic effects in a manner dependent on time and dose in epithelial tumor cells SK-BR-3 and A-431. In addition, EGCG was effective in inducing late apoptosis/necrosis, and genistein has blocked the progression of the cell cycle in G2/M phase. It was observed that the anti-proliferative effect of EGCG was mediated by 67LR. Flow cytometry measurements showed reduced levels of ErbB1 and ErbB2 protein expression after incubation of tumor cells in the presence of EGCG, but not in the presence of genistein.

Materials and methods

Chemicals and reagents

Quercetin, EGCG and genistein dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich, water soluble tetrazolium salts of 4- [3- (4-Iodophenyl) -2- (4-nitrofenyl) -2H-5-tetrazolio] -1, 3-benzene disulfonate (WST-1) from Roche Diagnostics GmbH, Mannheim, Germany, propidium iodide / RNase from BD Biosciences, Annexin V-FITC from Beckman Coulter, Brea, CA and 7-AAD from BD Biosciences, San Jose, CA. Modified Dulbecco medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin were from Sigma Aldrich and L-glutamine from Gibco - Life Technologies.

Cell cultures and antibodies

Cell lines of human breast cancer SK-BR-3 and human epidermoid carcinoma A-431 were obtained from the American Type Culture Collection (Rockville, MD) and were grown according to the respective specifications. Briefly, tumor cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO2 atmosphere.

There were used the following anti-ErbB1 antibodies: monoclonal antibody 528 (Mab528) produced by hybridoma cell line HB-8509 obtained from ATCC conjugated directly with AlexaFluor 546 or AlexaFluor 647; humanized anti-ErbB2 antibody, Herceptin (Roche, Budapest, Hungary) directly conjugated with AlexaFluor 546 or AlexaFluor 647.

Viability test

The cells SK-BR-3 and A-431 were cultured in triplicate at a density of $7 \cdot 10^3$ cells/sample in 96-well plates 24 h prior to the experiments. Treatment with various concentrations of quercetin for 24 h was performed in serum free medium (H. van der Woude & al. [35]). The final concentration of DMSO in the culture medium was 0.05%. Viability test was performed in triplicate using proliferation reagent WST-1 based on reduction of the tetrazolium salt WST-1 by mitochondrial dehydrogenases to formazan. The absorbance of the formazan was measured at 450 nm and 620 nm (correction) using a spectrophotometer for 96-well plates (ELISA reader)

Cell cycle analysis

After treatment with flavonoids (0, 5, 10, 50, and 75 µM quercetin) for 24 h in complete medium the cells were harvested by trypsinization, fixed in cold 70% ethanol (ice cold), and stored at -20°C. Ethanol was removed by centrifugation and the cells were washed in PBS after which 10^6 cells/sample were stained with propidium iodide/RNase for 15 minutes in the dark, according to the manufacturer's instructions. DNA content was analyzed using a FACSCalibur instrument (Becton Dickinson). For excitation at 488 nm we were using a laser with air cooled argon ion (15 mW) and data were collected from 10,000 events/sample with a band pass filter of 585-nm. Experiments were performed in triplicate. Data analysis was performed according to the percentages of cells in the phases G0/G1, S and G2/M of the cell cycle.

Assessment of apoptosis

Tumor cells were incubated with 0, 5, 10, 50, and 75 µM quercetin in complete medium for 24 to 72 hours. The cells were collected by trypsinization, washed twice in PBS and 10^6 cells/sample were double stained with Annexin V-FITC and 7-AAD according to the manufacturer's instructions. Samples were analyzed by flow cytometry in less than one hour with an instrument using a FACSCalibur (Beckton Dickinson San Jose, CA). The wavelength at which the fluorophores FITC and 7-AAD were excited was 488 nm (argon laser, 15 mW), and the data was recorded by means of bandpass type filter for FITC 530 nm and >670 nm 7-AAD. For data analysis, the cells Annexin V-FITC$^-$/7-AAD$^-$, Annexin V-FITC$^+$/7-AAD$^-$, Annexin V-FITC$^+$/7-ADD$^+$ and Annexin V-FITC$^-$/7-ADD$^+$ were considered viable, early apoptotic, late apoptotic and necrotic. Experiments were performed in triplicate.

Immunofluorescence labeling for flow cytometry

Trypsinized cells maintained for 24 h in serum-free medium were incubated for another 24 hours in the presence of various concentrations of flavonoids (also in serum-free medium). The primary anti-ErbB2 antibodies (Herceptin-Alexa Fluor 488), ErbB1 (528, Alexa Fluor 488) were used at a concentration of 20 µg/ml. Incubation with primary fluorescently labeled antibodies was carried out on ice for 30 minutes, followed by rinsing in cold PBS and fixation with 4% formaldehyde. Fluorescence of 20,000 cells was measured with FACSCalibur flow cytometer (Becton Dickinson).

Fluorescence resonance energy transfer (FRET)

FRET was measured by flow cytometry FACSArray. Antibodies conjugated directly with AlexaFluor 546 and AlexaFluor 647 were used as donor and acceptor. The fluorescence intensity of the donor, FRET and acceptor were measured for yellow, red and infrared spectra. Fluorescence for yellow and infrared were excited with 532 nm solid laser and detected using a band-pass filter of 585/42 nm and a long-pass filter of 635 nm. The fluorescence spectrum was excited using a red laser diode with emission at 635 nm, and its detection was performed using a 661/16 nm band-pass filter. Necessary control samples, calibration samples and evaluation principles have been described in another article (P. Nagy & al. [36]). Measured data for the 20,000 events were analyzed with the program reflex (G. Szentesi & al. [37]). FRET efficiency is inversely correlated with the sixth power of the donor and acceptor and is interpreted as a way of measuring the association between molecules (J. Szollosi & al. [38]).

Statistics

Data are presented as mean ± S.E.M. of three independent measurements unless otherwise specified. For statistical analysis t'Student test was used.

Results and discussions

Objective 1: To study the effects of polyphenols on the cell cycle, apoptosis and cell viability

The biphasic effect of quercetin in mammary and epidermoid tumor cell lines

Quercetin (Figure 1) has been administered to tumor cells SK-BR-3 and A-431 in various concentrations: 0, 2.5, 10, 20, 40, 80, 160 and 320 µM for 24 h. High concentrations of quercetin had an inhibitory effect on both tumor cell lines (Figure 2).

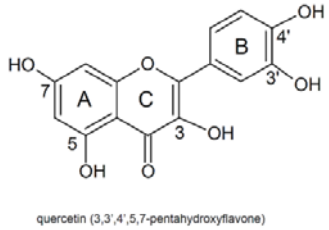


Figure 1. Chemical structure of quercetin (3,3'4 ', 5,7-pentahydroxyflavone).

In case of 24 hours incubation in the presence of quercetin, A-431 cell line has been shown to be more sensitive to the antiproliferative effect thereof as compared to the cell line SK-BR-3. On the other hand, lower concentrations of quercetin, in particular approx. 2.5 µM have induced an upward trend of viability in treated cells compared with control samples (Figure 2). Cell viability was increased by 15% and 16% after application of 2.5 µM in the cell line SK-BR-3 and A-431, respectively. The data may suggest a biphasic effect of quercetin in tumor cell lines SK-BR-3 and A-431

Blocking the S phase of the mammary cell line SK-BR-3 at high concentrations of quercetin

To identify the mechanism responsible for the inhibitory effect of quercetin in the cell line SK-BR-3 we evaluated its effect on DNA content, as measured by flow cytometry (Figure 3). In order to evaluate the progression of the cell cycle, namely the phase G0/G1, S and G2/M histograms recorded for DNA content were used.

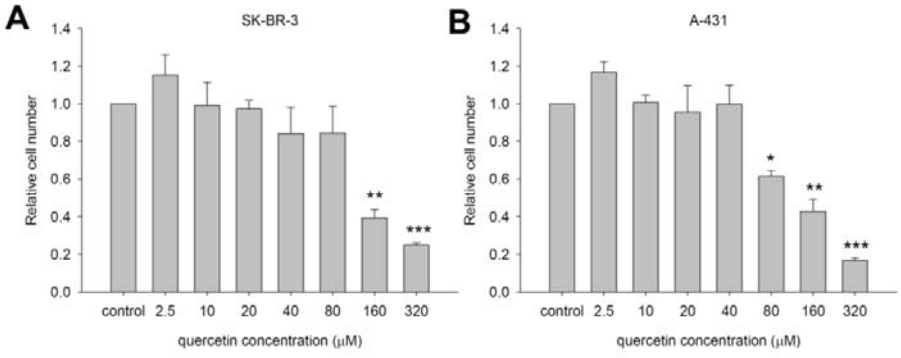


Figure 2. Inhibition of cell viability by the quercetin in breast cancer cells SK-BR-3 (A) and epidermoid carcinoma A-431 (B). Tumor cells were incubated with various concentrations of quercetin for 24 h and cell viability was measured by WST-1 assay. Data represent ± S.E.M. of the experiments carried out in triplicates. Significant differences from control: ***p <0.01, ***p <0.001*.

After incubation with 50 and 75 µM quercetin the number of SK-BR-3 cells in S-phase increased by 64% (p <0.01) and 78% (p <0.001) compared with 30% in control samples. As shown in Figure 3, the effect was

dose dependent. This was accompanied by a significant decrease in the cell population in the G0 / G1 from 63% to 6% of the control samples 50 and 75 µM quercetin ($p < 0.001$). At concentrations of 10 µM quercetin, S phase block was moderate. Small concentrations of quercetin (5 µM) did not lead to significant changes in cell cycle distribution of cell line SK-BR-3.

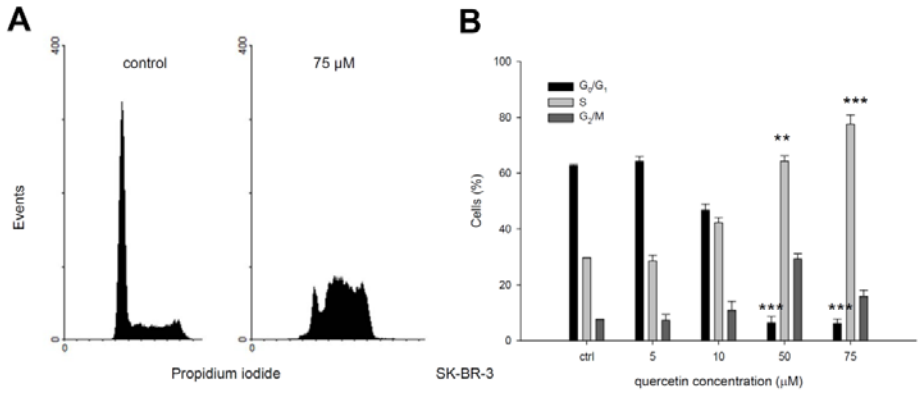


Figure 3. Effect of quercetin on the cell cycle on the tumor cell line SK-BR-3. Tumor cells after treatment with 0, 5, 10, 50, and 75 µM quercetin, were stained with propidium iodide/RNAse and DNA content was then analyzed by flow cytometry. (A) Representative histograms of DNA distribution. (B) Mean ± SD for phase G0/G1, S and G2/M with no treatment or treatment with quercetin (n = 3); significant differences from control: ***p*<0.01, ****p*<0.001.

Blocking of G0/G1 phase of epidermoid tumor cell line A-431 at low concentrations of quercetin

In the case of this cell line the effect of quercetin was dependent on concentration used (Figure 4). Low concentrations of quercetin, in the 5-10 µM induced cell cycle arrest in the G0/G1. Higher concentrations, 50-75 µM led to a moderate accumulation of tumor cells in the S phase in a dose-dependent manner. The results again suggested a biphasic dose-dependent effect of quercetin.

The effect of flavonoids (quercetin, EGCG, genistein) on apoptosis in cell lines SK-BR-3 and A-431

To investigate the effect of quercetin on apoptosis, cell lines SK-BR-3 and A-431 were stained with Annexin V-FITC and 7-AAD followed by flow cytometry measurements (Figure 5).

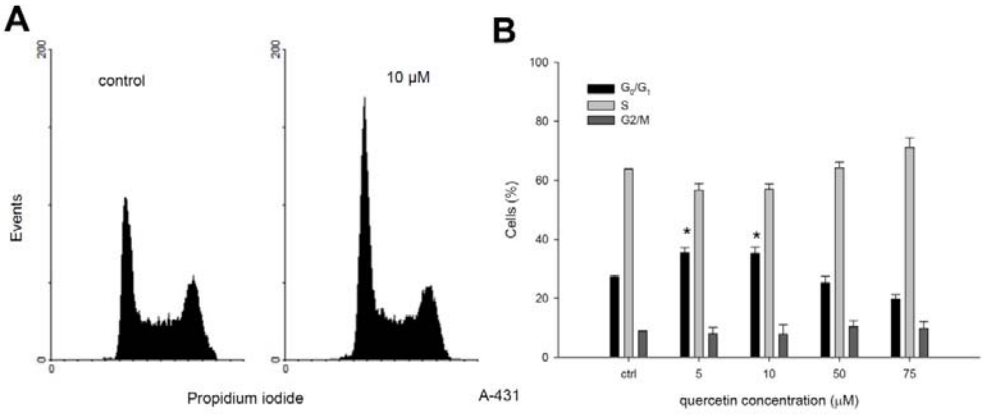


Figure 4. Effect of quercetin on cell cycle distribution in the A-431 epidermoid tumor cells. The cells were fixed in ethanol, stained with propidium iodide and measured by flow cytometry. (A) Representative histograms of tumor cells treated with 0 and

10 µM quercetin. (B) The data are mean ± SD (n = 3) and the asterisks indicate significant differences to the control sample, *$p < 0.05$*.

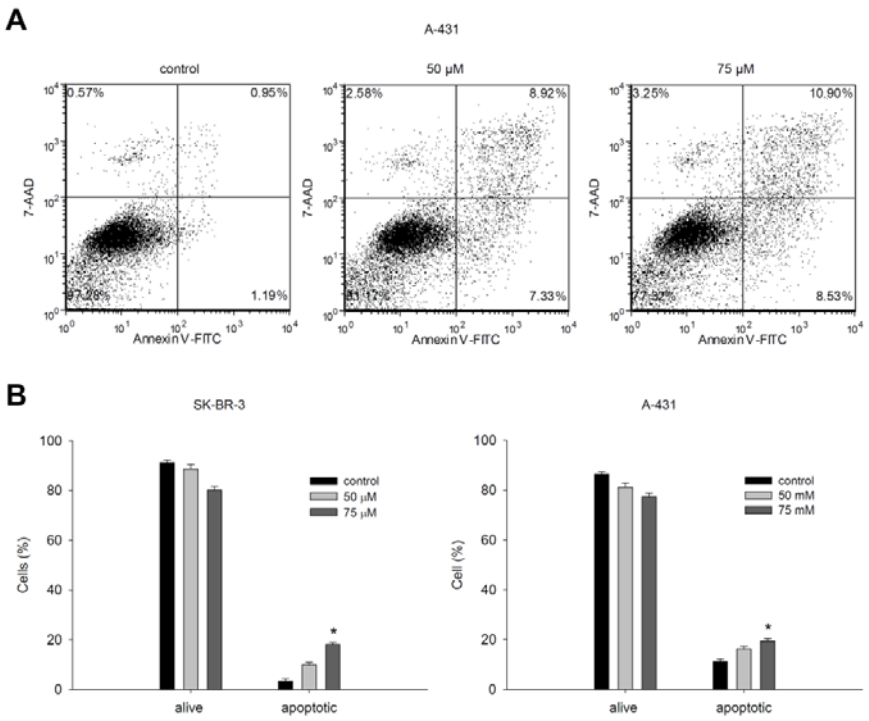


Figure 5. The effects of quercetin on apoptosis in tumor cells SK-BR-3 and A-431. After treatment for 72 h with quercetin, tumor cells were stained with Annexin V-FITC and 7-AAD and measured by flow cytometry within one hour. (A) Representative dot plots for A-431 cells. (B) Apoptotic cells, including both early (Ann V + / 7-AAD-) and late (Ann N / 7-AAD +) apoptotic cells is shown compared with living cells (Ann N / 7-AAD-). Data represent ± S.E.M. for triplicate experiments and asterisks indicate significant differences to the control sample, *$p < 0.05$.

The percentage of apoptotic cells was calculated from the amount of early (Annexin V + / 7-AAD-) and late (Annexin V - / 7-AAD +) apoptotic cells. In both tumor cell lines effects of quercetin were observed, moderate and dose-dependent, on the generation of apoptotic cells. Incubation with 75 µM quercetin for 72 h induced an increase in the apoptotic events from 3% to 18% in SK-BR-3 and from 3% to 19% of A-431. Lower incubation times (24 hours) in the presence of quercetin did not change the percentage of apoptotic cells.

To verify the induction of programmed cell death by EGCG and genistein, human tumor cell lines were stained with Annexin V-FITC and 7-AAD (Figure 6A). After incubation of tumor cells with 75 µM EGCG about 80% of tumor cells A-431 and about 40% of tumor cells SK-BR-3 cells were classified as late apoptotic/necrotic. Genistein induced late apoptosis/necrosis in cancer cells A-431, but no significant effect on tumor cells SK-BR-3. These experiments suggested that the A-431 cell line is more sensitive to the cytotoxic effect of EGCG, compared with the cell line SK-BR-3.

Inhibition of cell viability by flavonoids (quercetin, EGCG, genistein) could be explained by two different mechanisms of action: i) flavonoids (genistein and quercetin) can induce blocking of the cell cycle progression in a dose-dependent manner; ii) high doses of flavonoids on a longer period of time lead to apoptotic events.

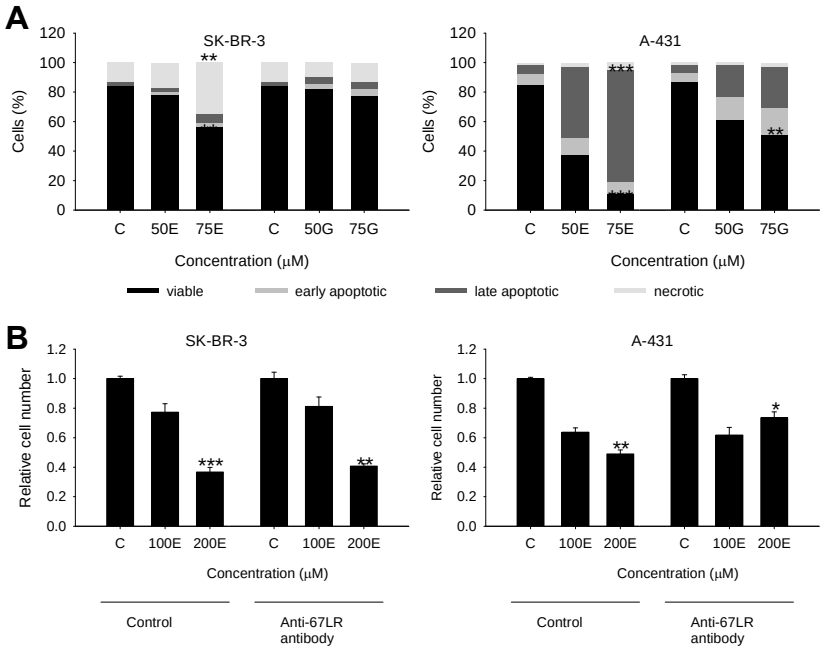


Figure 6. Induction of late apoptosis/necrosis in the tumor cell lines overexpress ErbB protein. (A) The cell lines SK-BR-3 and A-431 were incubated with 50 and 75 μM EGCG and genistein for 72 h in complete medium after which they were labeled with annexin V-FITC and 7-AAD for apoptosis measurements by flow cytometry. Stacked columns show the percentage of viable cells (Annexin V-FITC- / 7-add-), early apoptotic (Annexin V-FITC + / 7-add-), late apoptotic (Annexin V FITC + / 7 ADD +) and necrotic (Annexin V- FITC- / 7-ADD +). For flow cytometry measurements 10,000 events were analyzed (n = 3). (B) Cells pre-treated with the anti-67LR for 3 hours were incubated in the presence of the indicated concentrations of EGCG for 24 h. Cell proliferation was measured after removal of EGCG using WST-1 reagent and spectrophotometric measurements (n = 3, mean ± SEA). Asterisks indicate significant differences statistically compared with control samples (* P <0.05, ** P <0.01, *** P <0.001). E, EGCG; G, genistein.

Cell death induced by EGCG in tumor cells is mediated by 67LR

Previous studies have reported that 67LR is a EGCG receptor of A549 lung tumor cells (Tachibana H. & al. [39]). We were interested to investigate whether this phenomenon is confirmed in tumor cells that overexpress ErbB proteins. For this purpose the tumor cell was initially incubated with 20 μg/ml anti-67LR antibody, incubation followed by maintaining cells in the presence of EGCG and allowing them to stand there for 24 hours. Blocking of the extracellular region of laminin receptor reduced antiproliferative effect of EGCG on tumor cells SK-BR -3 and A-431 (Figure 6B). These results support the hypothesis that 67LR is a molecule involved in the mechanism of action of EGCG, tumor cell lines overexpressing ErbB protein.

Genistein produces cell cycle arrest in tumor cell lines that overexpress ErbB protein

Genistein has been effective in blocking progression of the cell cycle, in particular at concentrations of 50 and 75 μM. Breast tumor cells SK-BR-3 were blocked in G2/M after incubation with genistein (Figure 7A), and this increased the percentage of cells in G2/M phase which was correlated with the decrease in the percentage of cells in the G0/G1 phase. Epidermoid carcinoma cells A-431 showed a similar response to the action of genistein as mammary tumor cells (Figure 7B).

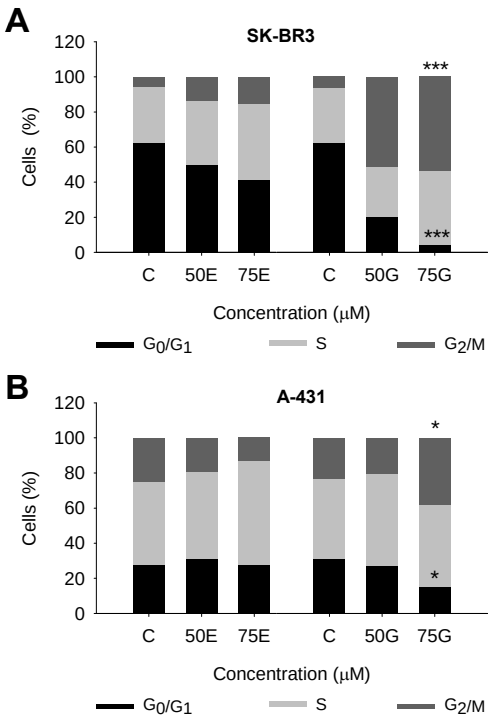


Figure 7. Analysis of the cell cycle in tumor cells SK-BR-3 and A-431 treated with genistein. Tumor cells SK-BR-3 (A) and A-431 (B) were treated with 50 and 75 μM EGCG and genistein for 36 h. The distribution of cell cycle phases was determined by measurement of flow cytometry after evaluation of DNA content labeled with propidium iodide (were evaluated 10000 events/sample, n = 3). The asterisk represents significant difference compared to control samples (* P <0.05, *** P <0.001). E, EGCG; G, genistein

The effect of EGCG on cell cycle progression was not as pronounced as that of genistein, suggesting that genistein is more effective than EGCG in blocking the cell cycle. Contradictory data have been reported in relation to cell cycle arrest in the G0/G1 by EGCG. While some results suggest an effect, other studies do not confirm such effects (JF Chess & al. [40]). Our data are in agreement with those who support EGCG inability to significantly influence the cell cycle phase distribution. On the other hand, the antiproliferative effect of genistein appear to rely almost exclusively on its property to block the progression of the cell cycle in G2 / M phase. These results are consistent with results reported by other research groups have found that cell cycle arrest in the G2 / M phase is correlated with overexpression of protein p21 and decrease of the expression of cyclin B1; also the molecular mechanism of action of genistein on cell cycle could involve its interaction with microtubules (S. Mukherjee & al. [41]).

Objective 2: Investigation of the effect of polyphenols on the expression of ErbB proteins

EGCG, but not genistein induces the reduction of the ErbB protein expression on the surface of tumor cells SK-BR-3 and A-431

Flow cytometric measurements were performed after incubation of tumor cells for 24 h in the presence of 50 μM of EGCG and genistein in the absence of serum. The cell line SK-BR-3 was fluorescent labeled for ErbB2 and A-431 cell line for ErbB1. EGCG induced decrease of 30% ErbB1 and ErbB2 protein expression level, while genistein did not affect significantly the expression of ErbB proteins (Figure 8). Treatment of tumor cells with flavonoids in the presence of serum did not significantly alter the expression of ErbB proteins, suggesting that this effect was enhanced by the absence of serum.

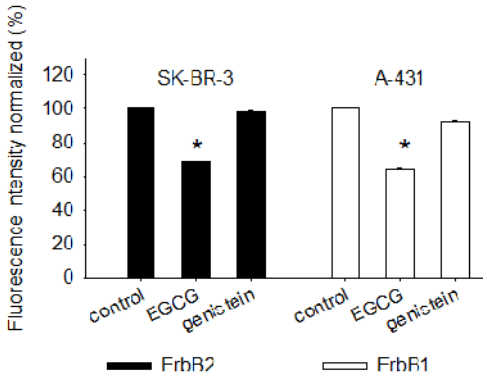


Figure 8. EGCG reduces ErbB protein expression in breast tumor lines and epidermoid carcinoma. (A) Measurement of flow cytometry to assess the expression level of the protein ErbB1 (cell line A-431) and ErbB2 protein (cell line SK-BR-3) after treatment with 50 µM EGCG and genistein for 24 h in medium without serum (n = 3).

A possible explanation for the protective role of serum from the effects of flavonoids on the expression of the ErbB protein in tumor cell lines could be related to the ability of some proteins, for example albumin, to bind flavonoids (Ishii T & al. [42]). It is known that ErbB2 is a protein resistant to the process of internalization (AM Hommelgaard & al. [43]) and flavonoids which can induce its internalization could be considered potential therapeutic agents.

Objective 3: The study of the effects of polyphenols on the association of ErbB proteins (interaction between ErbB proteins, β1-integrin, laminin receptor (67LR) homoassociation ErbB1, ErbB2)

Study of the effect of flavonoids on the relationship of the ErbB protein on the surface of tumor cells

Tumor epidermoid carcinoma cells A-431 were fluorescently labeled for FRET measurement using flow cytometry.

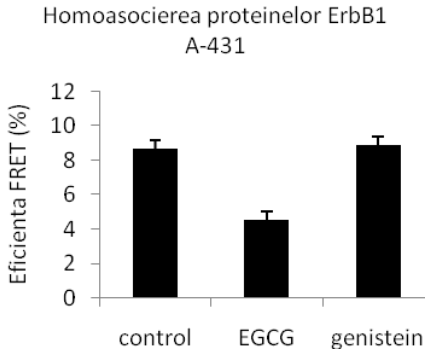


Figure 9: **Homoassociation of ErbB1 protein after treatment with flavonoids.** A-431 cells were grown in the absence of serum for 24 h and incubated with EGCG and genistein for an additional 24 h in complete medium. Cells were labeled with anti-ErbB1 (Mab528) conjugated with AlexaFluor 546 or 647. FRET efficiency was measured using flow cytometry.

Thus ErbB1 proteins were double labeled with anti-ErbB1 antibody conjugated with AlexaFluor 546 and AlexaFluor 647. The efficiency of energy transfer in the case of control samples, those treated with EGCG

and genistein have the following values (Figure 9) 8.7 ± 0 5% (control), 4.5 ± 0.3 (EGCG) and 8.9 ± 0.8 (genistein).

It can be seen a downward trend in energy transfer efficiency after incubation of tumor cells with EGCG for 24 h. In contrast genistein did not induce significant changes in the value of energy transfer efficiency for homoassociation of ErbB1 protein. The trend of diminishing energy transfer efficiency after treatment with EGCG is consistent with data on protein expression level of ErbB1, so just EGCG but not genistein influences homoassociation of ErbB1 protein.

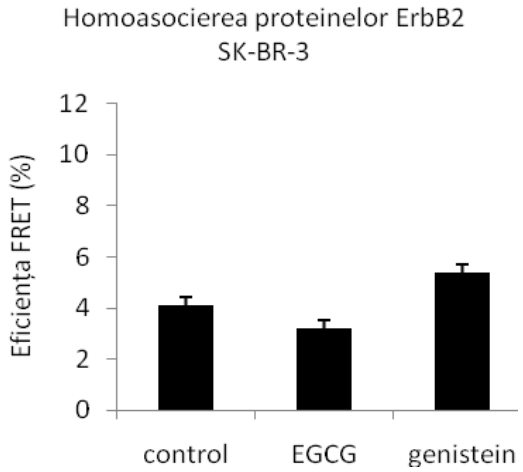


Figure 10: **Homoassociation of ErbB2 protein after treatment with flavonoids.** SK-BR-3 cells were cultured in the absence of serum for 24 h and incubated with EGCG and genistein for an additional 24 h in complete medium. Cells were labeled with anti-ErbB2 antibody (Herceptin) directly conjugated with AlexaFluor 546 or AlexaFluor 647. FRET efficiency was measured using flow cytometry.

Similarly FRET measurements were performed to assess the association of ErbB2 protein on the surface of tumor cells SK-BR-3 (Figure 10). ErbB2 proteins were double marked with a combination of anti-ErbB2 antibody conjugated with AlexaFluor 546 and AlexaFluor 647. Incubation of tumor cells SK-BR-3 with 50 μM EGCG and genistein did not result in significant changes compared to control samples: 4.1 ± 0.3% (control), 3.2 ± 0.4 (EGCG) and 5.4 ± 0.5 (genistein).

In the A-431 tumor cell line ErbB1 protein expression level is about 1.2 x 10^6 receptors/cell (Nagy P. & al. [44]), and tumor line SK-BR-3 level of expression of ErbB2 protein is approximately 0.8 x 10^6 receptors/cell (MM Mocanu & al. [11]). Although these cell lines express a large number of receptors on their surface, which could generate the conditions for the measurement of ErbB proteins homoassociation, however, the energy transfer efficiency values were low. Elevated energy transfer efficiency can easily measure when assessing two extracellular epitopes located in the same molecule, such as ErbB2, under these conditions the energy transfer efficiency being able to reach about 40% (MM Mocanu & al . [45]). Given these conditions, FRET measurements of 67LR and ErbB proteins β1-integrin or of ErbB proteins have not been conducted, taking into account the fact that the expression of β1-integrin or 67LR is much lower than that of ErbB proteins that are overexpressed in cells tumor. These measurements have been performed in cooperation with the Department of Biophysics and Cell Biology, University in Debrecen, Hungary, working team coordinated by Prof. Dr. Janos Szöllősi.

Conclusions

This study brings new evidence supporting the anti-proliferative activity of flavonoids (quercetin, EGCG, genistein) in two tumor cell lines of epithelial origin. Taken together these results support our center hypothesis on reducing cell viability by cell cycle progression by modulating flavonoids (quercetin, genistein) and by promoting pro-apoptotic effects (EGCG). In this study we showed that: i) while EGCG has rather a cytotoxic effect, genistein and quercetina are more effective in blocking cell cycle progression; ii) anti-proliferative effect of EGCG is blocked by anti-67LR; iii) EGCG and not genistein decreases ErbB1 and ErbB2 protein expression from the plasma membrane. Further studies will be required to investigate the effect of EGCG and genistein in combination with other therapeutic molecules to assess the impact of these anti-tumor flavonoids

FRET technique is based on sound physical principles and involves non-radiative energy transfer from an excited donor molecule to an acceptor molecule located at a maximum distance of 2 to 10 nm. Therefore, FRET has been referred to as "the spectroscopic ruler" (Jares-Erijman EA & al. [33], Nagy P. & al. [36] J. Szollosi & al. [38] L. Stryer [46]). Taking into account the results of the molecular association in case of ErbB1 and ErbB2 protein it may be suggested that EGCG causes a reduction trends in the homoassociation of ErbB1 protein on the surface of tumor epidermoid carcinoma cells. Such changes were not observed in ErbB2 protein or after treatment with genistein, which led us to stop investigating the association of ErbB proteins with β1-integrin or 67LR. We plan to investigate other aspects of localization of membrane receptors change in correlation with their association with regions of "lipid rafts" of the plasma membrane.

4. Phase IV: January 2013 – October 2014

Introduction

Study of polyphenols, focusing on epigallocatechin gallate (EGCG) extracted from green tea (*Camellia sinensis*) in breast adenocarcinoma (SK-BR-3) and epidermoid carcinoma (A431) cell lines was continued in order to meet the objectives of the project plan or objective 4: investigating the correlation between the activated status of ErbB proteins and polyphenols treatments and objective 5: to investigate the effect of polyphenols on protein ErbB signaling pathways.

Materials and methods

Cell culture, treatment with EGCG and growth factors. Breast tumor cells SK-BR-3 and epidermal A431 were grown to 60-70% confluence in DMEM with L-glutamine (Sigma) supplemented with 10% fetal bovine serum, 100 units penicillin/ml and 0.01 mg streptomycin/ml. Cell cultures were maintained at 37°C in an incubator with a humidified atmosphere and CO2 (5%). The medium was renewed at 2-3 days, and sub-cultivation was in the proportion of 1:2 to SK-BR-3 and 1:5 for A431. Adherent sub-confluent cells were treated with different concentrations of EGCG: 10, 50, 100 and 200 μM (Sigma Aldrich) dissolved in DMSO (up to 0.1%) for different periods of time. Treatments with 100 ng/ml epidermal growth factor (Sigma-Aldrich) have been performed after pre-treatment with EGCG for 10 minutes at 37°C.

Marking intracellular flow cytometry. Marking intracellular proteins was performed in 3.7% formaldehyde for fixation for 20 minutes at room temperature, followed by permeabilization with 0.2% Triton X-100 (cytoplasmic protein) and 0.1% Triton X-100 (membrane protein) for 15 minutes at room. Primary antibodies used to label were: pErbB1 (Y1173, Exbio) pErbB1 (Y1068, clone 1:12, Cell Signaling), pErbB2

(Y1428, Ab-18 clone PN2A, ThermoScientific), pAkt (S473) -PE (BD Bioscience), pERK (Y204)-FITC (Santa Cruz), pFAK (S910)-PE (BD Bioscience) and c-fos-PE (Santa Cruz). Secondary antibodies: Alexa Fluor 546 (Invitrogen/Life Technologies). The cells were incubated for 1 hour at 4°C, washed (10 minutes, 1500 rpm) and re-suspended in PBS for analysis.

Flow cytometric measurements were performed with a Beckman Coulter Gallios flow cytometer: the excitation being at 488 nm (10000 events/sample) while the emission was recorded using the following filters: 525/40 nm (FL-1, 525 BP 40) and 575/30 nm for FITC (FL-2, 575 BP 30) for PE. Also, part of the measurements have been performed with a FACS Array BD Bioscience cytometer with excitation at 532 nm and the emission was recorded with 585/42 nm bandpass filter for Alexa Fluor 546. Data analysis was performed with FCS Express and WinMDI 2.9.

Results

Objective 4: Investigating the correlation between the activated status of ErbB proteins and polyphenols treatments.

Recent studies have confirmed the anti-carcinogenic effect of polyphenols by modulating receptor tyrosine kinase phosphorylation. Molecular, biochemical results (Western blot) showed blockage of phosphorylation of EGFR, Akt and mTOR after administration of 72 and 256 µM EGCG to a lung adenocarcinoma cell line A-549 for 6, 12 and 24 hours (J. Relationships & al. [47]).

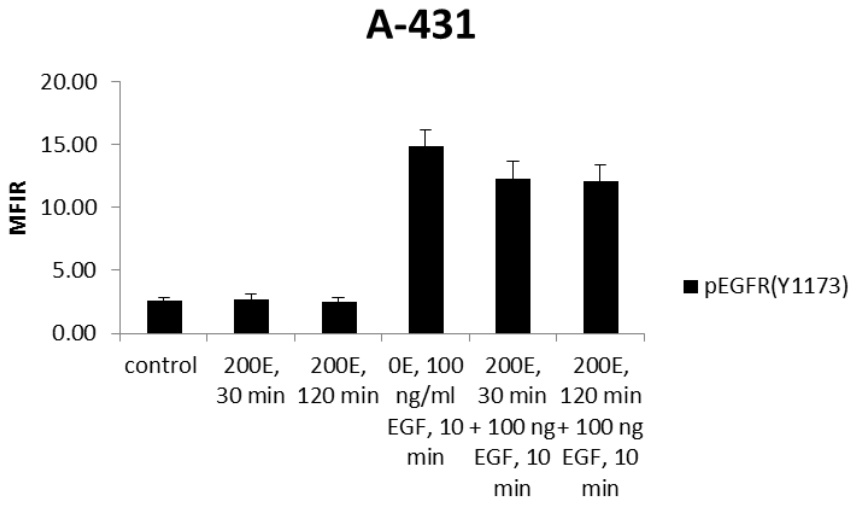


Figure 1: The effect of EGCG on the Y1173 phosphorylation site of the EGFR (ErbB1) was significantly increased after stimulation of the cells with epidermal growth factor (EGF). Variation of the incubation period with EGCG cell line A-431 for 30 or 120 minutes did not significantly modify EGGC effect on the phosphorylation of EGFR (Y1173).

To investigate the effect of EGCG on activation of the ErbB protein, these have been labeled with monoclonal antibodies recognizing the phosphorylated sites of proteins. Thus epidermal growth factor receptor (EGFR) was labeled for sites Tyr 1173 and Tyr 1068. Oncoprotein ErbB2 activation was assessed using an antibody that recognizes phosphorylated Tyr 1248. The expression level of intracellular phosphorylated tyrosine site at residues 1173 and 1068 of EGFR was investigated by flow cytometry and it was noticed the amplification of the phosphorylation after the treatment with EGF (Figure 1). Varying the incubation period between 30 and 120 minutes EGCG did not change the initial results, which resulted in

further experiments using a 30 minute incubation period. Increasing doses of EGCG induced reduction in the intracellular phosphorylation of Y1173 and Y1068 sites in a dose-dependent manner. Erlotinib, an inhibitor of EGFR phosphorylation was used as a negative control. The effect of EGCG was also investigated on the activation of the ErbB2 (Y1428) using increasing doses of polyphenols, and as negative control was used lapatinib, inhibitor of the phosphorylation of both EGFR and ErbB2. Since ErbB2 oncoprotein is considered to be a ligand orphan receptor, also existing in an extended conformation that mimics the ligand coupling (Hynes NE & al. [30]) there were used no growth factors to investigate the correlation between EGCG and growth factors in its case. It was observed that the effect of EGCG on activation of ErbB2 (Y1248) was not significant for incubation in the presence of flavonoids (data not shown).

Objective 5: **Investigation of the effect of polyphenols on protein ErbB signaling pathways.**

Two methods have been discussed for investigating the effect of EGCG on intracellular protein phosphorylation of proteins in the ErbB signaling pathways: i) short-term incubation (30 minutes) and ii) long-term incubation (48 h). As all of the tyrosine-kinase receptors in the ErbB protein family are activating the signaling pathway of mitogen-activated protein kinase (MAPK) one of the proteins of interest was the kinase regulated by extracellular signal (ERK) (Park JW & al. [2]).

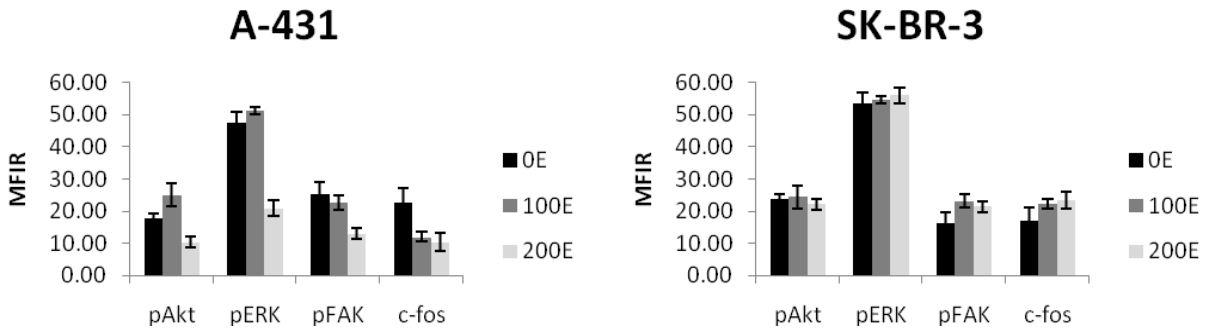


Figure 2: The effect of EGCG on signal paths at 30 minutes, in epidermoid carcinoma A-431 and breast adenocarcinoma SK-BR-3 cell line. EGCG reduces phosphorylation pAkt (S473), pERK (Y204), pFAK (S910), and the expression of nuclear transcription factor c-fos in a dose-dependent cell line A-431. Incubation of cell line SK-BR-3 in the presence of EGCG for 30 minutes did not significantly alter the phosphorylation status of proteins pAkt (S473), pERK (Y204) pFAK (S910) and does not induce changes on the transcription of factor c-fos. Both cell lines were pretreated with various concentrations of EGCG for 30 minutes in serum free medium after cell starvation overnight.

Another focal point of intracellular signaling pathways of ErbB protein is protein kinase B (PKB) or Akt protein. It is a protein kinase which acts on serine/threonine sites and is involved in the regulation of various cellular functions, including: apoptosis, survival or proliferation; also is active in tumorigenesis (Y. Gao & al. [48]).

Of interest is another protein: focal adhesion kinase (FAK) signaling pathways located at the intersection of ErbB proteins and extracellular matrix receptors, integrins. ErbB2 activation induces phosphorylation of FAK in tumorigenesis. In normal cells FAK is involved in survival, metabolism, and cell growth, gene deletion for FAK being lethal (Pentassuglia L. & al. [49]). It was also investigated the effect of EGCG on nuclear transcription c-fos factor. Fos transcription factor family includes many members, which together with the members of the Jun family form the group of AP-1 protein (activating protein-1) involved in tumorigenesis. AP-1 could regulate genes that are involved: proliferation, metastasis, apoptosis,

angiogenesis, differentiation (Milde K.-Langosch [50]). Short-term incubation of epidermoid carcinoma cell line A-431 with 100 and 200 µM EGCG resulted in partial inhibition of the phosphorylation of intracellular proteins pAkt (S473), pERK (Y204), pFAK (S910), and the expression of the transcription nuclear c-fos factor (Figure 2). Reducing the level of phosphorylation of intracellular proteins was observed to be dose dependent. In contrast, in the mammary adenocarcinoma line SK-BR-3, EGCG did not induce phosphorylation reduction after a short incubation. Maintenance of cell lines A-431 and SK-BR-3 in the presence of 50 µM EGCG concentrations for a longer period of time, 48 hours in complete medium, led to the reduction of the signal derived from the phosphorylation of intracellular proteins in both investigated lines.

Conclusions

The results show that the effect of epigallocatechin gallate on the activation of tyrosine kinases receptors from the family of ErbB2 protein and the phosphorylation of certain key proteins in these signaling pathways (pAkt, pERK, pFAK) depends on the investigated cell line, on the dose and the treatment period. These observations support the anti-carcinogenic effect of EGCG type polyphenols and combined therapies with monoclonal antibodies or other molecules with anti-tumor action could be a possible future experimental approach.

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Project web page:

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Principal Investigator,

Dr. Maria-Magdalena Mocanu
