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Exposure to nanographene oxide induces gene expression dysregulation in normal human astrocytes

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Objective. Nanographene oxide, an oxidation derivative of graphene, is considered to be one of the nanomaterials attractive for biomedical applications, although this nanomaterial is toxic. The increasing exploitation of graphene-based materials necessitates a comprehensive evaluation of the potential impact of these materials on the human health. Moreover, it is necessary to investigate in detail the mechanisms of its toxic effect on living cells particularly at the genome level. The present study aimed to evaluate the impact of low doses of nanographene oxide on the expression of key regulatory genes in normal human astrocytes.

Methods. Normal human astrocytes, line NHA/TS, were exposed to low doses of nanographene oxide (1 and 4 ng/ml) for 24 h. RNA was extracted from the cells and used for cDNA synthesis. The expression levels of NAMPT, TSPAN13, BCAR3, BRCA1, PTGS2, P4HA1, and P4HA2 mRNAs as well as microRNAs were measured by quantitative polymerase chain reaction.

Results. It was found that the low doses of nanographene oxide induced a dysregulation in the expression of the key regulatory genes in normal human astrocytes in dose-dependent (1 and 4 ng/ml) and gene-specific manner. Nanographene oxide also strongly suppressed the expression of *NAMPT*, *BCAR3*, and *TSPAN13* genes and significantly up-regulated *BRCA1*, *PTGS2*, *P4HA1*, and *P4HA2* ones with a more significant effect in P4HA1 and P4HA2 genes. The expression of miR-96-5p and miR-145-5p was also down-regulated in astrocytes treated with nanographene oxide in a dose-dependent manner.

Conclusion. The data obtained demonstrate that the low doses of nanographene oxide disturbed the genome functions by changing the expression levels of key regulatory genes in gene-specific and dose-dependent manner. Moreover, a higher dose of nanographene oxide induced more pronounced changes in expression of genes indicating for both genotoxic and neurotoxic possible effects in the normal human astrocytes.

Key words: nanographene oxide, NAMPT, BCAR3, BRCA1, P4HA2, mRNA expression, normal human astrocytes, genotoxicity

Nanographene and its oxide are nanomaterials, which have exceptional physiochemical properties currently considered to be very promising of their great application potential in different industries. Nanographene oxide, an oxidation derivative of graphene, is considered to be one of the nanomaterials attractive for biomedical applications in the field of drug delivery, cancer therapy, and diagnostics (Avitabile et al. 2018; Backes et al. 2021; Cellot et al. 2021; Cui et al. 2021; Dash et al. 2021). The increasing

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exploitation of nanographene-based materials, i.e., unique carbon nanoparticles, requires a comprehensive evaluation of their potential impact on the environment and the human health (Ema et al. 2017; Fadeel et al. 2018; Jia et al. 2019; Malhotra et al. 2020; Li et al. 2021). The hazardous impact of various carbon nanotubes (carbon nanomaterials with breakthrough properties significantly enhancing the physical properties of other materials) on the environment and the human health has also been recognized (Francis and Devasena 2017; Kavosi et al. 2018; Minchenko et al. 2018; Yan et al. 2019; Jiang et al. 2020; Rudnytska et al. 2021). Actually, the toxic potential of carbon nanotubes has been reported in numerous cell lines and animal models and their long-term toxicity has ever led to an increasing concern (Ahmadi et al. 2017; Eldawud et al. 2018; Yang and Zhang 2019).

It has also been shown that nanographene oxide and carbon nanotubes are able to penetrate over the air-blood and blood-brain barriers as well as the cell membranes and accumulate in the nucleus, which permits their use in the health sciences as a carrier of chemical compounds in cancer therapy and imaging probes (Mendonca et al. 2015; Aragon et al. 2017; Ema et al. 2017; Su et al. 2020). However, nanographene oxide is not biodegradable, which consequently restricts its use in medicine and biology since the raising concerns of its chronic genome toxicity (Ahmadi et al. 2017; Yang and Zhang 2019; Jiang et al. 2020). Previously, it was shown that carbon nanoparticles may interfere with an immune response, because of their strong suppressing effect on the expression of cell surface glycoproteins, such as HLA-DRA (major histocompatibility complex, class II, DR alpha) and HLA-DRB1. It also dysregulates the level of microRNAs, such as miR-190b and miR-7, which possibly contribute to the down-regulation of both HLA-DRA and HLA-DRB1 mRNA expressions (Minchenko et al. 2018). Furthermore, single walled carbon nanotubes deregulate the expression of LMNB1 (lamin B1), which participate in the control of genome stability. However, genotoxic effect of these nanoparticles has also been indicated in many other genes (Minchenko et al. 2018).

Recently, we have also shown that carbon nanotubes may affect the expression of genes associated with endoplasmic reticulum stress and cell proliferation, cell survival, and cancerogenesis in normal human astrocytes. These changes were dissimilar in magnitude and direction in the dysregulation of the genome stability (Rudnytska et al. 2021). At the same time, detailed mechanisms of the toxicity of this type of carbon nanoparticles have not been still fully identified. One of their possibly toxicity mechanisms may include signaling pathways of the endoplasmic reticulum stress as it has been shown for different nanoparticles (Boran and Ulutas 2016; Cao et al. 2017; Scherzad et al. 2017; Simon et al. 2017; Chen et al. 2020). There are data demonstrating that modulation of the endoplasmic reticulum stress by chemicals alters the toxicity of some nanoparticles (Simon et al. 2017; Cao et al. 2020). Chemical compound 4-phenylbutyric acid repressed most effects of nanoparticles, particularly the titanium dioxide nanoparticles, through the relief of some aspects of the endoplasmic reticulum stress signaling (Simon et al. 2017; Hu et al. 2018). However, the literature data dealing with the behavioral, reproductive, and developmental toxicity potential as well as genotoxicity of nanographene oxide particles are very limited (Ema et al. 2017; Fadeel et al. 2018; Jia et al. 2019; Malhotra et al. 2020; Li et al. 2021). However, it is very important to note that understanding of the molecular mechanism, by which the carbon nanoparticles may induce toxicity, is significant for both nanotoxicology and nanomedicine spheres.

For the present study, several genes encoding key regulatory proteins such as nicotinamide phosphoribosyltransferase (NAMPT) also known as pre-B-cell colony-enhancing factor (PBEF), tetraspanin 13 (TSPAN13), BCAR3 adaptor protein, NSP family member (BCAR3), breast cancer 1, early onset (BRCA1), prostaglandin-endoperoxide synthase 2 (PTGS2) also known as cyclooxygenase 2 (COX2), prolyl 4-hydroxylase subunit alpha 1 (P4HA1), and prolyl 4-hydroxylase alpha polypeptide II (P4HA2) were selected. The NAMPT is a rate-limiting component in the mammalian NAD biosynthesis pathway involved in many important biological processes including metabolism, stress response, and circadian clock function. It can also interact with TLR4 and many other proteins (Xu et al. 2017; Minchenko et al. 2020; Gasparrini et al. 2022). Furthermore, the NAMPT is a proinflammatory marker of adipose tissue associated with systemic insulin resistance (Chang et al. 2010). The TSPAN13 protein mediates the signal transduction events playing a role in the regulation of cell development, growth, and motility and has a relation to cancerogenesis (Jiang et al. 2019; Li et al. 2019; Qi et al. 2020). The breast cancer anti-estrogen resistance 3 protein (BCAR3) is an adapter protein downstream of several growth factor receptors. It promotes cell proliferation and migration and is also involved in the insulin signaling pathway (Oh et al. 2013; Pan et al. 2019; Zhang et al. 2021). There are data indicating

that BCAR3, as a SH2-containing signal transducer, is implicated in tumorigenesis and is related to the glioma pathological grade (Pan et al. 2019; Zhang et al. 2021). Protein BRCA1 is a nuclear phosphoprotein that plays an important role in maintaining the genomic integrity and stability and it has the E3 ubiquitin-protein ligase activity, which is required for its tumor suppressor function (Jhanwar-Uniyal 2003; Takaoka and Miki 2018; Tahir et al. 2020). The PTGS2 enzyme is associated with resistance to apoptosis and tumor growth, migration, and invasiveness (Lin and Wu 2018; Zhu et al. 2020; Ravinder et al. 2022). The prolyl 4-hydroxylase is a key enzyme in collagen synthesis and deposition. In collagen and related proteins, the prolyl 4-hydroxylase catalyzes the formation of 4-hydroxyproline that is essential to the regulation of metabolism through post-translational modification of the human proteome and is also required for the tumor growth and metastasis (Chakravarthi et al. 2014; Wang et al. 2019; Cao et al. 2020; Zhou et al. 2020).

The microRNAs (miRNAs) are a class of endogenous small single-stranded non-protein coding RNA molecules, which play a critical role in the control of gene expression at the posttranscriptional level mainly through the interaction with the 3'-untranslated regions of target messenger RNAs and subsequent induction of their degradation or translational repression. It is important to note that microRNAs play an important role in the regulation of all metabolic processes and neurodevelopment (Li et al. 2019; Ma et al. 2019; Pan et al. 2019; Yapijakis 2020).

This study was aimed to reveal the impact of low doses of nanographene oxide on the expression of key regulatory genes such as *NAMPT*, *TSPAN13*, *BCAR3*, *BRCA1*, *PTGS2*, *P4HA1*, and *P4HA2* as well as microRNAs in normal human astrocytes line NHA/TS.

Materials and methods

Cell culture and treatment. NHA cells (normal human astrocytes; Cambrex Bio Science, Walkersville, MD, USA) were immortalized by Sasai et al. (2007). The immortalized normal human astrocytes (NHA/TS cell line) were received from Dr. K. Sasai and Dr. S. Tanaka (Japan) and left grown at 37°C under a humidified atmosphere of 95% air and 5% CO_2 (Sasai et al. 2007). These astrocytes were treated with nanographene oxide (1 and 4 ng/ml) for 24 h. Nanographene oxide (2 mg/ml, dispersion in water) was received from Sigma-Aldrich Chemie GmbH, Germany.

RNA extraction and cDNA synthesis. Total RNA was extracted from NHA/TS cells using TRIZOL reagent as described previously (Auf et al. 2013; Rudnytska et al. 2021). The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification the RNA samples were re-precipitated with 95% ethanol containing sodium acetate and pellets were dissolved again in nuclease-free water. RNA quantity as well as spectral characteristics were measured using NanoDrop One (Thermo Scientific). Thermo Scientific Versco cDNA Synthesis Kit was used for the mRNA reverse transcription. For polyadenylation and microRNA reverse transcription, a Mir-X miRNA a First-Strand Synthesis Kit (Takara, Japan) was used.

Real-time quantitative polymerase chain reaction. The expression levels of mRNAs and microRNAs were measured by real-time qPCR using "QuantStudio 5 Real-Time PCR System" (Applied Biosystems) using Absolute qPCR SYBRGreen mix for mRNAs and Mir-X miRNA qRT-PCR SYBR Kit (Takara, Japan) for microRNAs. For analysis of mRNA expressions, four different RNA samples for each experimental group as well as pairs of the specific primers (Table 1) received from Sigma-Aldrich (St. Louis, MO, USA) were used. For analysis of microRNA expressions, a universal qPCR reverse primer and specific for each miRNA forward primers were used (Table 1). Polymerase chain reaction was performed in triplicates. The quality of amplification products was analyzed by melting curves and electrophoresis in 2% agarose gel and visualized by SYBR* Safe DNA Gel Stain (Life Technologies, Carlbad, CA, USA). Quantitative PCR analysis was performed using a special computer program "Differential Expression Calculator". The values of mRNA and microRNA expression were normalized to the level of ACTB and U6 RNA, respectively, and presented as a percentage of control (100 %).

Statistical analysis. All values are expressed as mean ± SEM obtained from triplicate measurements performed in 4 independent experiments. Statistical analysis was performed according to Student's t-test using Excel program as described previously (Rudnytska et al. 2021). A value of $p<0.05$ was considered statistically significant.

Results

We investigated the impact of nanographene oxide on the genome function by measuring the expression level of mRNAs, which encode regulatory proteins and enzymes related to the control of

Table 1.
Primers characteristics used in the quantitative real-time polymerase chain reaction.

Gene symbol	Gene name	Primer's sequence	Nucleotide number in sequence	GeneBank accession number
NAMPT (PBEF)	Nicotinamide hosphoribosyltransferase (pre-B-cell colony-enhancing factor)	F: 5'- gttacaagttgctgccacct R: 5'- agacgttaatcccaaggcca	1118–1137 1339–1320	NM_005746.3
TSPAN13	Tetraspanin 13	F: 5'- gcaaccacaggttccaagat R: 5'- ctcggagactggaaatcagc	196–215 346–327	NM_014399.3
BCAR3	BCAR3 adaptor protein, NSP family member	F: 5'- cctcccataaggtcctgtga R: 5'- gtccatgatgtgcctctcct	426–445 635–616	NM_003567.4
BRCA1	Breast cancer 1, early onset	F: 5'- tgaagaaagaggaacgggct R: 5'- tggctcccatgctgttctaa	4145–4164 4384–4365	NM_007294.4
PTGS2 (COX2)	Prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2)	F: 5'- tgaaacccactccaaacaca R: 5'- gagaaggcttcccagctttt	333–352 519–500	NM_000963.4
P4HA1	Prolyl 4-hydroxylase subunit alpha 1	F: 5'- ggcagccaaagctctgttac R: 5'- ggcttgttccatccacagtt	520–539 703–684	NM_000917.4
P4HA2	Prolyl 4-hydroxylase alpha polypeptide II	F: 5'- gggtttccaaaagctcctgg R: 5'- ttccccgtccctaaatgctt	1552–1571 1759–1740	NM_004199.3
ACTB	beta-actin	F: 5'- catccgcaaagacctgtacg R: 5'- cctgcttgctgatccacatc	948–967 1165–1146	NM_001101.5
miR-96	microRNA-96-5p	F: 5'- tttggcactagcacatttttgct	9–31	NR_029512.1
miR-145	microRNA-145-5p	F: 5'- gtccagttttcccaggaatccct	16–38	NR_029686.1

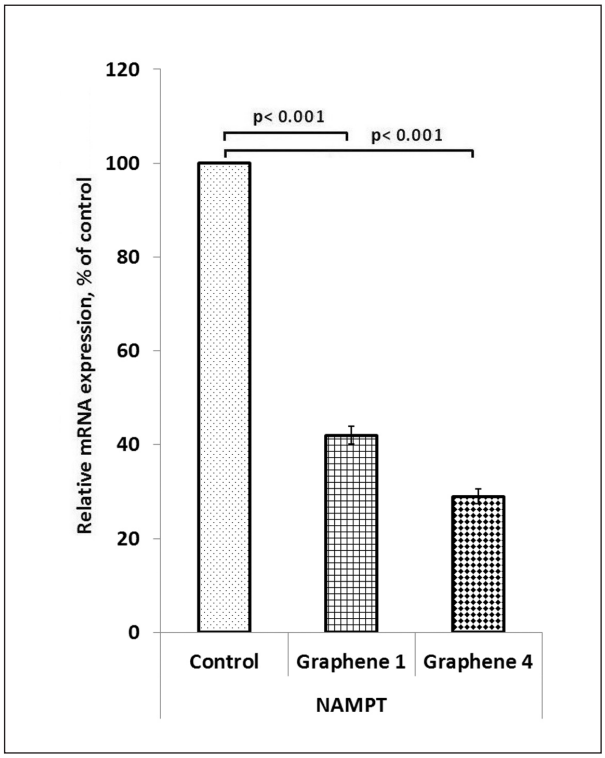

Figure 1. The impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the nicotinamide phosphoribosyltransferase-NAMPT mRNA (known also as a pre-B-cell colony-enhancing factor-PBEF) expression in normal human astrocytes line NHA/TS versus not treated (control) cells. Values of this mRNA expression were normalized to the ACTB mRNA level; n=4.

metabolism, cell proliferation and cancerogenesis. As shown in Figure 1, the 24-h exposure of normal human astrocytes line NHA/TS to 2 different doses of nanographene oxide (1 and 4 ng/ml) led to a significant down-regulation of the NAMPT mRNA expression levels by –58% and –71%, respectively. The suppressive effect of nanographene oxide was also shown on the TSPAN13 mRNA expression levels (Figure 2). The exposure of normal human astrocytes to a lover nanographene oxide dose (1 ng/ml) decreased the TSPAN13 mRNA expression by –37%, but their exposure to a higher dose (4 ng/ml) of these nanoparticles induced more significant changes at the level of the above-mentioned mRNA expression (–53%). The impact of nanographene oxide on the expression of breast cancer, anti-estrogen resistance 3 (BCAR3) mRNA was also dose-dependent, decrease by –49% for 1 ng/ml and –62% for 4 ng/ml (Figure 3). At the same time, strong dose-dependent up-regulation was detected in the expression of BRCA1, PTGS2, P4HA1, and P4HA2 mRNAs in the normal human astrocytes 24 h after the nanographene oxide treatment (Figures 4–7). The expression level of BRCA1 mRNA in astrocytes treated with 1 and 4 ng/ml of nanographene oxide was significantly increased by +87 and +123%, respectively (Figure 4). The impact of nanographene oxide exposure on the expression of prostaglandin-endoperoxide synthase 2 was also dose-dependent, but less pronounced: at

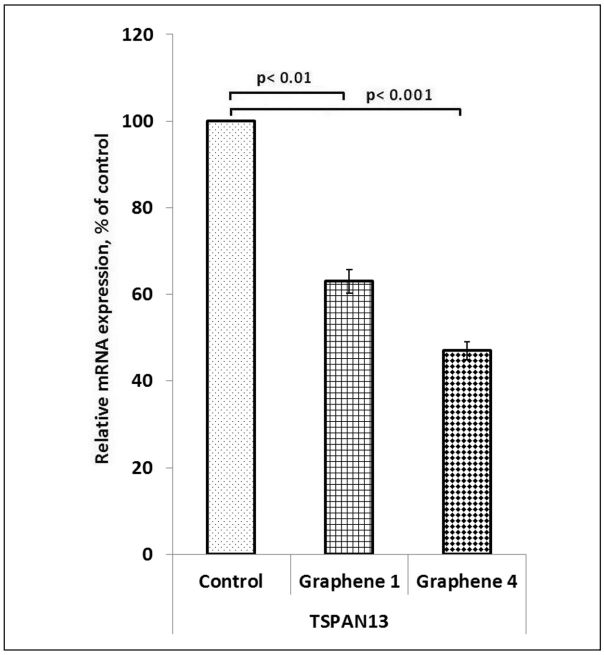

Figure 2. Dose-dependent impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the tetraspanin 13 (TSPAN13) mRNA expression in the normal human astrocytes versus control cells. TSPAN13 mRNA expression values were normalized to the ACTB mRNA levels; n=4.

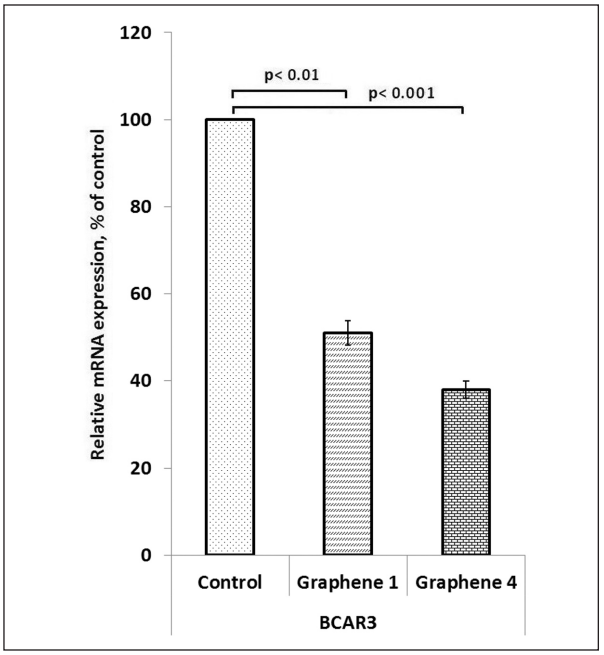

Figure 3. The impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the expression of BCAR3 adaptor protein, NSP family member (BCAR3) mRNA in NHA/TS cells versus control cells. Values of BCAR3 mRNA expression were normalized to the level of ACTB mRNA; n=4.

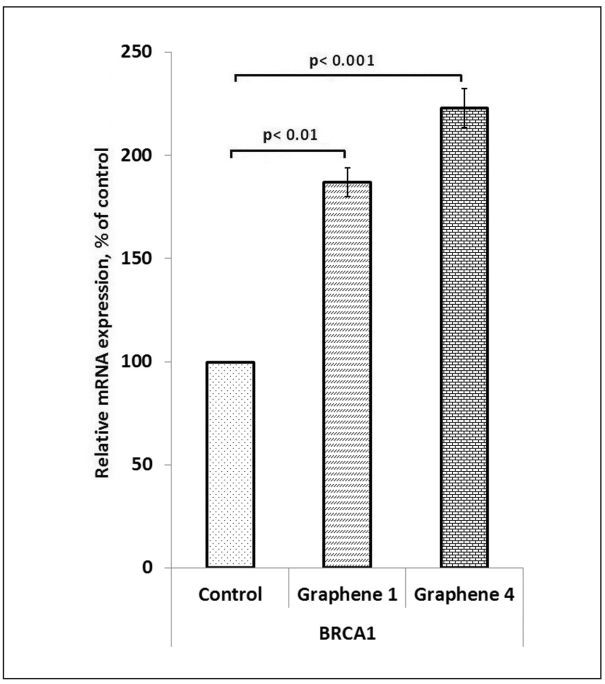

Figure 4. The impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the expression of the breast cancer 1, early onset (BRCA1) mRNA in normal human astrocytes versus control cells. Values of BRCA1 mRNA expression were normalized to the level of ACTB mRNA; n=4.

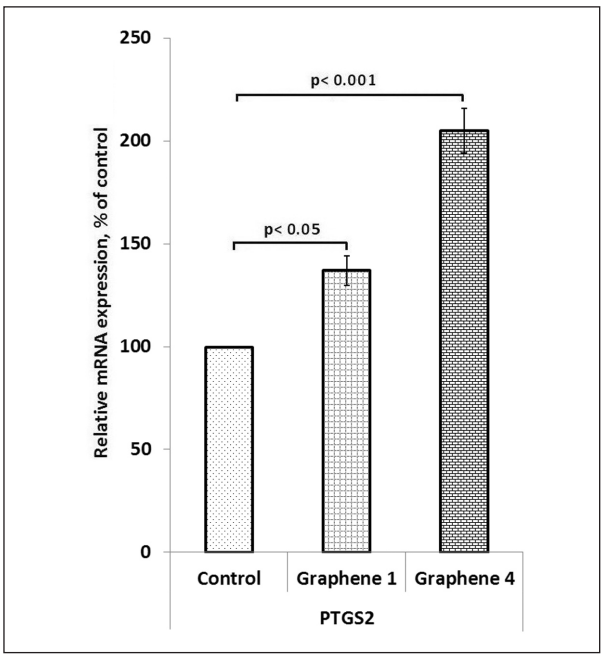

Figure 5. Dose-dependent impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the expression of prostaglandin-endoperoxide synthase 2 (PTGS2) also known as cyclooxygenase 2 (COX2) mRNA in normal human astrocytes versus control cells. Values of PTGS2 mRNA expression were normalized to the level of ACTB mRNA; n=4.

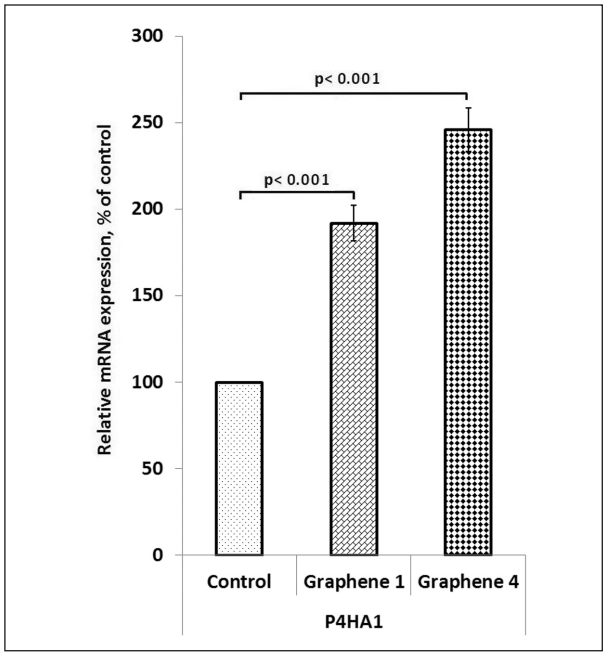

Figure 6. The impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the expression of prolyl 4-hydroxylase subunit alpha 1 (P4HA1) mRNA in NHA/TS cells versus control cells. Values of P4HA1 mRNA expression were normalized to the level of ACTB mRNA; n=4.

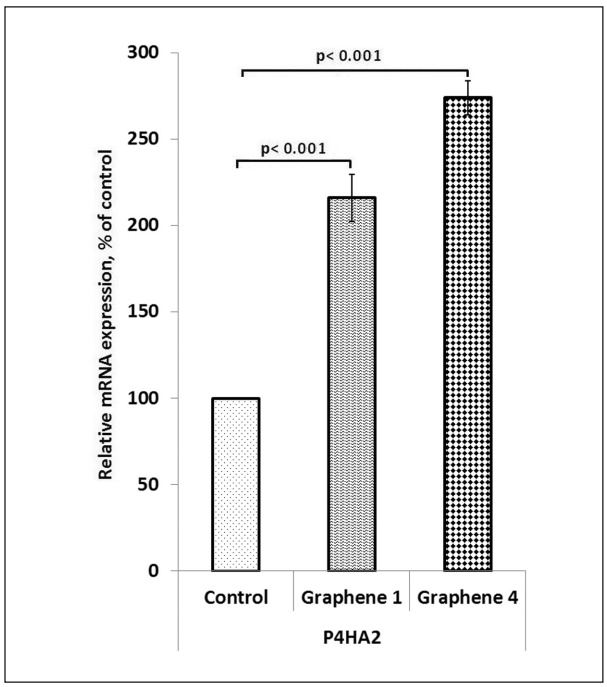

Figure 7. The impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the expression level of prolyl 4-hydroxylase alpha polypeptide II (P4HA2) mRNA in the normal human astrocytes versus no treated cells (control). Values of this mRNA expression were normalized to the level of ACTB mRNA; n=4.

dose of 1 ng/ml increased by 37%, and at a higher dose (4 ng/ml) – by 105% (Figure 5). Analysis of P4HA1 and P4HA2 mRNAs expression showed a sharp induction of both mRNAs in normal human astrocytes due to the action of nanographene oxide at both lower (1 ng/ml: +92 and +146%, respectively) and higher (2 ng/ml: +116 and +174%, respectively) doses (Figures 6, 7). Nanographene oxide particles at very low quantities dysregulate the expression of mRNA, which encode important regulatory enzymes and proteins in normal human astrocytes in a dose-dependent and gene-specific manner.

We also studied the impact of nanographene oxide on the expression of microRNAs including miR-96-5p and miR-145-5p, which have binding sites in 3'-untranslated regions of NAMPT and TSPAN13 mRNAs, respectively. As shown in Figure 8, the exposure of normal human astrocytes to lower dose (1 ng/ml) of nanographene oxide led to a significant down-regulation of the miR-96-5p expression level by +45%. More pronounced increase in the expression of this microRNA (+98%) was detected in astrocytes treated with higher dose of these carbon nanoparticles (Figure 8). As shown in Figure 9, the lower dose of nanographene oxide (1 ng/ml) increased the level of miR-145-5p by 56% and the larger dose (4 ng/ml) by 161%. The exposure of normal human astrocytes to low doses of nanographene oxide had strong impact on the expression of both miR-96-5p and miR-145-5p genes in dose-dependent manner.

Discussion

In this study, we investigated a set of genes, which encode important regulatory enzymes and proteins related to the control of metabolism, cell proliferation, and cancerogenesis. It was found that the exposure of normal human astrocytes to very low quantities of nanographene oxide particles significantly alters their mRNA as well as microRNA expression, which participate in the post-transcriptional regulation of corresponding mRNA levels. The results of this investigation clearly demonstrate that exposure of normal human astrocytes to nanographene oxide particles dysregulates the genome function that leads to significant dose-dependent and gene-specific changes in the expression of important regulatory factors and enzymes including NAMPT, TSPAN13, BCAR3, BRCA1, PTGS2, P4HA1, P4HA2, and microRNAs.

The observed dose-dependent changes in the expression of the studied genes in the normal human astrocytes treated by the nanographene oxide

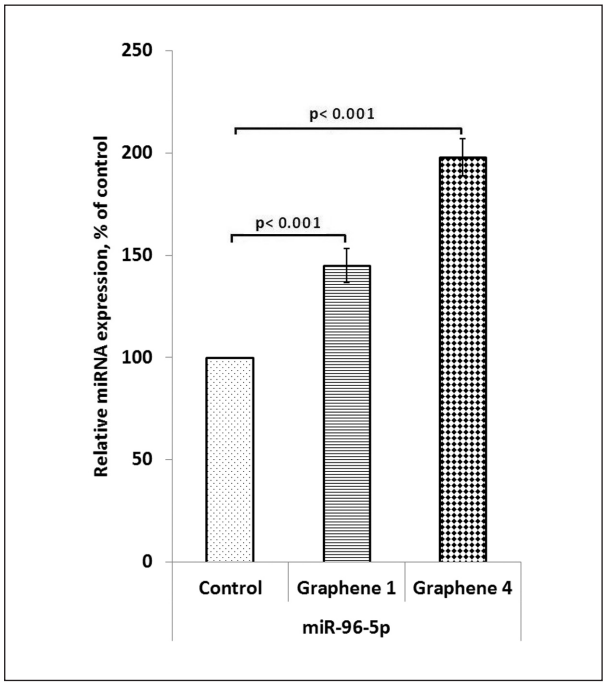

Figure 8. The impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the expression of microRNA miR-96-5p in NHA/TS cells versus control cells. Values of this microRNA expression were normalized to the level of U6 RNA; n=4.

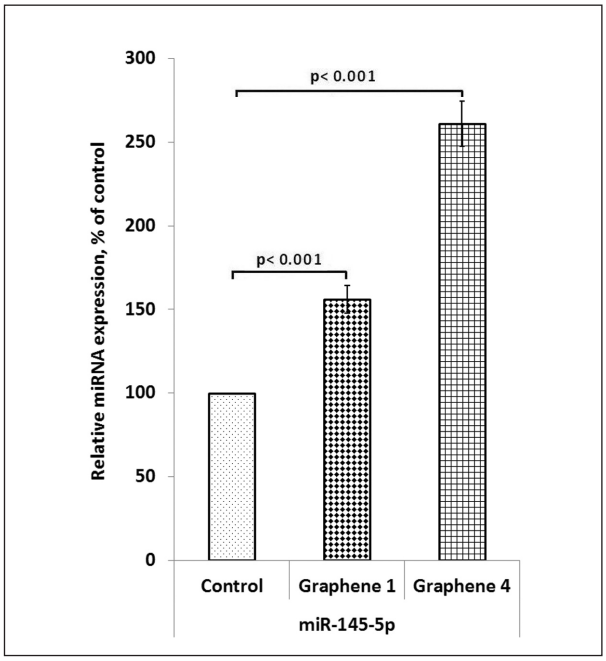

Figure 9. The impact of nanographene oxide (1 and 4 ng/ml, treated for 24 h) on the expression level of microRNA miR-145-5p in the normal human astrocytes line NHA/TS versus control cells. Values of miR-145-5p expression were normalized to the level of U6 RNA; n=4.

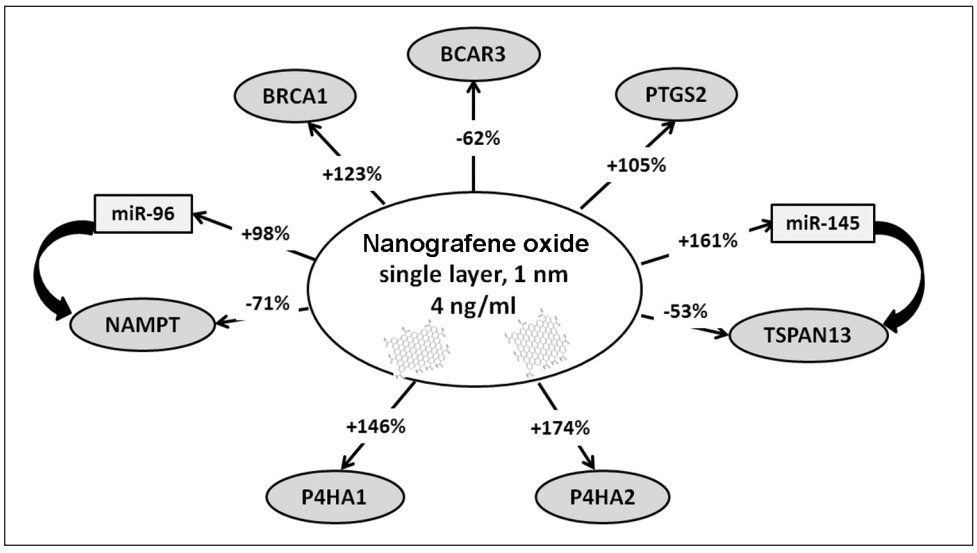

Figure 10. Schematic demonstration of changes in the expression profile of *NAMPT*, *TSPAN13*, *BCAR3*, *BRCA1*, *PTGS2*, *P4HA1*, *P4HA2* genes and miR-96-5p and miR-145-5p microRNAs in normal human astrocytes line NHA/TS treated with nanographene oxide (4 ng/ml) for 24 h.

particles indicate for their possible toxic effect that is in agreement with the data of other authors dealing with the toxicity of graphene-based nanomaterials (Ou et al. 2016; Ema et al. 2017; Fadeel et al. 2018; Jia et al. 2019; Chen et al. 2020; Li et al. 2021). It is possible that the toxic effect of nanographene oxide particles might be realized through the endoplasmic reticulum stress, which is consistent with the numerous data

concerning the molecular mechanisms of various nanoparticles impact on the genome function (Simon et al. 2017; Hu et al. 2018; Cao et al. 2020). Since the functional activity of all studied genes is altered in the most malignant tumors, there is a great probability that it may be associated with the endoplasmic reticulum stress (Li et al. 2019; Pan et al. 2019; Cao et al. 2020; Minchenko et al. 2020; Qi et al. 2020; Tahir et al. 2020; Zhou et al. 2020; Zhang et al. 2021; Ravinder et al. 2022).

In this study, we showed that the expression of *NAMPT*, *TSPAN13*, and *BCAR3* genes is down-regulated in normal human astrocytes treated with the nanographene oxide particles, but the expression of other genes including *BRCA1*, *PTGS2*, *P4HA1*, and *P4HA2* were significantly up-regulated (Figure 10). It is noteworthy that *PTGS2*, *P4HA1*, and *P4HA2* genes encode proteins with pro-proliferative and pro-oncogenic properties. However, the *BRCA1* gene, which expression is also up-regulated in cells treated by nanographene oxide, operates preferentially as a tumor suppressor and play an important role in maintaining the genomic stability (Jhanwar-Uniyal 2003; Takaoka and Miki 2018). At the same time, the expression of *NAMPT*, *TSPAN13*, and *BCAR3* genes is significantly suppressed, whereas proteins encoded by these genes exhibit pro-proliferative properties (Oh et al. 2013; Xu et al. 2017; Jiang et al. 2019; Li et al. 2019; Pan et al. 2019; Zhang et al. 2021 Gasparrini et al. 2022). The NAMPT and BCAR3 proteins are involved in the insulin signaling pathway (Chang et al. 2010; Minchenko et al. 2020; Qi et al. 2020).

Most of the proteins encoded by the above-mentioned genes are known as multifunctional proteins that are involved in various cell functions. In this regard, it is difficult to determine the functional significance of those gene expression changes that we found in the normal astrocytes induced by the nanographene oxide particles. We assume that in normal human astrocytes, very low quantity of the nanographene oxide particles may dysregulate the expression of mRNAs and microRNAs, encoding important regulatory enzymes and proteins, in dose-dependent manner. It is presumable that the gene-specific response observed in the present study induced by nanographene oxide may reflect functional dysregulation of this genome. Similar results have also been reported with carbon nanotubes (Minchenko et al. 2018; Rudnytska et al. 2021).

We also found that nanographene oxide particles affected the expression of miR-96-5p and miR-145-5p microRNAs in normal human astrocytes. The binding site for miR-96-5p was identified in 3'-untranslated regions of NAMPT mRNA and for miR-145-5p in TSPAN13 mRNA. Results of this study demonstrated significant up-regulation of both microRNAs in astrocytes treated with nanographene oxide particles, which correlate with down-regulation of NAMPT and TSPAN13 mRNA expression (Figure 10). It is possible that these microRNAs participate in the post-transcriptional regulation of NAMPT and TSPAN13 mRNAs expression. These data are in agreement with the results of other researchers data (Lin and Wu 2018; Minchenko et al. 2018; Goldewski et al. 2019; Li et al. 2019; Ma et al. 2019; Rudnytska et al. 2021), who have demonstrated that microRNA plays an essential role in the regulation of gene expression and thus regulates different physiological and pathophysiological processes in various cells and tissues including the brain tissue.

Many mechanisms regarding diversity of the carbon nanoparticle toxic effects have been described, but mechanisms of toxicity of the nanographene oxide particles are still unidentified. One of the possible signaling pathways might include the endoplasmic reticulum stress as it has already been shown in different nanoparticles (Boran and Ulutas 2016; Cao et al. 2017; Scherzad et al. 2017; Simon et al. 2017; Chen et al. 2020). It has been demonstrated that modulation of the endoplasmic reticulum stress by chemicals may alter the toxicity of some nanoparticles (Simon et al. 2017; Cao et al. 2020). It is possible that most of the changes introduced by nanographene oxide are mediated by signaling pathways of the endoplasmic reticulum stress reprogramming the genome function. miRNAs have also emerged as key regulators of the endoplasmic reticulum homeostasis and important players in the unfolded protein response-dependent signaling (Maurel and Chevet 2013). Finally, the fact that endoplasmic reticulum stress can regulate the miRNA expression supports the assumption that endoplasmic reticulum stress is an important component of the nanographene oxide outcome similar to other nanoparticles (Maurel and Chevet 2013; Simon et al. 2017; Chen et al. 2020).

In conclusion, we revealed that very small quantities of the nanographene oxide particles exert a dose-dependent genotoxicity in normal human astrocytes and disturb their genome function through up- or down-regulation of genes encoding the key regulatory proteins and enzymes. Currently, little is known about the genotoxicity of the nanographene oxide or many other nanoparticles. Better understanding of the detailed molecular

mechanisms of the nanographene oxide, so unique carbon nanomaterial, is important not only from the view of its promising therapeutic application, but also its potential risk associated with use for human health. The present data indicate that likewise in other sorts of nanoparticles, more caution is also needed in the nanographene oxide biomedical applications.

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References

Ahmadi H, Ramezani M, Yazdian-Robati R, Behnam B, Razavi Azarkhiavi K, Hashem Nia A, Mokhtarzadeh A, Matbou Riahi M, Razavi BM, Abnous K. Acute toxicity of functionalized single wall carbon nanotubes: A biochemical, histopathologic and proteomics approach. Chem Biol Interact 275, 196–209, 2017.

Aragon MJ, Topper L, Tyler CR, Sanchez B, Zychowski K, Young T, Herbert G, Hall P, Erdely A, Eye T, Bishop L, Saunders SA, Muldoon PP, Ottens AK, Campen MJ. Serum-borne bioactivity caused by pulmonary multi-walled carbon nanotubes induces neuroinflammation via blood-brain barrier impairment. Proc Natl Acad Sci USA 114, E1968–E1976, 2017.

Auf G, Jabouille A, Delugin M, Guerit S, Pineau R, North S, Platonova N, Maitre M, Favereaux A, Vajkoczy P, Seno M, Bikfalvi A, Minchenko D, Minchenko O, Moenner M. High epiregulin expression in human U87 glioma cells relies on IRE1a and promotes autocrine growth through EGF receptor. BMC Cancer 13, 597, 2013.

Avitabile E, Bedognetti D, Ciofani G, Bianco A, Delogu LG. How can nanotechnology help the fight against breast cancer? Nanoscale 10, 11719–11731, 2018.

Backes C, Behera RK, Bianco A, Casiraghi C, Doan H, Criado A, Galembeck F, Goldie S, Gravagnuolo AM, Hou HL, Kamali AR, Kostarelos K, Kumar V, Lee WH, Martsinovich N, Palermo V, Palma M, Pang J, Prato M, Samori P, Silvestri A, Singh S, Strano M, Wetzl C. Biomedical applications: general discussion. Faraday Discuss 227, 245–258, 2021.

Boran H, Ulutas G. Genotoxic effects and gene expression changes in larval zebrafish after exposure to ZnCl2 and ZnO nanoparticles. Dis Aquat Org 117, 205–214, 2016.

Cao Y, Long J, Liu L, He T, Jiang L, Zhao C, Li Z. A review of endoplasmic reticulum (ER) stress and nanoparticle (NP) exposure. Life Sci 186, 33–42, 2017.

Cao Y, Han Q, Li J, Jia Y, Zhang R, Shi H. P4HA2 contributes to cervical cancer progression via inducing epithelial-mesenchymal transition. J Cancer 11, 2788–2799, 2020.

Cellot G, Franceschi Biagioni A, Ballerini L. Nanomedicine and graphene-based materials: advanced technologies for potential treatments of diseases in the developing nervous system. Pediatr Res 2021, Sep 3. doi: 10.1038/s41390-021-01681-6. Online ahead of print.

Chakravarthi BV, Pathi SS, Goswami MT, Cieslik M, Zheng H, Nallasivam S, Arekapudi SR, Jing X, Siddiqui J, Athanikar J, Carskadon SL, Lonigro RJ, Kunju LP, Chinnaiyan AM, Palanisamy N, Varambally S. The miR-124-prolyl hydroxylase P4HA1-MMP1 axis plays a critical role in prostate cancer progression. Oncotarget 5, 6654–6669. 2014.

ChangYC, ChangTJ, LeeWJ, Chuang LM. The relationship of visfatin/pre-B-cell colony-enhancing factor/nicotinamide phosphoribosyltransferase in adipose tissue with inflammation, insulin resistance, and plasma lipids. Metab Clin Exp 59, 93–99, 2010.

Chen L, Li J, Chen Z, Gu Z, Yan L, Zhao F, Zhang A. Toxicological evaluation of graphene-family nanomaterials. J Nanosci Nanotechnol 20, 1993–2006, 2020.

Cui G, Wu J, Lin J, Liu W, Chen P, Yu M, Zhou D, Yao G. Graphene-based nanomaterials for breast cancer treatment: promising therapeutic strategies. J Nanobiotechnology 19, 211, 2021.

Dash BS, Jose G, Lu YJ, Chen JP. Functionalized reduced graphene oxide as a versatile tool for cancer therapy. Int J Mol Sci 22, 2989, 2021.

Eldawud R, Wagner A, Dong C, Stueckle TA, Rojanasakul Y, Dinu CZ. Carbon nanotubes physicochemical properties influence the overall cellular behavior and fate. NanoImpact 9, 72–84, 2018.

Ema M, Gamo M, Honda K. A review of toxicity studies on graphene-based nanomaterials in laboratory animals. Regul Toxicol Pharmacol 85, 7–24, 2017.

Fadeel B, Bussy C, Merino S, Vazquez E, Flahaut E, Mouchet F, Evariste L, Gauthier L, Koivisto AJ, Vogel U, Martín C, Delogu LG, Buerki-Thurnherr T, Wick P, Beloin-Saint-Pierre D, Hischier R, Pelin M, Candotto Carniel F, Tretiach M, Cesca F, Benfenati F, Scaini D, Ballerini L, Kostarelos K, Prato M, Bianco A. Safety assessment of graphene-based materials: focus on human health and the environment. ACS Nano 12, 10582–10620, 2018.

Francis A, Devasena T. Toxicity of carbon nanotubes: a review. Toxicol Ind Health 34, 200–210, 2018.

Gasparrini M, Mazzola F, Cuccioloni M, Sorci L, Audrito V, Zamporlini F, Fortunato C, Amici A, Cianci M, Deaglio S, Angeletti M, Raffaelli N. Molecular insights into the interaction between human nicotinamide phosphoribosyltransferase and Toll-like receptor 4. J Biol Chem 298, 101669, 2022.

Goldewski J., Lenart J, Salinska E. MicroRNA in brain pathology: Neurodegeneration the other side of the brain cancer. Non-coding RNA 5, 20, 2019.

Hu H, Li L, Guo Q, Zong H, Yan Y, Yin Y, Wang Y, Oh Y, Feng Y, Wu Q, Gu N. RNA sequencing analysis shows that titanium dioxide nanoparticles induce endoplasmic reticulum stress, which has a central role in mediating plasma glucose in mice. Nanotoxicology 12, 341–356, 2018.

Jhanwar-Uniyal M. BRCA1 in cancer, cell cycle and genomic stability. Front Biosci 8, s1107–1117, 2003.

Jia PP, Sun T, Junaid M, Yang L, Ma YB, Cui ZS, Wei DP, Shi HF, Pei DS. Nanotoxicity of different sizes of graphene (G) and graphene oxide (GO) in vitro and in vivo. Environ Pollut 247, 595–606, 2019.

Jiang L, Zhang X, Geradts J, Wei Q, Hochwald S, Xu H, Huang H. Expression of tetraspanins NET-6 and CD151 in breast cancer as a potential tumor biomarker. Clin Exp Med 19, 377–384, 2019.

Jiang T, Amadei, CA, Gou N, Lin Y, Lan J, Vecitis CD, Gu AZ. Toxicity of single-walled carbon nanotubes (SWCNTs): effect of lengths, functional groups and electronic structures revealed by a quantitative toxicogenomics assay. Environ Sci Nano 7, 1348–1364, 2020.

Kavosi A, Noei SHG, Madani S, Khalighfard S, Khodayari H, Mirzaei M, Kalhari MR, Yavarian M, Alizadeh AM, Falahati M. The toxicity and therapeutic effects of single- and multi-wall carbon nanotubes on mice breast cancer. Sci. Reports 8, 8375, 2018.

Li P, Dong M, Wang Z. Downregulation of TSPAN13 by miR-369-3p inhibits cell proliferation in papillary thyroid cancer (PTC). Bosn J Basic Med Sci 19, 146–154, 2019.

Li J, Zeng H, Zeng Z, Zeng Y, Xie T. Promising graphene-based nanomaterials and their biomedical applications and potential risks: A comprehensive review. ACS Biomater Sci Eng 7, 5363–5396, 2021.

Lin Y, Wu Z. MicroRNA-128 inhibits proliferation and invasion of glioma cells by targeting COX-2. Gene 658, 63–69, 2018.

Ma Q, Zhang L, Pearce WJ. MicroRNAs in brain development and cerebrovascular pathophysiology. Am J Physiol Cell Physiol 317, C3–C19, 2019.

Malhotra N, Villaflores OB, Audira G, Siregar P, Lee JS, Ger TR, Hsiao CD. Toxicity studies on graphene-based nanomaterials in aquatic organisms: current understanding. Molecules 25, 3618, 2020.

Maurel M, Chevet E. Endoplasmic reticulum stress signaling: the microRNA connection. Am J Physiol Cell Physiol 304, C1117–C1126, 2013.

Mendonça MC, Soares ES, de Jesus MB, Ceragioli HJ, Ferreira MS, Catharino RR, da Cruz-Höfling MA. Reduced graphene oxide induces transient blood-brain barrier opening: an in vivo study. J Nanobiotechnology 13, 78, 2015.

Minchenko OH, Tsymbal DO, Minchenko DO, Hnatiuk OS, Prylutskyy YI, Prylutska SV, Tsierkezos NG, Ritter U. Single-walled carbon nanotubes affect the expression of genes associated with immune response in normal human astrocytes. Toxicology in Vitro 52, 122–130, 2018.

Minchenko DO, Tsymbal DO, Luzina OY, Riabovol OO, Danilovskyi SV, Minchenko OH. Silencing of NAMPT leads to up-regulation of insulin receptor substrate 1 gene expression in U87 glioma cells. Endocr Reg 54, 31–42, 2020.

Oh MJ, Yi SJ, Kim HS, Kim JH, Jeong YH, van Agthoven T, Jhun BH. Functional roles of BCAR3 in the signaling pathways of insulin leading to DNA synthesis, membrane ruffling and GLUT4 translocation. Biochem Biophys Res Commun 441, 911–916; 2013.

Ou L, Song B, Liang H, Liu J, Feng X, Deng B, Sun T, Shao L. Toxicity of graphene-family nanoparticles: a general review of the origins and mechanisms. Particle Fibre Toxicol 13, 57, 2016.

Pan Z, Zhu Q, You W, Shen C, Hu W, Chen X. Silencing of Mig-7 expression inhibits in-vitro invasiveness and vasculogenic mimicry of human glioma U87 cells. Neuroreport 30, 1135–1142, 2019.

Qi Y, Li H, Lv J, Qi W, Shen L, Liu S, Ding A, Wang G, Sun L, Qiu W. Expression and function of transmembrane 4 superfamily proteins in digestive system cancers. Cancer Cell International 20, 314, 2020.

Ravinder D, Rampogu S, Dharmapuri G, Pasha A, Lee KW, Pawar SC. Inhibition of DDX3 and COX-2 by forskolin and evaluation of anti-proliferative, pro-apoptotic effects on cervical cancer cells: molecular modelling and in vitro approaches. Med Oncol 39, 61, 2022.

Rudnytska OV, Khita OO, Minchenko DO, Tsymbal DO, Yefimova YV, Sliusar MY, Minchenko OH. The low doses of SWCNTs exhibit a genotoxic effect on the normal human astrocytes by disrupting the functional integrity of the genome. Curr Res Toxicol 2, 64–71, 2021.

Sasai K, Akagi T, Aoyanagi E, Tabu K, Kaneko S, Tanaka S. O6-methylguanine-DNA methyltransferase is down-regulated in transformed astrocyte cells: implications for anti-glioma therapies. Mol Cancer 6, 36, 2007.

Scherzad A, Meyer T, Kleinsasser N, Hackenberg S. Molecular mechanisms of zinc oxide nanoparticle-induced genotoxicity short running title: genotoxicity of ZnO NPs. Materials 10, 1427, 2017.

Simon M, Saez G, Muggiolu G, Lavenas M, Le Trequesser Q, Michelet C, Deves G, Barberet P, Chevet E, Dupuy D, Delville M-H, Seznec H. In situ quantification of diverse titanium dioxide nanoparticles unveils selective endoplasmic reticulum stress-dependent toxicity. Nanotoxicology 11, 134–145, 2017.

Su S, Wang J, Qiu J, Martinez-Zaguilan R, Sennoune SR, Wang S. In vitro study of transportation of porphyrin immobilized graphene oxide through blood brain barrier. Mater Sci Eng C Mater Biol Appl 107, 110313, 2020.

Tahir DE, Rehman MS, Rehman MU. An overview of cancer genetics with focus on involvement of BRCA1/2 genes in breast carcinomas. J Pak Med Assoc 70, 1240–1247, 2020.

Takaoka M, Miki Y. BRCA1 gene: function and deficiency. Int J Clin Oncol 23, 36–44, 2018.

Wang T, Fu X, Jin T, Zhang L, Liu B, Wu Y, Xu F, Wang X, Ye K, Zhang W, Ye L. Aspirin targets P4HA2 through inhibiting NF-kappaB and LMCD1-AS1/let-7g to inhibit tumor growth and collagen deposition in hepatocellular carcinoma. EBioMedicine 45, 168–180, 2019.

Xu R, Yuan Z, Yang L, Li L, Li D, Lv C. Inhibition of NAMPT decreases cell growth and enhances susceptibility to oxidative stress. Oncol Rep 38, 1767–1773, 2017.

Yan H, Xue Z, Xie J, Dong Y, Ma Z, Sun X, Kebebe Borga D, Liu Z, Li J. Toxicity of carbon nanotubes as anti-tumor drug carriers. Int J Nanomedicine 14, 10179–10194, 2019.

Yang M, Zhang M. Biodegradation of carbon nanotubes by macrophages. Front Mater 6, 225, 2019.

Yapijakis C. Regulatory role of microRNAs in brain development and function. Adv Exp Med Biol 1195, 237–247, 2020.

Zhang Z, Wang Y, Wang Y, Wang C, Shuai Y, Luo J, Liu R. BCAR3 promotes head and neck cancer growth and is associated with poor prognosis. Cell Death Discov 7, 316, 2021.

Zhou H, He Y, Li L, Wu C, Hu G. Overexpression of P4HA1 is correlated with poor survival and immune infiltrates in lung adenocarcinoma. Biomed Res Int 2020, 8024138, 2020.

Zhu Y, Shi C, Zeng L, Liu G, Jiang W, Zhang X, Chen S, Guo J, Jian X, Ouyang J, Xia J, Kuang C, Fan S, Wu X, Wu Y, Zhou W, Guan Y. High COX-2 expression in cancer-associated fibroblasts contributes to poor survival and promotes migration and invasiveness in nasopharyngeal carcinoma. Mol Carcinog 59, 265–280, 2020.