Effect of flavonoid extract, green tea extract, chinese bayberry extract, coffee extract, olive oil and trans-fatty acid on DMBA-induced LINE-1 DNA methylation

Doctoral (PhD) thesis

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1. Introduction

According to WHO data, in 2018, the number of newly registered malignant cancer cases worldwide was approximately 18.1 million and the number of deaths, despite the increasing availability of screening tests, advances in diagnostic methods and the use of targeted therapies, was nearly 9.6 million. Thus, early diagnosis and prevention of malignant cancers is still the most effective strategy to reduce incidence and mortality. In prevention, a healthy diet and the avoidance of smoking, and in early diagnosis the use of appropriate biomarkers are of key importance.

Some carcinogenic foods and tobacco smoke both contain the carcinogenic compound 7,12-dimethylbenz[a]anthracene (DMBA), which can be used in animal experiments of cancer models. In addition, trans-fatty acids (TFAs) also have a significant diet-related harm. From a tumour biology point of view, several chemopreventive compounds, such as polyphenol-rich parts of plants or extra virgin olive oil (*Oleaeuropaea*), green tea (*Camellia sinensis*), chinese bayberry (*Myrica rubra*) and coffee (*Coffee arabica*), exert a protective effect against it.

The methylation status of the promoter region of the Long Interspersed Element-1 (LINE-1) retrotransposon DNA segment is a representative biomarker of these factors.

However, despite this established hypothesis, to date there have been few studies on DMBA carcinogen-induced LINE-1 DNA hypomethylation.

Based on what is described above, the topic of my thesis is the effect of dietary carcinogens and chemopreventive molecules on the methylation pattern of the LINE-1 transposon.

A further importance of the topic is, that as part of prevention strategies, chemopreventive agents may reverse harmful early epigenetic changes.

2. Objective

In my study, I examined the DNA methylation pattern of LINE-1 in the liver, spleen and kidneys of DMBA pretreated mice in animal models. The aim of the experiment was to determine how these carcinogenic/chemopreventive effects are reflected in the

DNA methylation patterns of LINE-1, to what extent the chemopreventive agents studied are able to prevent DMBA-induced hypomethylation, and whether they can be used as potential biomarkers of these effects. A further aim was to examine the effect of TFA on DMBA-induced LINE-1 DNA hypomethylation, to determine whether it was able to enhance it or not?

3. Materials and methods

In our experiment eight groups (n = 6) of 12-week-old female CBA/Ca mice were used. The untreated control and the DMBA-treated control groups received no pre-feeding, while prior to the DMBA treatment:

1. one group received 4 mg/day/animal green tea (*C. sinensis*) extract (catechin content 80%) (Xi'an Longze Biotechnology Co. Ltd.)
2. one group received 2.5 mg/day/animal chinese bayberry (*M. rubra*) extract (myricetin [3,5,7,3 ', 4', 5'-hexahydroxy-flavon] content 80 %) Xi'an Longze Biotechnology Co. Ltd.)
3. one group received 30 mg/day/animal flavonoid extract (common wine grape (*Vitis vinifera 'Cabernet Sauvignon'*) seed and peel, blackberry 'thornfree (*Rubus fruticosus „Thornfree"*) seed and peel, blackcurrant (*Ribes nigrum*) seed and peel with added resveratrol of 4 grams in 100 ml FruitCafe^{TM} (SC Vita Crystal Research SRL),
4. one group received 30 mg/day/animal (150 ml) coffee (*C. arabica*) extract,
5. one group received 300 mg/day/animal olive oil (Agraria Riva Del Garda SCA), and
6. one group received 300mg/day/animal TFA (trans-3-hexadecenoic acid) (Sigma Aldrich)

for two weeks in addition to their usual diet.

With the exception of the untreated control (negative control) group, the other seven groups received 20 mg/kg bw DMBA intraperitoneally (Sigma-Aldrich), dissolved in 0.1 ml corn oil. (The untreated control group also received 0.1 ml of corn oil intraperitoneally). After 24 h of DMBA exposure the organs to be examined (liver, kidneys and spleen) were removed after cervical dislocation.

3.1. Isolation of DNA

DNA isolation was performed with High Pure PCR Template Preparation Kit (Roche, Madison, WI, USA), according to the manufacturer's instructions.

3.2. Analysis of LINE-1 DNA methylation

EpiTect Bisulfite kit (Qiagen, Hilden, Germany) was used for bisulfate-conversion, according to the manufacturer's instructions. This process resulted in the conversion of unmethylated cytosines into uracil. Subsequently, a high-resolution melting (HRM) analysis was performed, which, based on the melting point difference, was able to distinguish between uracil and methylated cytosine bases. If the DNA contains highly methylated regions, the bisulfite conversion and subsequent amplification results in a higher melting point.

For the HRM analysis, primers targeting CpG rich region of LINE-1 were used (Newman, 2012), the sequences were as follows: forward: 5'-GGT TGA GGT AGT ATT TTG TGT G-3', reverse: 5'- TCC AAA AAC TAT CAA ATT CTC TAA C-3'. The amplification was performed in 96 well plates, in a Roche LightCycler480 qPCR instrument (Roche, Madison, WI, USA). The reaction mix contained 20 ng bisulfite treated DNA, 0.75–0.75 μM forward and reverse primers, 1x LightCycler 480 High Resolution Melting Master (Roche, Madison, WI, USA) in 20 μl final volume (Bray, 2018). The PCR parameters were as follows: heating to 95°C for 5 min was followed by 35 cycles: 1. 95°C for 20 sec, 2. 60°C for 30 sec, 3. 72°C for 20 sec. Subsequently the melting point/melting curve analysis was performed between 73°C and 84°C with temperature steps of 0.1°C/2 sec.

For the purpose of positive and negative controls Mouse high methylated genomic DNA (Epigen Dx, Hopkinton, MA, USA) and Mouse low methylated genomic DNA (Epigen Dx, Hopkinton, MA, USA), and their mixtures in different proportions were used, in order to allow the quantification of the methylation levels of our samples.

3.3. Calculation and statistical analysis

The relative LINE-1 methylation levels of LINE-1 expression levels were calculated and compared using the $2^{-\Delta\Delta CT}$ method. To examine the distribution of results we used the Kolmogorov-Smirnov test, and then to compare the averages following the Levene's *F*-test we used a *T*-test. IBM SPSS 21 statistical software was used for calculations and analysis, and we determined the level of statistical significance at a p value of <0.05.

Average DNA methylation levels were expressed as percentages of untreated animals (negative controls).

4. Results

I examined the combined effect of DMBA and the administered substances on LINE-1 methylation compared to the control treated with DMBA alone.

4.1. Changes in mehylation patterns in the spleen

In the case of spleen, flavonoid extract, green tea extract and chinese bayberry extract, co-administered with DMBA, prevented DMBA-induced LINE-1 hypomethylation, but induced just the opposite – hypermethylation – change. A slight hypomethylation was observed for coffee extract (Figure 1).

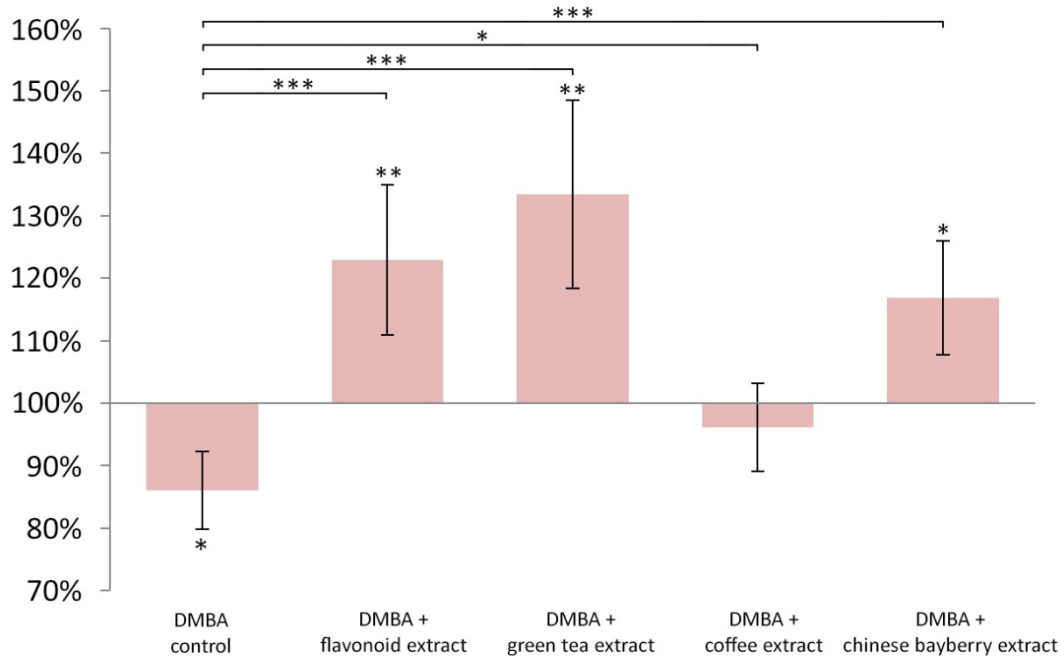

Figure 1: LINE-1 methylation pattern in the spleen of CBA/Ca female mice (n=6) exposed to the effects of DMBA, and to the effects of flavonoid extract, green tea extract, coffee extract or chinese bayberry extract co-administered with DMBA, considering untreated control to be 100 % (* p<0.05; ** p<0.01; *** p<0.001 – the indications above and below the columns show the significance level of the comparison with the untreated control, the indications at the horizontal lines show the significance level of the comparison with the DMBA-treated group).

EVOO co-administered with DMBA significantly reduced, while TFA significantly enhanced DMBA-induced LINE-1 hypomethylation in the spleen (Figure 2).

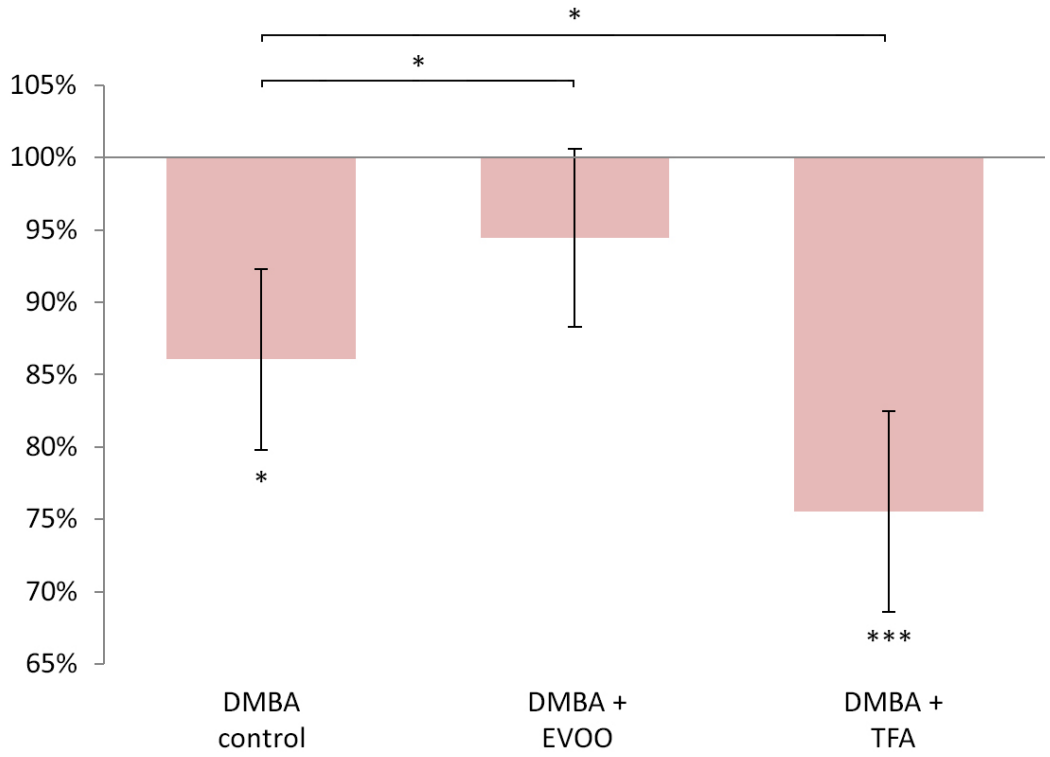

Figure 2: LINE-1 methylation pattern in the spleen of CBA/Ca female mice (n=6) exposed to the effects of DMBA and to the effects of EVOO or of TFA co-administered with DMBA, considering untreated control to be 100 % (* $p<0.05$; *** $p<0.001$ – the indications below the columns show the significance level of the comparison with the untreated control, the indications at the horizontal lines show the significance level of the comparison with the DMBA-treated group).

4.2. Changes in methylation patterns in the liver

Flavonoid extract and green tea extract co-administered with DMBA successfully prevented DMBA-induced LINE-1 hypomethylation and even resulted in hypermethylation in the liver. Chinese bayberry extract and coffee extract also showed statistically significant protective effects, with coffee showing a slight hypomethylation (Figure 3).

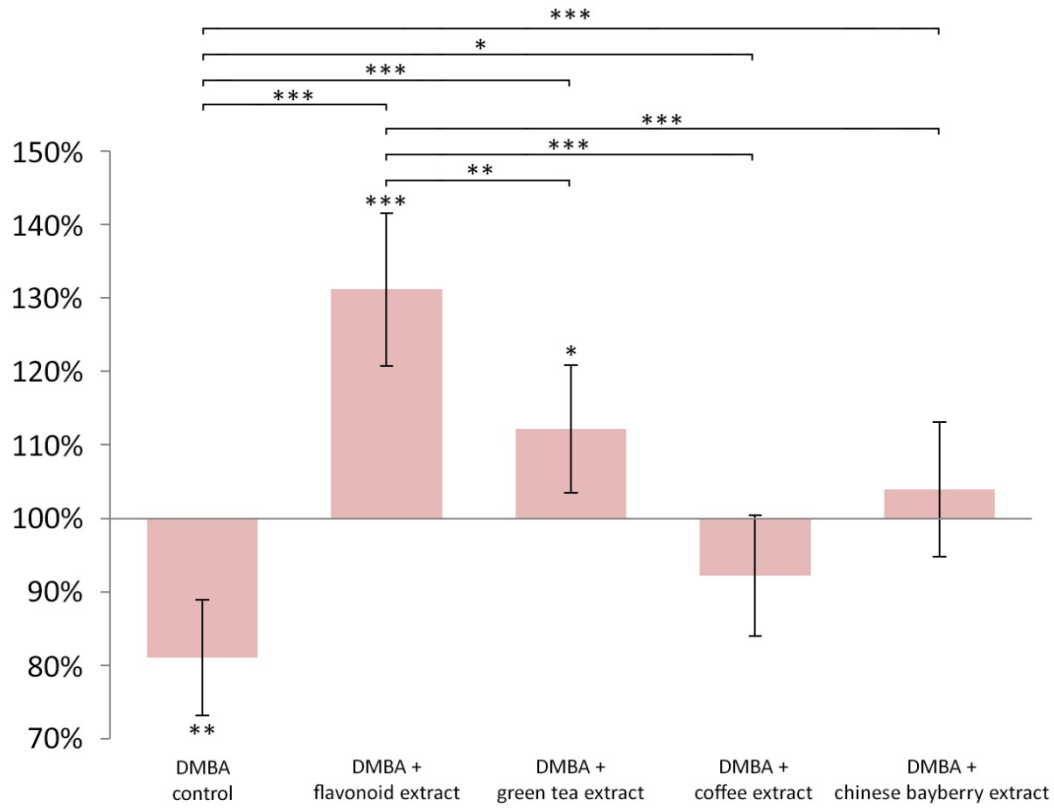

Figure 3: LINE-1 methylation pattern in the livers of CBA/Ca female mice (n=6) exposed to the effects of DMBA and to the effects of flavonoid extract, green tea extract, coffee extract or chinese bayberry extract co-administered with DMBA, considering untreated control to be 100 % (* p<0.05; ** p<0.01; *** p<0.001 – the indications above and below the columns show the significance level of the comparison with the untreated control, the indications at the horizontal lines show the significance level of the comparison with the DMBA-treated group).

As in spleen, EVOO co-administered with DMBA prevented LINE-1 hypomethylation in liver, while TFA further enhanced it (Figure 4).

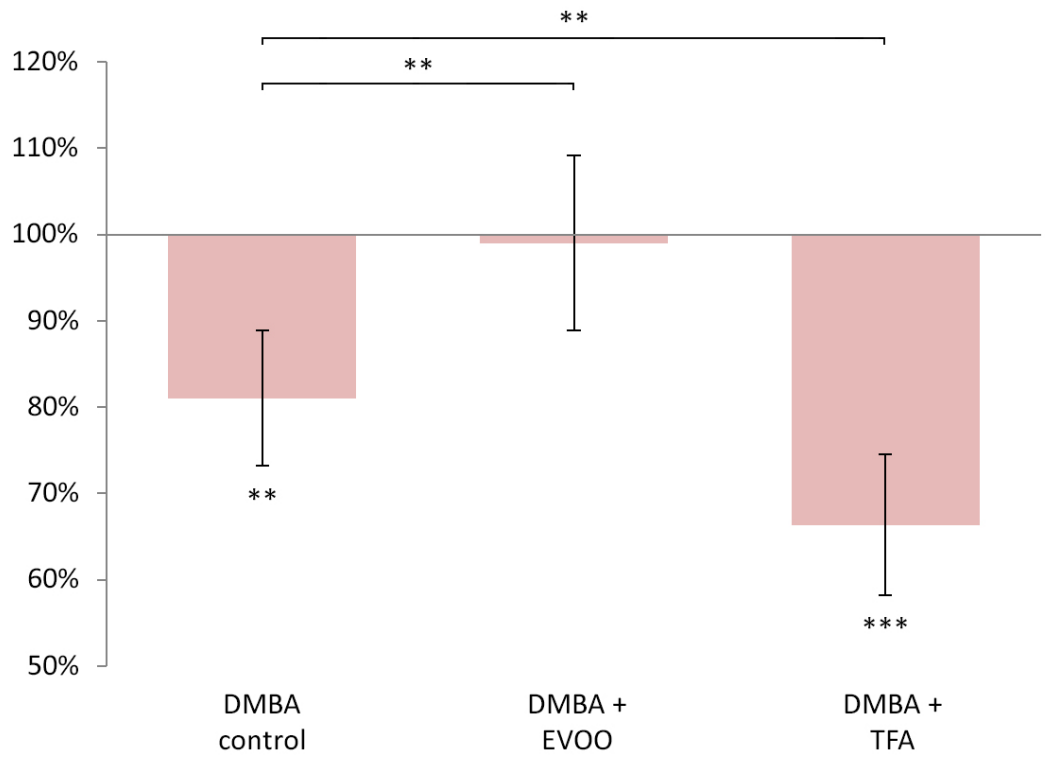

Figure 4: LINE-1 methylation pattern in the livers of CBA/Ca female mice (n=6) exposed to the effects of DMBA and to the effects of EVOO or TFA co-administered with DMBA, considering untreated control to be 100 % (p<0.01; *** p<0.001 – the indications below the columns show the significance level of the comparison with the untreated control, the indications at the horizontal lines show the significance level of the comparison with the DMBA-treated group).**

4.3. Changes in methylation patterns in the kidneys

Flavonoids, coffee extract and green tea extract prevented DMBA-induced LINE-1 hypomethylation, while chinese bayberry extract caused its small, statistically non-significant reduction in the kidneys. Flavonoid extract and coffee extract also caused hypermethylation even when compared to the untreated control, which was statistically significant for coffee (Figure 5).

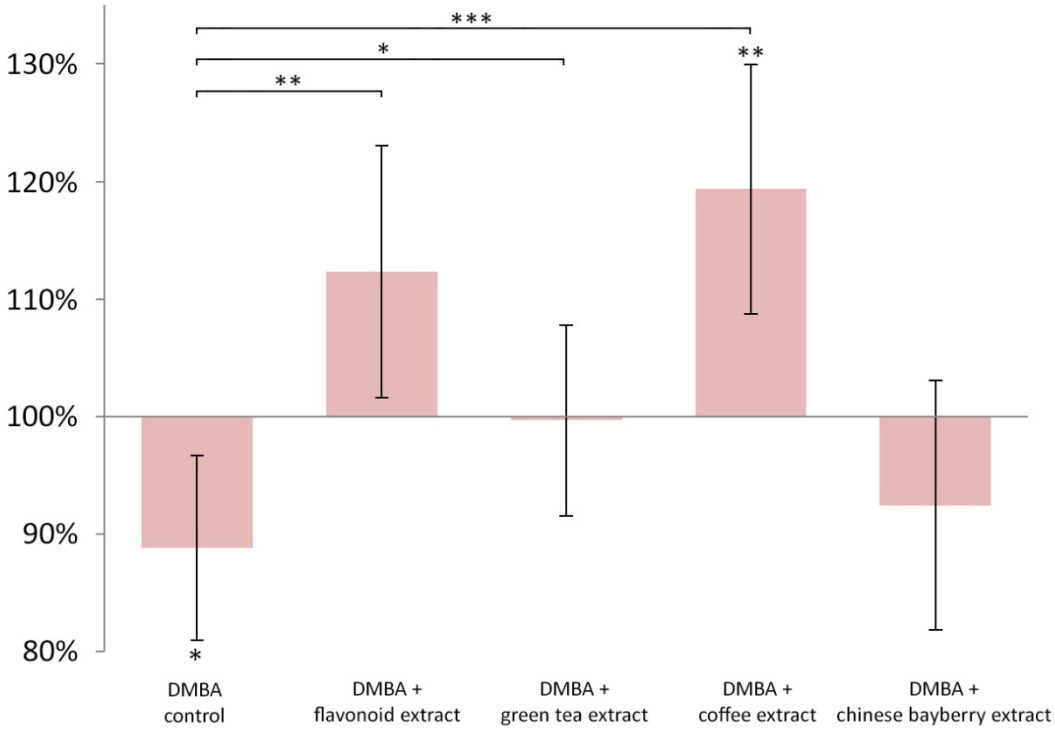

Figure 5: LINE-1 methylation pattern in the kidneys of CBA/Ca female mice (n=6) exposed to the effects of DMBA and to the effects of flavonoid extract, green tea extract, coffee extract or chinese bayberry extract co-administered with DMBA, considering untreated control to be 100 % (* p<0.05; ** p<0.01; *** p<0.001 – the indications above and below the columns show the significance level of the comparison with the untreated control, the indications at the horizontal lines show the significance level of the comparison with the DMBA-treated group).

EVOO co-administered with DMBA reduced and TFA enhanced LINE-1 hypomethylation in the kidneys. Figure 6 shows the ratios and that none of the components caused statistically significant changes compared to the DMBA-treated group.

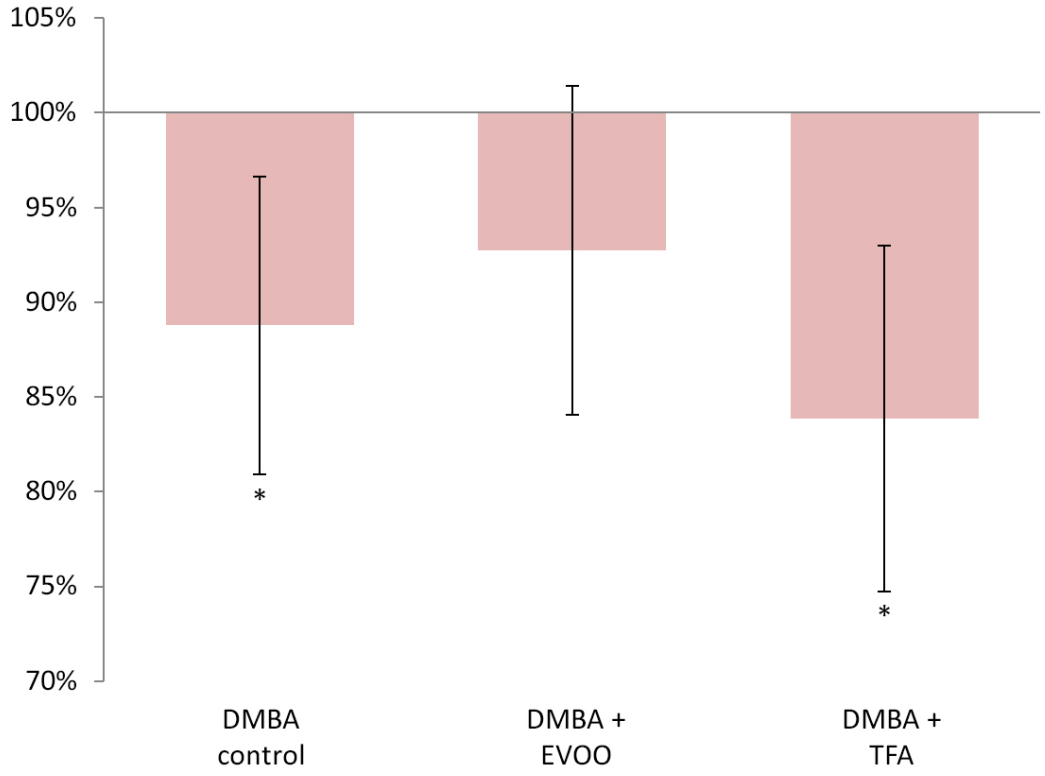

Figure 6: LINE-1 methylation pattern in the kidneys of CBA/Ca female mice (n=6) exposed to the effects of DMBA and to the effects of EVOO or TFA co-administered with DMBA, considering untreated control to be 100 % (* $p<0.05$ – the indications below the columns show the significance level of the comparison with the untreated control).

4.4. Summary of results

In summary, the flavonoid extract and green tea extract showed the strongest effects to prevent LINE-1 hypomethylation in the spleen and liver. In the kidneys, coffee extract showed the highest hypermethylation. EVOO either reduced or prevented DMBA-induced hypomethylation, but did not induce hypermethylation. As could be expected, TFA increased LINE-1 hypomethylation in all three organs, enhancing the effect of DMBA.

5. Discussion

5.1. Effect of DMBA on LINE-1 methylation pattern

DMBA may have either direct effects, such as oxidative stress on cellular components (DNA, proteins, membranes, etc.), or direct mutagenic and epigenetic (e.g. gene and microRNA expression, global DNA hypomethylation) effects on DNA. On the other hand, DMBA also exerts indirect effects by altering the regulation of cell division in a direction that promotes oncogenesis, or by triggering and stimulating pro-inflammatory secondary signalling processes, or by depleting antioxidants (such as glutathione [GSH]), etc. Oxidative damage may also have a positive feedback by activating secondary signalling pathways. For example, in DMBA-treated mice, reactive oxygen species (ROS) are released, which increase the levels of interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor (TNF) and these with the nuclear factor kappaB (NF-κB) mutually activate each other – and even generate additional ROS. The significance of this is, that ROS, in addition to triggering local inflammatory signalling, indirectly increase the likelihood of malignant transformation, and ultimately lead to global DNA hypomethylation – the biomarker of which is the LINE-1 methylation pattern. LINE-1 hypomethylation of the organs examined is presumably the result of the aggregation of these effects.

Thus, beside NF-κB, DMBA also activates the mitogen-activated protein kinase (MAPK) and Janus kinase (JAK) signalling pathways, which stimulate the proinflammatory, and indirectly the mentioned oncogenesis-promoting interleukins, and also the NF-κB in a positive feedback loop – which thus also acquires a direct proinflammatory effect –, and may also be associated with malignant transformation processes.

DMBA-induced damage triggers the early steps of carcinogenesis through the oncogenic activation of the mutation of the RAS proto-oncogene family and through the enhancement of oncogenic expression of *C-MYC*. The activated K-RAS protein is able to hypermethylate transcription factors of tumour suppressor genes (e.g. *INK4-ARF*) in several colorectal carcinoma cell lines and thereby epigenetically silence the expression of these tumour suppressor genes. Activated K-RAS also inhibits the degradation of the

transcription factor ZNF304, which is a transcriptional regulator of the tumour suppressor β1 integrin. So these secondary signalling mechanisms mainly promote carcinogenesis, as well.

The significant hypomethylating effect of DMBA on L1-RTP DNA might have occured through the inhibition of the DNMT enzyme. In addition, DMBA, also by affecting the methylation pattern of these CpG islands of DNMT enzymes, causes hypomethylation of oncogenes (e.g. *HA-RAS*) and hipermethylation of tumour suppressor genes (e.g. *P53*), which consequently lead to enhanced proliferation, thus increasing the likelihood of carcinogenesis.

5.2. Harmful effects of TFA on the LINE-1 methylation pattern

TFA is incorporated into the phospholipid layer of the cell membrane and indirectly contributes to oxidative damages and also exerts pro-inflammatory, tumorigenic effects through secondary signal transduction pathways. In addition, TFA can inhibit antioxidant enzymes such as superoxide dismutase (SOD) and deplete antioxidant molecules (e.g. GSH) – which contribute to hepatotoxic processes – and this indirectly promotes the mentioned inflammation and tumour formation, as well.

Feeding with TFA reduced the levels of n-3 polyunsaturated fatty acids (PUFA) of adipose tissue in cell membranes and thus increased its rigidity in animal models. A contributory factor is that TFA – containing at least one trans-configurated conjugated bond and therefore (at least partially) linear in shape – also stiffens the membrane structure. F2-isoprostanes formed in association with TFA also contribute to this. The decrease in membrane fluidity shows a positive association with the increase in ROS activity within the phospholipid bilayer of the membrane, as well as with the probability of inflammation and malignant transformation. Thus, TFA directly contributes to oxidative damages and even to inflammation and tumour formation via secondary signal transduction mechanisms, as well. Furthermore, in association with TFA damage an increase in cholesterol synthesis was observed in animal models in both hepatocytes and adipocytes, which indirectly activates the RAS gene family. The literature data are consistent with our findings which show that kidneys tend to incorporate smaller amounts of TFA compared to the liver.

TFA consumption also enhanced the activity of intercellular adhesion molecule-1 (ICAM-1) and of vascular cell adhesion molecule-1 (VCAM-1), as well as of matrix metalloproteinases (MMPs), which, in addition to generation of oxidative stress, indirectly inhibit the expression of the antitumor miR-134 and *P53* genes, as well.

5.3. Effecs of polyphenols on DMBA-induced LINE-1 hypomethylation

Resveratrol and myricetin can reduce these mentioned harmful effects by inhibiting CYP1A1 enzymes which activate the procarcinogenic form of DMBA to a carcinogenic form. Polyphenol extracts are rich in 4-hydroxybenzoic acid, 4-hydroxycinnamic acid, flavanol, flavonol, anthocyanidin and stilbene polyphenols, which are antioxidants, anti-inflammatory (e.g. they inhibit TNF-α, IL-1β, IL-6), anticarcinogenic, for example they regulate cell proliferation – in a synergistic manner.

Resveratrol inhibits both oxidative and inflammatory damage-induced decreases in *DNMT1, DNMT3a, DNMT3b* and *SIRT2* expression and thus prevented LINE-1 hypomethylation induced by these damages. Chlorogenic acid and hydroxycinnamic acid (4-hydroxycinnamic acid) content of coffee, catechins in green tea, flavonoids and resveratrol have free radical scavenging effects that reduce damage caused by ROS. Moreover, these substances also induce the antioxidant enzymes SOD and glutathione S-transferase (GST).

The studied polyphenols also influence the methylation pattern through secondary signalling pathways, for example resveratrol increases the expression of phosphatase and tensin homolog gene (*PTEN*) leading to the inhibition of DNMT1, which is anticancerous since it hypermethylates the promoter region of the DNMT1 tumour suppressor genes. The epigallocatechin gallate (EGCG) polyphenol of green tea is also an inhibitor of DNMT1 enzyme and increases the expression of tumor suppressor gene *P21* by hypomethylation of the P21 promoter region *in vivo*, independently of the P53 tumor suppressor-induced signalling pathway.

5.4. Effects of EVOO

The oleocanthal phenolic compound and the oleuropein polyphenol are water-soluble substances of olive oil, and are antioxidants and mainly protect cell membranes, e.g.

inhibit the activation of NF-κB, reduce the expression of the *mTOR* gene and inhibit the synthesis of IL-6, IL-1β and TNF-α.

In addition, oleocanthal increases intracellular levels of GSH, which otherwise are reduced by free radicals.

The monounsaturated fatty acid (MUFA), omega-9 oleic acid content of EVOO and the polyunsaturated fatty acid (PUFA) content of linoleic acid increase cell membrane fluidity and its LINE-1 methylation enhancing effect exerted via the above mentioned secondary signalling pathways is obvious. Interestingly, despite the fact that palmitic acid content of saturated EVOO increases cell membrane rigidity via induction of the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator gene in vitro, it induced global hypermethylation. Furthermore, both omega-9 oleic acid and palmitic acid exerts chemopreventive effects through the PPARα pathway as well, which ultimately increases the LINE-1 methylation. In septic mice, omega-9 oleic acid decreased the expression of the inflammatory interleukins TNF-α and IL-1β and increased the level of the anti-inflammatory IL-10.

PUFA, through its direct β-catenin inhibitory effect, significantly reduces the expression of *C-MYC* gene. This is of great significance for the DNA methylation pattern, as C-MYC also affects the expression of the *ten-eleven translocation methylcytosine dioxygenase* (TET) genes, and TET1 enhances tumor proliferation, and has DNA demethylating effect, as well.

5.5. Effect of other substances on the LINE-1 methylation pattern

The decrease in GSH (and other carcinogenic effects) caused by trace amounts of acrylamide and furan present in coffee were found to be negligible with respect to the results of the LINE-1 methylation pattern, as they were probably masked by strong beneficial effects.

Caffeine (1,3,7-trimethylpurine-2,6-dione) is present in both coffee and green tea extracts and activated the *PTEN* gene both in human osteosarcoma MG63 cells and in fibrosarcoma HT1080 cells by increasing intracellular cAMP levels. This led to the inhibition of the PI3-K/AKT/mTOR signalling pathway, which has a proapoptotic

effect. In addition, caffeine induces P53-dependent apoptosis in JB6 cells by activating BAX and caspase-3 signalling pathways, as well.

In contrast, caffeine induces LINE-1 hypomethylation (via inhibition of DNMT3) in the cellular tumor model HL-60. This may certainly be through a different mechanism than the previously mentioned anticarcinogenic effect of resveratrol and of EGCG inhibiting DNMT1.

Thus, the chemopreventive effect of caffeine is questionable just because of its hypomethylation-inducing effect on LINE-1, while all other molecular biological signalling pathways support an anticarcinogenic effect. In addition, an epidemiological study has shown that there is no association between the dose of caffeine intake and the risk of the cancers studied (prostate, lung, colorectal and ovarian cancer).

6. Summary of results, conclusion

The flavonoid extract prevented DMBA-induced LINE-1 hypomethylation in the liver, spleen and kidneys of female CBA/Ca mice and caused significant hypermethylation in the liver and spleen compared to untreated controls.

The green tea extract prevented DMBA-induced LINE-1 hypomethylation in the liver, spleen and kidneys of female CBA/Ca mice and caused significant hypermethylation in the liver and spleen compared to untreated controls.

Chinese bayberry extract prevented DMBA-induced LINE-1 hypomethylation in the liver and spleen of female CBA/Ca mice and caused significant hypermethylation in the spleen compared to untreated controls.

Coffee extract prevented DMBA-induced LINE-1 hypomethylation in the liver, spleen and kidneys of female CBA/Ca mice and caused significant hypermethylation in the kidneys compared to untreated controls.

Extra virgin olive oil prevented DMBA-induced LINE-1 hypomethylation in the liver and spleen of female CBA/Ca mice.

TFA further enhanced DMBA-induced LINE-1 hypomethylation in the liver and spleen of female CBA/Ca mice.

The DNA methylation changes we studied may contribute to the anticarcinogenic and, in the case of TFA, to the carcinogenic effects of the chemopreventive agents, and are significant representatives of these processes as biomarkers. Furthermore, the LINE-1 DNA hypomethylation pattern testing system can be considered as a suitable molecular epidemiological biomarker for the study of the epigenetic impact of other, potentially chemopreventive agents.

LIST OF PUBLICATIONS

List of publications used as a source for the dissertation

Szabo L, Molnar R, Tomesz A, Deutsch A, Darago R, Varjas T, Ritter Z, Szentpeteri JL, Andreidesz K, Mathe D, Hegedüs I, Sik A, Budan F, Kiss I. Olive Oil Improves While Trans Fatty Acids Further Aggravate the Hypomethylation of LINE-1 Retrotransposon DNA in an Environmental Carcinogen Model. Nutrients. 2022 Feb 21;14(4):908.

IF: 6.706

Szabo L, Molnar R, Tomesz A, Deutsch A, Darago R, Nowrasteh G, Varjas T, Nemeth B, Budan F, Kiss I. The effects of flavonoids, green tea polyphenols and coffeeon DMBA induced LINE-1 DNA hypomethylation. PLoSOne. 2021 Apr 20;16(4):e0250157.

IF: 3.752

Conference participations

Andreidesz K, Szabo L, Molnar R, Tomesz A, Darago R, Deutsch A, Varjas T, Ritter Zs, Szentpeteri LJ, Mathe D et al. A transz-zsírsavak súlyosbítják a LINE-1 retrotanszpozon DNS hipometilációt DMBA környezeti karcinogén modellben. 51st MEMBRANE TRANSPORT CONFERENCE POSTERS ABSTRACTS 2022.

Budan F, Szabo L, Molnar R, Tomesz A, Varjas T, Ritter Zs, Szentpeteri LJ, Mathe D, Hegedus I, Sik A et al. Az extraszűz olíva olaj csökkenti a karcinogén DMBA l1-RTP DNS hipometiláló hatását. 51st MEMBRANE TRANSPORT CONFERENCE POSTERS ABSTRACTS 2022.

Other publications

Tomesz A, Szabo L, Molnar R, Deutsch A, Darago R, Raposa BL, Ghodratollah N, Varjas T, Nemeth B, Orsos Z, Pozsgai E, Szentpeteri JL, Budan F, Kiss I. Changes in miR-124-1, miR-212, miR-132, miR-134, and miR-155 Expression Patterns after 7,12-Dimethylbenz(a)anthracene Treatment in CBA/CaMice. Cells. 2022 Mar 17;11(6):1020. IF: 7.666

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