




Effects of matcha tea extract on cell viability and peroxisome proliferator-activated receptor γ expression on T47D breast cancer cells

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Received: 14 October 2021 / Accepted: 23 December 2021 / Published online: 25 January 2022
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Abstract

Purpose In the following work, we investigated the nuclear peroxisome proliferator-activated receptor gamma (PPAR γ)-dependent proliferation behavior of breast cancer cells after stimulation with matcha green tea extract (MTE).

Methods T47D cells were stimulated with MTE at concentrations of 5, 10 and 50 $\mu\text{g/ml}$. Cell viability was assessed using a WST-1 assay after an incubation time of 72 h. PPAR γ expression was quantified at the gene level by real-time polymerase chain reaction (PCR). A western blot (WB) was carried out for the qualitative assessment of the expression behavior of on a protein level.

Results The WST-1 test showed a significant inhibition of viability in T47D cells after 72 h at 5, 10 and 50 $\mu\text{g/ml}$. The PCR showed an overexpression of PPAR γ in T47D cells in all concentrations. At the concentration of 50 $\mu\text{g/ml}$ the expression was significantly increased ($p < 0.05$). The WB demonstrated a significant quantitative increase of PPAR γ at protein level with MTE concentrations of 10 and 50 $\mu\text{g/ml}$. In addition, there was a negative correlation between the overexpression of PPAR γ and the inhibition of proliferation.

Conclusion MTE decreases the cell viability of T47D cells and furthermore leads to an overexpression of PPAR γ on protein and mRNA level.

Keywords Matcha tea extract · MTE · T47D · PPAR γ · WST-1 · PCR · Western blot

Introduction

Breast cancer is the most frequent cancer in women worldwide. Increases in incidence over the last decades have been linked to the implementation of screening programs [1]. In 2018, around 2.1 million new cases were diagnosed worldwide and 626,679 women died of breast cancer [2]. Modern multimodal therapeutic concepts have enabled cure rates of

70–80% [3]. To achieve further improvements, the investigation of mechanisms and possible substances that may inhibit cancer proliferation and invasion remains of interest. Second only to water, tea is the most commonly consumed beverage worldwide [4] and in western countries its popularity is increasing further. Like black and green tea (GT), matcha tea (MT) is obtained from the leaves of the tea plant *Camellia sinensis*. While with GT the tea leaves are removed from the tea before consumption, with MT they are ground into a fine powder, dissolved in boiling water and ingested in their entirety; therefore, the intake of various substances contained in the leaves is much higher [4–6]. MT contains polyphenols, including the subdivisions of flavonoids, flavanols and catechins. Up to 30% of the dry mass of fresh tea leaves is made up of phenolic compounds, 90% of which are catechins. Of the catechins found in MT, epigallocatechin gallate (epigallocatechin-3-gallate, EGCG) is most abundant [7]. Other catechins such as epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) only make up

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10%. The remaining dry mass components in tea leaves are proteins (15%), amino acids (4%), caffeine (4%), raw fibers (26%), lipids (7%), other carbohydrates (7%), pigments, such as chlorophyll and carotenoids (2%) and minerals (5%) [8]. 100 ml of GT contains approximately 20–100 mg of EGCG [9]. The main limitation for broad therapeutic application of polyphenols is that after oral intake and metabolism only as little as 0.024% are bioavailable [10, 11]. Results of pharmacokinetic studies concluded that after ingestion, EGCG concentrations in human serum may reach no more than the high nanomolar range [12]. The concentrations we used in vitro in the study presented here are deliberately lower than those used in previous experiments, but still substantially higher than serum concentrations measured in vivo after consumption and metabolism by humans. EGCG has been extensively investigated for its tumor inhibitory effects in breast and prostate cancer [13–16]. Different mechanisms by which EGCG can influence the cell cycle and proliferation have been described, such as the inhibition of matrix metalloproteinases [17], Wnt signaling [18], methylation [19] and induction of peroxisome proliferator-activated receptors (PPAR) [20–22]. The PPAR family of essential nuclear receptors includes three subtypes (α , β/δ , and γ) that bind directly to certain DNA regions and regulate the expression of target genes [23]. Previous research suggests that PPAR γ has a pivotal role in the pathophysiology of multiple diseases and possibly their treatment. For instance, PPAR γ is one of the major transcription factors activated by thiazolidinediones [24]. Research on PPAR γ in tumor cells has yielded divergent results; in some studies it acted as a tumor suppressor, but others reported growth-promoting effects [25–28]. Investigations regarding breast cancer report mostly tumor suppressing effects [27, 29, 30]. With current data supporting that *Camellia sinensis* has a tumor suppressing effect on breast cancer cells, we wanted to elucidate a possible involvement of PPAR γ . With diverging results of a possible cell inhibitory effect of EGCG on T47D [31, 32], we, therefore, investigated the effect of MTE in different concentrations on T47D breast cancer cells. Cell proliferation was analyzed using the WST-1 array. PPAR γ expression was measured via real-time PCR and PPAR γ protein levels were determined via western blot.

Materials and methods

Cell cultivation and cell stimulation

To mimic ductal breast carcinoma, the T47D cell line was used. Cells were grown on 80% monolayer in a cell culture bottle and cultivated in Dulbecco's Modified Eagle Medium (DMEM; 3.7 g/L NaHCO₃, 4.5 g/L D-glucose, 1.028 g/L stable glutamine, and sodium pyruvate; Biochrom, Berlin,

Germany). Then 10% heat-inactivated fetal calf serum (FCS; Biochrom) was added to the medium and incubated with atmospheric concentrations of CO₂ of 5% at 37 °C. For further use they were trypsinized and counted.

Preparation of the matcha tea extract (MTE)

Matcha tea was purchased commercially (Houjo Matcha Tea, harvested in Hoshino, Yame prefecture, Japan). For each test, the tea extract preparation was done as described in each test below.

WST-1 assay

T47D cells were cultivated in a 96-well plate at a density of 10,000 cells per well in 50 μ l DMEM with 10% FCS. After 4 h, the medium was replaced with DMEM without FCS. FCS is known to contain substances affecting cell proliferation and maintenance such as growth factors, hormones, vitamins, and transport proteins. By using DMEM without FCS, a possible effect of FCS components on cell proliferation was reduced [33, 34]. Cells were incubated for a further 12 h. 27.1 mg tea extract were dissolved in 100 μ l pure ethanol and diluted 1:1000 with DMEM without FCS. A control solution was prepared in the same way without adding MTE. Afterwards, different amounts of the MTE solution and DMEM without FCS were added to achieve the desired concentrations (5, 10 and 50 μ g/ml), resulting in a total of 100 μ l per well. For the control group, instead of the MTE solution, the control solution and DMEM without FCS were added to each well. The wells were then incubated for 72 h. After incubation, the WST-1 reagent (water soluble tetrazolium, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; Sigma-Aldrich, St. Louis, MO, USA) was added to detect the activity of mitochondrial succinate dehydrogenase cleaving the tetrazolium salt to formazan. Cell viability was measured after incubating for 30 min with the use of a multi-well spectrophotometer (wavelength: 420–480 nm). Three independent measurements with three technical replicates were performed.

PCR

T47D cells were incubated on a 12-well plate at a density of 500,000 cells per well for 4 h with 500 μ l DMEM with 10% FCS. Then the medium was replaced with 500 μ l DMEM without FCS and the cells were incubated for a further 12 h. 27.1 mg tea extract were dissolved in 100 μ l pure ethanol and diluted 1:1000 with DMEM without FCS. A control solution was prepared in the same way without adding MTE. Afterwards, different amounts of the MTE solution and DMEM without FCS were added to achieve the desired concentrations (5, 10 and 50 μ g/ml), resulting in a total of

500 µl per well. For the control group, instead of the MTE solution, the control solution and DMEM without FCS were added to each well. The wells were then incubated for two hours. This time was selected because changes in mRNA levels are expected within hours after stimulation and the half-life of mRNA also being in the range of hours. Longer incubation times may, therefore, be unsuitable for detection of short-term changes in mRNA levels. The excess liquid was removed. The wells were washed with phosphate-buffered saline (PBS) and RA-1 buffer (Macherey–Nagel, Düren, Germany) was added for cell lysis. For RNA isolation, the NucleoSpinRNAII (Macherey–Nagel, Düren, Germany) was used. For the reverse transcription of RNA, the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used. 10 ng of RNA were added. The temperature protocol phases were 10 min at 25 °C, 2 h at 37 °C, 5 s at 85 °C and stopped with a cooling phase at 4 °C. For the TaqMan® PCR, 96-well plates with 20 µl well volume were used. Each well was filled with 10 µl of TaqMan® Universal PCR Master Mix 2X (Thermo Fisher Scientific), 8 µl of distilled water treated with 0.1% diethyl pyrocarbonate (DEPC), 1 µl of TaqMan® Gene Expression Assay 20X (Thermo Fisher Scientific; target: ACTB, assay ID Hs99999903_m1; target: PPARG, assay ID Hs01115513_m1; sequences of the primers not revealed by manufacturer), and 1 µl of cDNA sample. The PCR assay was performed using the ABI Prism 7500 Fast (Thermo Fisher Scientific). Thermal cycling was started for 20 s at 95 °C and was followed by 40 cycles of amplification at 95 °C for 3 s, and 60 °C for 30 s. To analyze the results, the comparative $2^{-\Delta\Delta CT}$ method was used [35]. As endogenous control for the ΔCT -values, β -actin was used. Three independent measurements with two technical replicates were performed.

Western blot

T47D cells were cultivated in a 12-well plate at a density of 500,000 cells per well in 1000 µl DMEM with 10% FCS. After 4 h, the medium was replaced with 1000 µl DMEM without FCS and the cells were incubated for a further 12 h. 20 mg tea extract were dissolved in 100 µl pure ethanol and diluted 1:1000 with DMEM without FCS. The dilution for the control cells was prepared in the same way without the addition of MTE. Afterwards, different amounts of the MTE solution and DMEM without FCS were added to achieve the desired concentrations (5, 10 and 50 µg/ml), resulting in a total of 1000 µl per well. For the control group, instead of the MTE solution, 250 µl control solution and 750 µl DMEM without FCS were added to each well. Cells were incubated for 48 h, then washed with phosphate-buffered saline (PBS). For cell lysis, 200 µl of a buffer solution consisting of a 1:100 of protease inhibitor (Sigma-Aldrich, St.

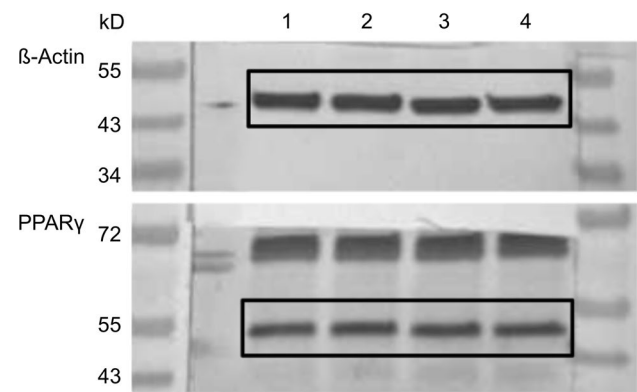


Fig. 1 Example of western blot membranes after stimulation with different concentrations of MTE or the control group and incubation with β -actin and PPAR γ antibodies. The respective bands are numbered [(1) 0 µg/ml; (2) 5 µg/ml; (3) 10 µg/ml; (4) 50 µg/ml] and marked with black boxes.

Louis, MO, USA) in RIPA buffer (radioimmunoprecipitation assay buffer; Sigma-Aldrich) was added to each well before incubating for 30 min at 4 °C. After centrifuging the lysates, a Bradford protein assay of the supernatant was performed. With western blotting, the proteins were separated depending on their molecular weight with the use of SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany). After blocking the PVDF membrane for 1 h in a receptacle containing a casein solution 1× (Vector Laboratories, Burlingame, CA, USA) to prevent a nonspecific binding of the antibodies, the primary antibodies anti- β -actin (clone AC-15, mouse IgG; Sigma-Aldrich Co., St. Louis, Missouri, USA) and anti-PPAR γ (polyclonal IgG, rabbit, Abcam, Cambridge, UK) were diluted in a 1×casein solution and afterwards laid onto the membrane for 16 h at 4 °C. After washing the membranes with tris-buffered saline (TBST), membranes were incubated with biotinylated anti-rabbit IgG antibody and ABC-AmP reagent (VECTASTAIN ABC-AmP Kit for rabbit IgG, Vector Laboratories) according to the manufacturer's protocol. Specific bands on the membrane were visualized using the BCIP/NBT chromogenic substrate (Vectastain ABC-AmP Kit, Vector Laboratories), detected with the Bio-Rad Universal Hood II (Bio-Rad Laboratories, Hercules, CA, USA) and the specific color densities of the bands were quantified with the Bio-Rad Quantity One software (Bio-Rad Laboratories) (Fig. 1). For statistical evaluation, the color intensity of PPAR γ bands clustered pixels were set in relation to the β -actin bands clustered pixels. The western blots were repeated independently five times.

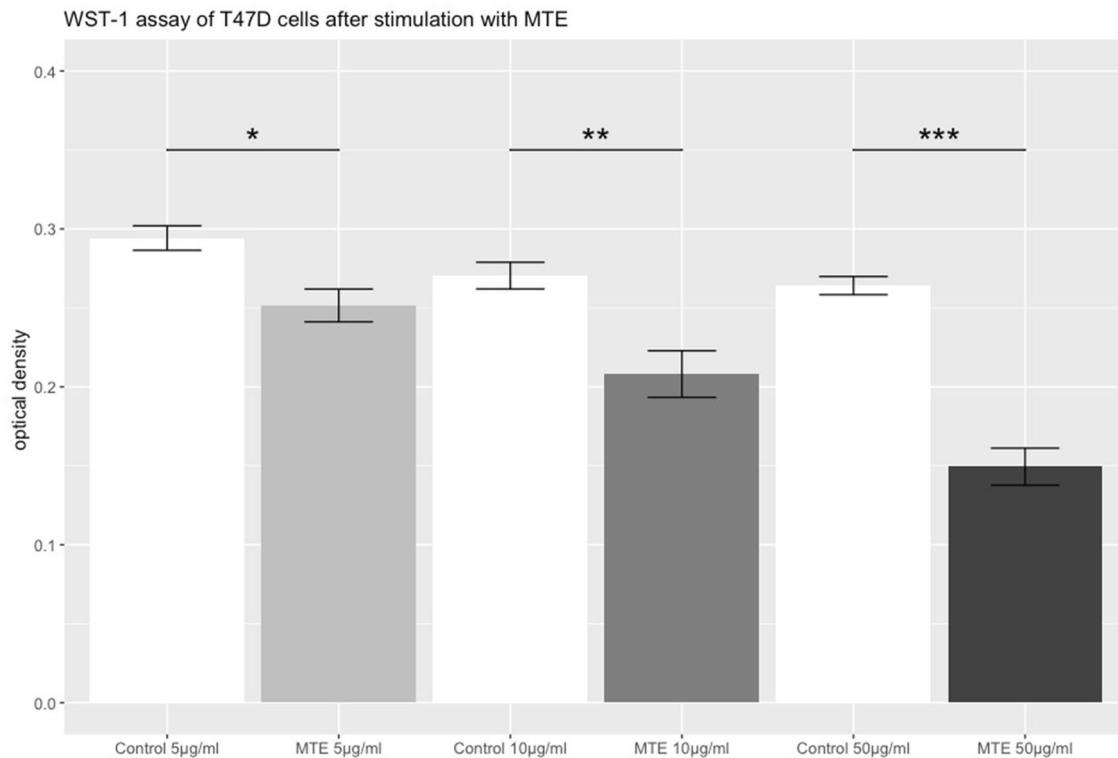


Fig. 2 WST-1 assay of T47D cells stimulated with MTE. The grey bars represent the optical density of T47D cells after the incubation with different concentrations of MTE (5, 10 and 50 µg/ml) for 72 h. The white bars represent the control group. The top of each bar represents the mean ± standard error (SE).

MTE induced a significant reduction of cell proliferation at every concentration. Significant results are linked and marked with asterisks ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$)

Statistical analysis

The statistical programming environment R, version 4.0.2 [36], was used for processing and statistical analysis of the collected data. Findings with p values < 0.05 were considered significant. Shapiro–Wilk tests were used to check normality of distributions. Adapted to the specific design of experiment we applied different statistical tests to compare stimulated and control samples. The paired t test was used for the WST-1 and PCR assay. To account for repeated measurements in several Western-blot analyses we fitted a linear random effects model.

Results

WST-1 proliferation assay

To detect changes in T47D cellular viability after incubation, a WST-1 proliferation assay was performed. A significant inhibitory effect in cell viability was observed ($p = 0.017$) already at the concentration of 5 µg/ml MTE. As the concentrations of MTE increased, higher levels of significance

where observed (10 µg/ml MTE: $p = 0.002$; 50 µg/ml MTE: $p < 0.001$). Results are shown in Fig. 2.

PCR for PPAR γ expression on mRNA level

To detect changes of PPAR γ mRNA expression after incubation of T47D cells with different MTE concentration, TaqMan[®] PCR was performed. To analyze the results, the comparative $2^{-\Delta\Delta C_q}$ method was used. The x fold expression of mRNA in comparison to the control increased in a dose-dependent manner. Whereas at the concentrations of 5 µg/ml and 10 µg/ml mRNA expression of PPAR γ was numerically higher than control, this increase was only statistically significant at the highest concentration of 50 µg/ml MTE (5 µg/ml MTE: $p = 0.539$; 10 µg/ml MTE: $p = 0.163$; 50 µg/ml MTE: $p = 0.029$). Results are shown in Fig. 3.

Western blot for PPAR γ on protein level

To investigate PPAR γ expression on a protein level, a western blot was applied. At all MTE concentrations PPAR γ expression increased in a dose-dependent manner in comparison to the control cells. PPAR γ expression was significantly elevated at MTE concentrations of 10 and 50 µg/ml

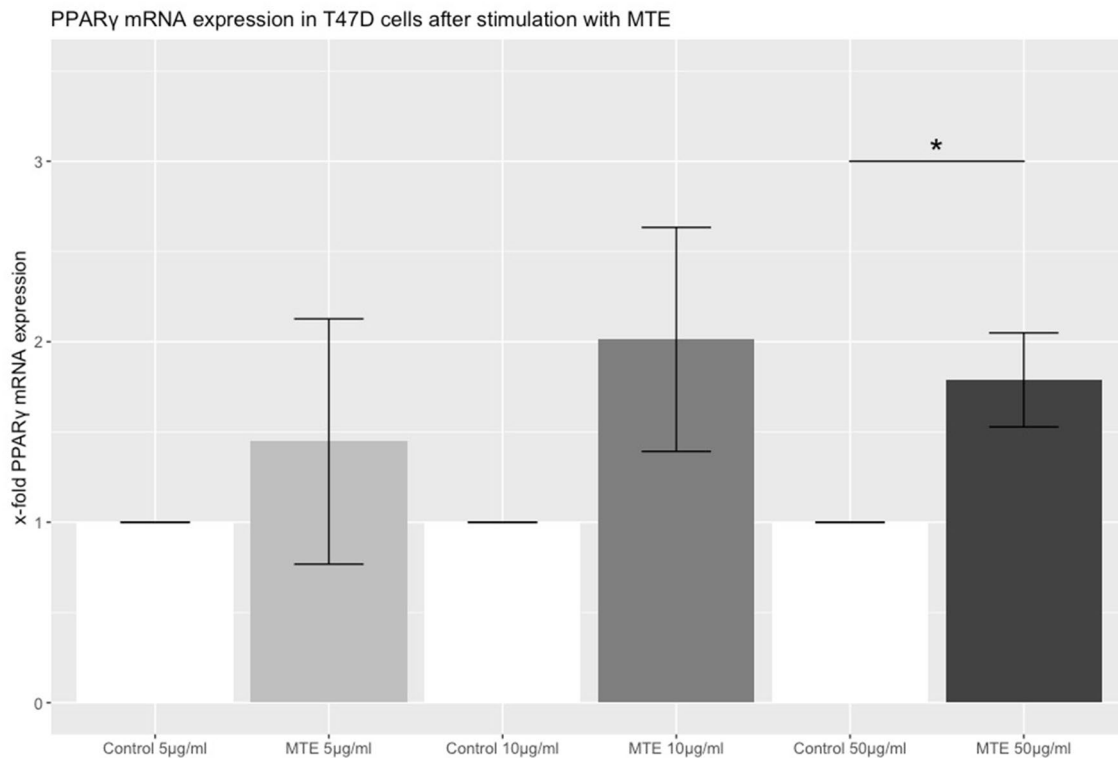


Fig. 3 The bar chart represents the relative PPAR γ expression on mRNA level in T47D cells after incubation with three different concentrations of MTE (5, 10 and 50 μ g/ml) or the control solution for 2 h. mRNA levels were detected via TaqMan[®] real-time PCR. The

values on the y axis are show ratios of stimulated and control expression levels. The top of each bar represents the mean \pm SE. Statistical significance was achieved at the highest concentration of MTE ($p=0.029$) and is marked with one asterisks

(5 μ g/ml MTE: $p=0.315$; 10 μ g/ml MTE: $p<0.001$; 50 μ g/ml MTE: $p<0.001$). Results are shown in Fig. 4.

Discussion

To our knowledge, this is the first study investigating the effect of MTE on PPAR γ expression in T47D breast cancer cells. Natural substances have a long history in the prevention and treatment of neoplasms [37]. Green tea and matcha tea—with their chemical compounds including vitamins, phenolic acids, quercetin and catechins—have been associated with health benefits such as anti-inflammatory and anti-carcinogenic effects [38]. EGCG is the most abundant catechin in matcha tea. Its potent growth inhibitory effects have been demonstrated in various cell lines [39, 40]. A possible anti-carcinogenic effect of EGCG on the estrogen receptor positive, HER2 negative, T47D cell line has been investigated in the past yielding controversial results. While Moradzadeh et al. were able to demonstrate decreased cell viability after treatment with EGCG [31], Zeng et al. could not find an anti-carcinogenic effect of EGCG on T47D cells alone [32]. We therefore wanted to investigate the potential antiproliferative effects and effects on PPAR γ expression

of matcha tea extract on T47D cells. In this study, we used an extract instead of its individual active compounds to evaluate the cumulative and synergistic anti-carcinogenic effect of all active compounds in MT. In previous studies, we could demonstrate anti-carcinogenic effects on both ER/PR receptor-positive and -negative breast cancer cells using GT, MT, EGCG and quercetin [40]. Our hypothesis in this trial was that PPAR γ induction may be responsible for some of the observed anti-carcinogenic effects.

It has been proposed that MTT/MTS-based assays may underestimate an antiproliferative effect of EGCG. EGCG has been described to induce an increased activity of the mitochondrial dehydrogenase and furthermore has an intrinsic potential to reduce MTT and MTS and therefore increase the formation of formazan [41–43]. The significant reduction in cell growth by all used concentrations underlines the anti-carcinogenic effect of matcha tea extract on T47D cells in vitro. The main limitations of the methods used in this in vitro study are the use of concentrations not achievable in vivo by oral ingestion of MT and that MTE has not been subjected to prior digestion, intestinal absorption, and first pass metabolism. In 1997, Nagakawa and Miyazawa demonstrated that due to the complexity of digestion, absorption and metabolism, the maximum serum plasma concentration

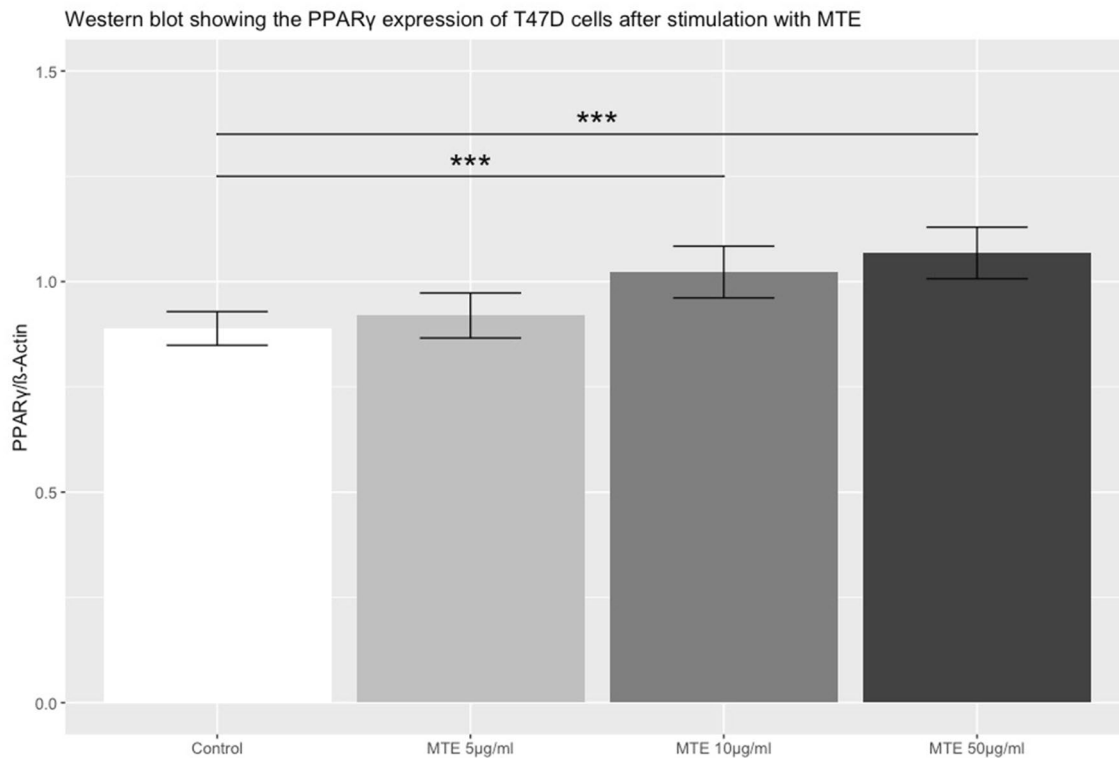


Fig. 4 The bar chart shows the PPAR γ protein expression in T47D cells after stimulation with different concentrations of MTE compared to control. The top of each bar represents the mean \pm SE. MTE induced a significant upregulation of PPAR γ expression on protein

level at MTE concentrations of 10 μ g/ml and 50 μ g/ml. Significant differences between groups are linked and marked with asterisks ($p < 0.001^{***}$)

of EGCG is only 0.024% of the amount ingested [10]. In a study by Yang et al. 18 individuals consumed 3 g of decaffeinated GT (EGCG content: 73 mg/g) which resulted in an EGCG plasma concentration of no more than 326 ng/ml. Whereas higher amounts of GT consumption (4.5 g) did not lead to increased plasma concentrations, an increase from 1.5 to 3 g resulted in a triple EGCG plasma concentration [44]. The lowest concentration used in our study was 5 μ g/ml, equal to 5000 ng/ml MTE. Weiss et al. described concentrations of EGCG after extraction with water of 0.32 mg per gram of MT [45]. Transferring these findings, the amount of EGCG at the given concentrations of 5, 10 and 50 μ g/ml are higher than those that are achievable by oral ingestion of MT. EGCG metabolism such as methylation, glucuronidation, sulfation, and oxidative degradation further weakens its efficacy. For example, the catechol-*O*-methyltransferase (COMT) reduces the amount of EGCG available. Carriers of the lesser potent COMT-L allele benefit more from catechin activity than carriers of the highly potent COMT-H allele [46]. To overcome low bioavailability, several different approaches have been investigated. A simple method is the application of EGCG capsules where dosages of 800 mg resulted in plasma levels of up to 439 ng/ml [47]. Approaches that are more complex include modification of

the EGCG molecule. For example, it has been demonstrated that the addition of peracetate protections to EGCG hydroxy groups leads to higher bioavailability and higher concentrations in breast cancer cells [48]. Moreover, recently several different advanced nanovehicle-enabled delivery systems of EGCG have been tested in various cell and animal models that demonstrate better bioavailability and promising results in the context of cancer therapy [49]. Due to the facts described above, it becomes clear that although relevant results regarding PPAR γ induction were demonstrated, this in vitro study cannot simulate the conditions encountered in human physiology. Animal studies might provide more insight.

Despite the methodological limitations mentioned above, the strength of our study lies in the fact that while many in-vitro studies have until now extensively investigated steroid receptor interaction and resulting downstream cellular cascades after MT and EGCG incubation, our study has demonstrated a different target of phenol action in T47D breast cancer cells: the PPAR γ receptor. Clinical studies have identified PPAR γ expression as a positive prognostic factor in ductal breast cancer [50] and higher concentrations of PPAR γ seem to correlate inversely with grading, size and TNM stage of breast

cancer [51, 52]. Until now, studies investigating a potential association between PPAR γ and the anti-carcinogenic effect of MTE on breast cancer cells are lacking. Therefore, we investigated the PPAR γ expression on protein and mRNA level after being exposed to matcha tea extract at concentrations of 5, 10, and 50 $\mu\text{g/ml}$). With the use of western blots, we could demonstrate a positive correlation between PPAR γ expression and an increase in MTE concentration. Similar results emerged at the mRNA level, where an increased concentration of MTE leads to higher expression rates of PPAR γ , but statistical significance was only reached at the highest MTE concentration.

As a nuclear receptor, the activity of PPAR γ is depending on its intracellular localization and the activation through ligands, which can be natural agonists like fatty acids or prostanoids or synthetic ones like thiazolidinedione drugs [29]. Current data from in vitro and in vivo studies suggest an involvement of PPAR γ in various cell signaling pathways. Via the stimulation of PPAR γ ligands an upregulation of the expression of syndecan-1 and the activation of FAS ligand has been reported, which lead to an induction of cell apoptosis [53, 54]. Moreover, ligand activated PPAR γ can interfere in the regulation of cell growth and cell cycle via the activation of promoter gene p53 and the upregulation of p53 and the target gene p21 [55]. This also triggers the intrinsic apoptotic pathway. Of special interest regarding a possible interference between PPAR γ and the cell growth inhibitory effects induced by the compounds of matcha tea is the downregulation of the cyclin D1 promoter by PPAR γ ligands. The reduced expression leads to the induction of a cell cycle arrest [56, 57]. Hong et al. could demonstrate that treatment of breast cancer cells with EGCG also leads to an inhibition of growth via downregulation of β -catenin, phosphorylated Akt and cyclin D1 [58]. With the dose dependent anti-carcinogenic effect and simultaneous upregulation of PPAR γ , the influence of PPAR γ via the downregulation of cyclin D1 seems plausible.

As our study seems to be one of the first investigating a potential involvement of PPAR γ in the anti-carcinogenic effect of MTE in breast cancer, there is a lack of evidence regarding the alteration of cellular pathways. Moreover, as we analyzed an extract, the substrate responsible for the observed results still remains unknown. From other experiments of our group, we know that matcha tea also contains fatty acids, which could be responsible for PPAR γ upregulation. In the future the experiment could be repeated with EGCG and fatty acids to evaluate their effects. In addition, decreased viability of T47D cells after MTE stimulation could be investigated in detail by immunohistochemistry or reverse transcriptase quantitative (RTQ)-PCR quantification of apoptosis-induced markers (for example, p53, p21, BCL2, Caspase 8/9). MTE incubation of estrogen receptor negative breast cancer cells (e.g. BT-20) could evaluate the

effect of possible estrogen receptor interaction. Finally, our results may serve as a motivation for further investigations on matcha tea induced PPAR γ alterations in cancer cell or animal models.

Conclusions

In this in vitro study, we could demonstrate that incubation of T47D cells with different concentrations of MTE results in an increased expression of PPAR γ and a dose dependent reduction of cellular viability. As PPAR γ expression can result in anti-carcinogenic effects, more research in this field is needed to evaluate the observed results.

Acknowledgements We want to thank everybody involved in realizing the project. Special thanks to Constantin Tilgener and Simone Hofmann for their laboratory work.

Author contributions S K: manuscript writing/editing, Data analysis; C T: data collection or management, protocol/project development; U J: protocol/project development, data analysis, supervision; S H: data collection or management, protocol/project development; T V: manuscript writing/editing; T K: manuscript writing/editing; H H: manuscript writing/editing; F B: manuscript writing/editing; S M: project supervision; L S: protocol/project development, manuscript writing/editing.

Funding Open Access funding enabled and organized by Projekt DEAL.

Availability of data and materials The datasets used and analyzed during the study are available from the corresponding author on request.

Declarations

Conflict of interest S. Mahner has received research support, advisory board, honoraria and travel expenses from AstraZeneca, Clovis, Eisai, GlaxoSmithKline, Medac, MSD, Novartis, Olympus, PharmaMar, Roche, Sensor Kinesis, Teva and Tesaro. All other authors declare to have no conflict of interest.

Consent for publication All authors have analyzed and interpreted the data and read and agreed to the published version of the manuscript.

Ethics approval Not applicable.

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