Use of the Comet Assay and Molecular Docking Techniques to Evaluate the Effect of Nicotine in Human Breast Cancer Cell Line

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Abstract
The harmful effects of tobacco use and nicotine are well known. There is a strong epidemiological evidence for smoking as a risk factor for lung cancer and oral cavity cancer. The evidence for second-hand smoke as a risk factor for breast cancer is rapidly accumulating. The single-cell gel electrophoresis (Comet) assay has been widely used to measure DNA damage in human breast cancer cells in a variety of physiological and pathological conditions. These damages occured in DNA are some structural damages such as single- and double- strand breaks, oxidative base damage, and DNA-DNA, DNA-protein, DNA- Drug crosslinking and mutations in DNA. Single cell gel electrophoresis (SCGE) or so-called "Comet assay" is a rapid, simple and very sensitive fluorescent microscopic method to examine DNA damage at individual cell level in living populations. We investigated the effects of nicotine on breast cancer cells (MCF-7) *in vitro* and examined breast cancer cells line by using the Comet assay. This was seen most clearly when changes in the Comet tail length were measured when there were discrete undamaged and damaged populations. After increasing doses of nicotine, an increasing proportion of cells were found in the damaged population. These observations led to the hypothesis that nicotine might be playing a direct role in the promotion and progression of human breast cancers. Here, we study on the role and the effects of nicotine on breast cancer cell growth and physiology. However, the mechanisms by which nicotine promotes cancer development and metastasis are not fully understood. We determined the interaction between nicotine and the receptor is a protein (galectin-3) by molecular docking technique. It has recently been determined that development of breast cancer is originated from

cancer stem cells, which are a minor population of breast cancer. In the present study; it is thought that the DNA damage is related to cancer stem cell number increase.

Keywords: Nicotine, breast cancer, docking, comet assay, DNA damage.

Introduction

DNA, which allows genetic information to be transmitted from generation to generation, is a molecule that can easily be damaged [1]. The genome is exposed to a number of endogenous and external factors that cause DNA damage [2, 3]. The genotoxic effects of tobacco carcinogens are known to be long lasting; the contribution of tobacco components to carcinogenesis by cell surface receptor signaling has not been fully explored [4]. The International Agency for Research on Cancer (IARC) is expected to be one of the leading causes of deaths in the world, predicting 7.6 million deaths and 12.7 million new cases of cancer a year. Breast cancer is a type of cancer with a high rate in modern societies and this type of cancer, especially in women, is a great threat to human health[5,8].

In epidemiological and clinical studies comparing smokers with non-smokers; it has been determined that the risk increase in breast cancer development is related to smoking [9, 10]. There are over 4000 components in the cigarette, and it is known that the most important factor in these components is nicotine [11]. Nicotine (C5H4N) CH(CH2) 3N(CH3) is a natural alkaloid found in tobacco leaves and roots [12, 13]. The harmful effects of cigarette smoking are known to be one of the main factors for human tobacco dependence [14]. Tobacco addiction, long-term smoking is the leading cause of premature death, contributing clinically to cardiovascular disease and cancer development, and is one of the most important public health problems of our time [15, 16]. There are many studies in the literature showing that nicotine, a major addictive compound in tobacco smoke, promotes the development and progression of certain cancers, such as lung [17], head [18], neck [19], pancreas [20] and breast cancer [21].

When the primary tumors from breast cancer patients were investigated, it was seen that Nicotine induced galectin-3 (an anti-apoptotic β-galactoside-binding lectin) expression in breast cancer cell lines [22]. Galectin-3, which is a member of at least fifteen β-galactoside-binding soluble lectins family, is involved in tumor cell adhesion, angiogenesis, cancer progression and metastasis [23, 24]. The normal and cancer tissues compared to each other, it was shown that, Galectin-3 expression was significantly increased in breast, gastric, liver, lung, bladder and head and neck cancers and was found to be associated with the formation of metastases [25, 26]. In addition, a change in the cellular localization of galectin-3 was observed during the progression of various cancers [27, 28, 29].

The increased galectin-3 expression in apoptosis resistance in cancer cells were reported by recent studies [30], but unfortunately there was no studies show the link between galectin-3 and nicotine. Here we present evidence that nicotine binds to galectin-3 in breast cancer cells and supports galectin-3 expression, thanks to our docking calculations. Nicotine was increased galectin-3 expression in breast cancer cells and this support chemoresistance which was known cancer stem cell like properties. For this reason galectin-3 may be a potential target for the prevention of nicotine-induced chemoresistance in breast cancer, so it has been taken as the target protein in the docking studies. Molecular docking is a technique used to determine the preferred possible binding poses of ligand-receptor complexes [31] where the ligand is usually a small molecule (Nicotine) and the receptor is a protein (galectin-3). The determination of the interaction between the ligand and the receptor, disclosure of the ligand and protein binding mechanism, and estimation of protein activation or inhibition [32].

Cells' DNA damage's can be repaired with "DNA repair mechanism" [33]. DNA damage cannot be repaired as a result of mutations and genomic instability, causes cancer and aging [34]. The rate of DNA repair is dependent on many factors, including the cell type, the age of the cell, and the extracellular environment [35]. Experimental animals with genetic deficiencies in DNA repair often show decreased life span and increased cancer incidence [36].

Long-term smoking of tobacco clinically contributes to the development of cardiovascular disease and also to the development of cancer [37]. While smoking, 80-90% of inhaled nicotine (~1.0 mg per cigarette) is systemically absorbed [38]. Nicotine is thought to have a role in the formation of various types of cancer and has been demonstrated to increase angiogenesis in previous proliferation model studies [39, 40, 41, 42, 43, 44].

The deleterious effects of smoking and tobacco use have been well documented [45]. Tobacco use is well recognized as the major cause of lung, head and neck cancers [46]. Evidence for breast cancer risk from active and passive smoking is accumulating [47, 48]. There is increasing evidence that the use of tobacco can decrease the efficacy of cancer treatment and increase the risk of recurrence [49]. Recent studies

have shown that nicotine inhibits apoptosis of cancer cells treated with a variety of chemotherapeutic drugs [50, 51].
In the present study, we have evaluated activities of nicotine in human breast cancer cell line (MCF-7), by the comet assay. We think that the structure of the DNA molecule will change as the concentration of nicotine increases and it will result from the fact that various mutations, different phenotypes or diseases arise from this because it causes changes in DNA codes and therefore changes in protein expression.

Materials and Methods

Cell Culture

MCF-7 human breast cancer cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 gg/mL streptomycin. Cells were subcultured twice weekly and maintained as exponentially growing monolayers in a humidified 5% carbon dioxide air atmosphere at 37°C [52].

Comet Analysis and Scoring of Slides

To detect DNA double-strand breaks in a single cell by using Comet assay, alkaline lysis and then alkaline gel electrophoresis were used [52]. Briefly, cells were incubated with various concentrations of nicotine up to 48 h. The cells were treated with trypsin to detach cells from the dish and from each other, and then they were suspended in phosphate-buffered saline (PBS). DNA breakage was observed using single-cell gel electrophoresis (comet assay) as described by Lynn et al [52] with some modification. Briefly, MCF-7 cells after nicotine treatment were harvested and embedded in 1٪ low melt agarose (Fermentas company) gel at a density of 1×10^6 cells/ ml, and spread on a microscopic slide previously coated with normal melting point agarose. The slides were immersed in ice-cold lysis buffer (10 mM Tris-HCl, 2.5M NaCl, 100 mM Na2EDTA, 1٪ sodium N-lauryl sarcosinate, pH 10) for 1 hr at 4 ºC. Cellular DNA was denatured in electrophoresis buffer (300 mM NaOH AND 1 mM Na2EDTA) for 20 min at room temperature then electrophoresis was performed for 20 min at constant voltage (25 V). All the procedure was carried out under indirect light, and then the slides were washed in distilled water and renatured in 0.4 M Tris-HCl (pH 7.5). The slides were stained with ethidium bromide (2 µg/mL) and examined under the fluorescence microscope (Olympus, BX51). Quantitative image analysis was performed using an intensified solid state CCD camera (Sony CCD-IRIS; I.S.S. Group, Manchester, U.K.) attached to the microscope and linked to the Comet score analysis software. Samples were run in duplicate, and 50 cells were randomly analyzed per slide for a total of 100 cells per sample and scored for Comet tail parameters. Comet tail length is the maximum distance the damaged DNA migrates from the center of the cell nucleus, the percentage of tail DNA is total DNA that migrates from the nucleus into the comet tail, and the tail moment is a product of the tail length which gives a more integrated measurement of overall DNA damage in the cell.

Molecular Docking and ADME Properties

Using the Glide SP (standard precision) module of the Maestro version 11.4 in the Schrodinger Software program (Schrödinger Release 2017-4: Maestro, Schrödinger, LLC, New York, NY, 2017) [53, 54, 55] the docking calculations were performed. To prepare ligand for docking calculations, first the most stable conformation of Nicotine was generated from the result of the molecular dynamic calculation, and then was prepared to optimization by the Lig Prep tool in the Maestro 11.4 version of the Schrödinger Software program using the OPLS force field [56].
After selecting the ionization states at pH 7.0 ± 2.0, possible stereoisomers were produced for the ligand. Because of nicotine induces the expression of galectin-3 in breast cancer cell line and in primary tumors from breast cancer patients, we choose galectin-3 [22] (PDB code: 2XG3) which could be a potential target to prevent nicotine induced chemo resistance in breast cancer. By using SWISS-MODEL server, the crystal structure obtained from the protein data bank was arranged to get a better protein homology model [57]. All waters were removed, polar hydrogen were added, bond orders were assigned, charges were defined using PROPKA [58] at pH 7.0, and galectin-3 was optimized by using the Protein Preparation Wizard tool [59]. Energy minimization was carried out by preferring 0.3A˚ RMSD and the OPLS3 force field was used to converge heavy atoms. To generate a grid, receptor grid generation tool was used; a cubic box was formed at centered on the centroid of the ligand with specific dimensions. A ligand docking tool was employed to achieve ligand-receptor docking by defining routable groups of an active site. Absorption, distribution, metabolism, and excretion (ADME) properties were also calculated using the Qik-Prop module of the Schrodinger software to specify the physicochemical and biological

functional properties such as the molecular weight (MW), percent human oral absorption, predicted octanol/water partition coefficient (QPlogPo/w), polar surface area (PSA), and number of violations of Lipinski's rule of five [60], which is important for generating an effective drug in new drug development.

Statistical Analysis

SPSS V16 was used in all statistical analyzes. The Student-T test was used to compare the two groups and the one-way ANOVA (one-way ANOVA) was used to compare more than two groups. Datas are given using mean standard deviation (mean ± SD), minimum and maximum values. Correlation of the results obtained with the measurements was compared with Pearson correlation analysis. $p < 0.05$ and $p < 0.01$ were considered to be statistically significant.

Results

Molecular Docking Results

The docking score obtained for Nicotine in the active side of the Galectin-3 protein was -4.23 kcal/mol, as shown in **Table 1.** and **Figure 1**. The binding pocket of the Galectin-3 (2XG3.pdb) protein has hydrophobic and negative charged regions as indicated by the green and red line, respectively. The most likely binding position between Galectin-3 protein and Nicotine was determined by hydrogen bonds represented by the purple line and were picturized in the 2D ligand interaction graph in **Figure 2**. In the active site of protein, Nicotine was bound with strong hydrogen bond by the residue GLU-72, (1.65 Å) and a salt bridge with the same amino acid residue was established and stability was achieved. At the same time, the $\pi-\pi$interaction with the TRP-69 residue has also increased its stabilization in active site. The binding interactions of Nicotine with GLU-72, (1.65 Å) and TRP-69 residues were also shown in **Figure. 3**. The electrostatic potential map surfaces of the Nicotine and Galectin-3 protein were also constituted to define the regions that were electron-rich and electron-poor. The lowest potential (electron-rich) regions were expressed in red; while those with the highest potential (electron poor) were shown in blue. The oxygen atoms in the carbonyl group with the red region in GLU-72 interacted with the blue region in Nicotine (high potential), while the hydrogen atoms in the TRP-69 residue tended to create $\pi-\pi$ interaction with NH_2 region with Nicotine. (**Figure 4)**. The pharmacokinetic parameters of drugs such as their permeability towards QPlogP for octanol/gas, QPlogP for octanol/water, PlogS for aqueous solubility, P log K hsa Serum Protein Binding, Predicted CNS Activity, Caco-2 Permeabilitiy, skin permeability, the blood–brain barrier, percentage oral absorption were also calculated and tabulated using the Qik Prop application of the Maestro software package in **Table 2.** Absorption of nicotine molecule, has a high oral absorption rate of 77 percent, in Caco-2 cells which called is a human colon epithelial cancer cell line that forms the human intestinal absorption model of drugs and other compounds, is as high as 304 nm / sec. Madin-Darby canine kidney epithelial cell lines permeability (MDCK) of nicotine is 167 nm / sec and as high as Caco-2 cells permeability. In addition to affecting heart movements, the HERG K+ Channel also modulate the functions of some cells of the nervous system and produce cancer-like properties in leukemic cells, and the nicotine molecule is highly effective with -4.454 value (concern below -5) in these channels. Nicotine has also a high capacity to cross an important barrier, such as a brain barrier.

Comet analysis of slides results

DNA double-strand breaks in a single cell by using Comet assay show us nicotine is effectide on breast cancer cells. The most effective nicotine concentration is 1 uM which concentration is also effect on cancer stem cell increase of proliferation rate. As shown in Figure 5, stimulation with 0.01- 0.05- 01- 1-10 nicotine increased the ratio of DNA double-strand breaks. Comet tail length was measured with compare control cells and in the presence of increasing nicotine breast cancer cells results after 24-48 h shown at Table 3.

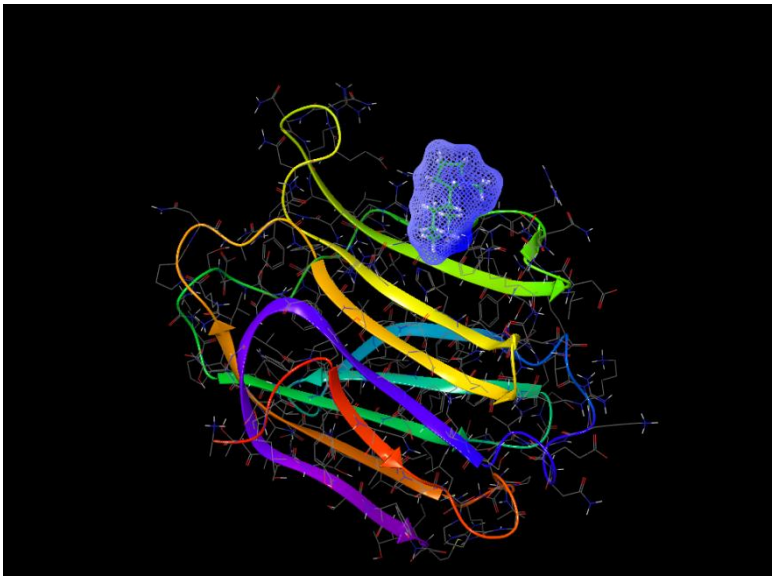

Figure 1. The docked molecular structure of Nicotine and Galectin-3.

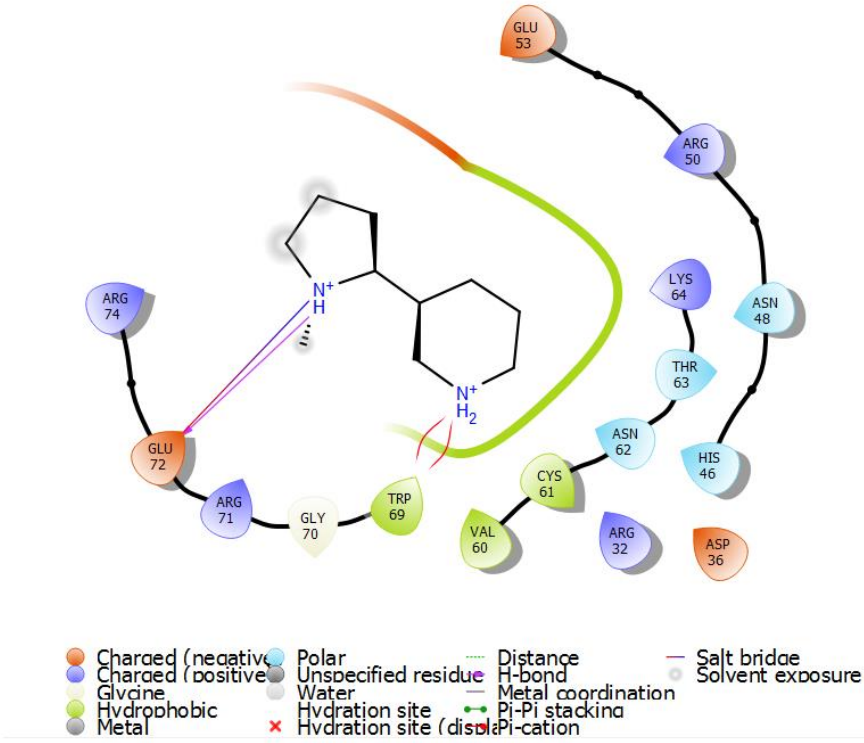

Figure 2. 2D ligand interaction of Nicotine in the active side of the Galectin-3

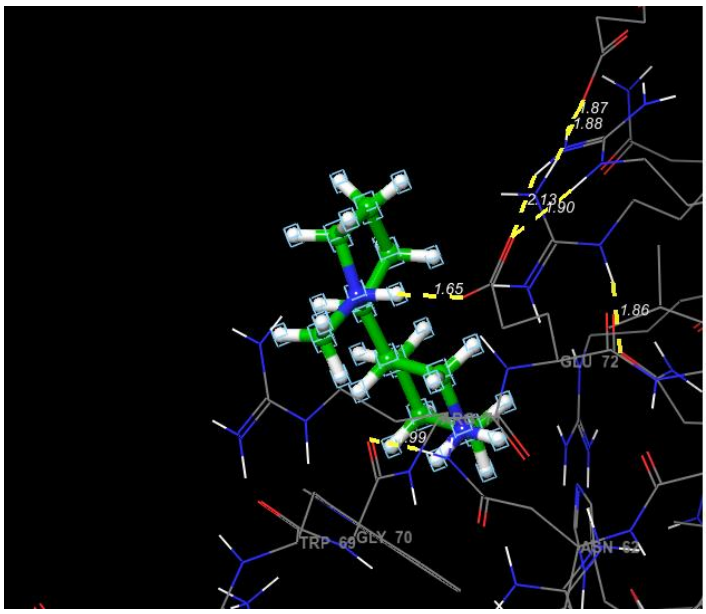

Figure 3. The binding interaction of Nicotine in the active site of the Galectin-3 with GLU and TRP-69 residues

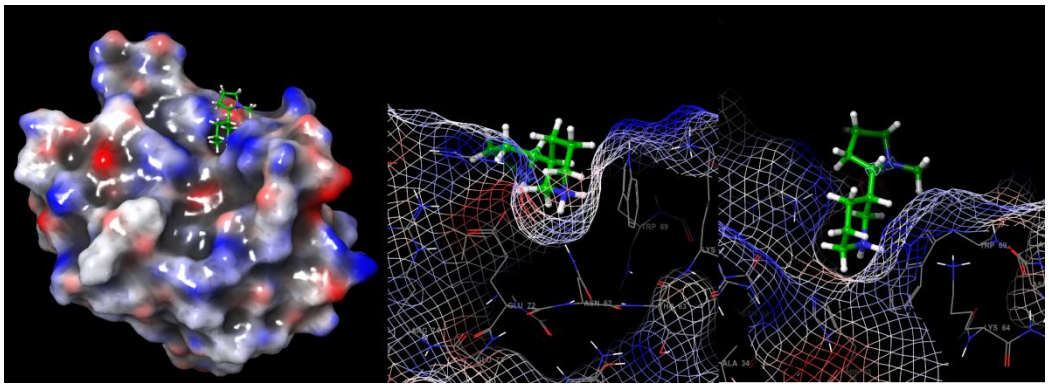

Figure 4. The electrostatic potential of Galectin-3 and Nicotine

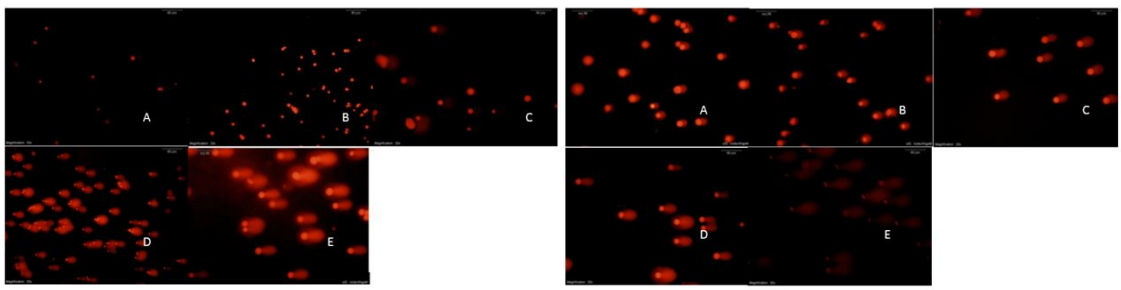

Figure 5. Imagine of comet analysis of nicotine induced breast cancer cells with fluorescence microscopy (20x). A-0.01 uM, B-0.05 uM, C-0.1 uM, D-1 uM, E-10 uM. (1) Imagining of nicotine induced cells after 24h, (2) Imagining of nicotine induced cells after 48h.

Table 1. The conformation and docking score energies

Ligand	Docking Score (kcal/mol)
1	-4.231
2	-4106
3	-3.950
4	-3.853
5	-3.828
6	-3.791
7	-3.729
8	-3.463

Table 2. The ADME properties of Nicotine

Property		**Value**	**Recommended**
Solute	Molecular Weight	168.281	(130.0 / 725.0)
Solute	Dipole Moment (D)	2.029	(1.0 / 12.5)
Solute	Total SASA	416.248	(300.0 /1000.0)
Solute	Hydrophobic SASA	383.98	(0.0 / 750.0)
Solute	Hydrophilic SASA	32.267	(7.0 / 330.0)
Solute	Carbon Pi SASA	0	(0.0 / 450.0)
Solute	Weakly Polar SASA	0	(0.0 / 175.0)
Solute	Molecular Volume (A^3)	692.251	(500.0 /2000.0)
Solute	vdW Polar SA (PSA)	20.935	(7.0 / 200.0)
Solute	No. of Rotatable Bonds	0	(0.0 / 15.0)
Solute as Donor - Hydrogen Bonds		1	(0.0 / 6.0)
Solute as Acceptor - Hydrogen Bonds		3.5	(2.0 / 20.0)
Solute Globularity (Sphere 1)		0.909	(0.75 / 0.95)
Solute Ionization Potential (eV)		9.101	(7.9 / 10.5)
Solute Electron Affinity (eV)		-2.232	(-0.9 / 1.7)*
Predictions for Properties:			
QP Polarizability (Angstroms^3)		21.55	(13.0 / 70.0)
QP log P for hexadecane/gas		5.268	(4.0 / 18.0)
QP log P for octanol/gas		9.545	(8.0 / 35.0)
QP log P for water/gas		5.852	(4.0 / 45.0)
QP log P for octanol/water		0.903	(-2.0 / 6.5)
QP log S for aqueous solubility		-0.272	(-6.5 / 0.5)
QP log S - conformation independent		0.692	(-6.5 / 0.5)
QP log K hsa Serum Protein Binding		-0.129	(-1.5 / 1.5)
QP log BB for brain/blood		1.1	(-3.0 / 1.2)
No. of Primary Metabolites		1	(1.0 / 8.0)
Predicted CNS Activity (-- to ++)		++	
HERG K+ Channel Blockage: log IC50		-4.454	(concern below -5)
Apparent Caco-2 Permeability (nm/sec)		304	(<25 poor. >500 great)
Apparent MDCK Permeability (nm/sec)		167	(<25 poor. >500 great)
QP log Kp for skin permeability		-6.239	(Kp in cm/hr)
Jm. max transdermal transport rate		0.052	(micrograms/cm^2-hr)

Lipinski Rule of 5 Violations	0	(maximum is 4)
Jorgensen Rule of 3 Violations	0	(maximum is 3)
% Human Oral Absorption in GI (+-20%)	77	(<25% is poor)
Qual. Model for Human Oral Absorption	HIGH	(>80% is high)

Table 3. Analysis of single strand break in MCF-7 monolayer cell populations. (a) Cells were treated nicotine and the relative degree of single-strand breakage was determined by alkaline single-cell gel electrophoresis (comet assay) immediately after 24-48 hours.

Nicotine concentration (µM)	**(24 h) comet tail length (mm)**	**(48 h) comet tail length (mm)**
0,01	0,081	0,09
0,05	0,088	0,294
0,1	0,43	1,565*
1	1,69*	1,739*
10	1,77*	1,864*

Discussion

Our previous study showed that nicotine induced a dose-dependent increase in proliferation of breast cancer cells. We demonstrated that nicotine increases the cancer stem cell population in MCF-7 cells [61].

In addition, several lines of evidence indicate that cigarette smoking correlates with increased metastasis of lung, pancreatic, breast and bladder cancers [62, 63, 64]. Although cigarette smoke is a complex mixture of over 4000 compounds, nicotine has been shown the major addictive component of cigarettes [11]. Nicotine, while not carcinogenic by itself, has been shown to induce proliferation [65]; these effects occurred at concentrations normally found in the blood stream of smokers (10^{-8} M to 10^{-7}M). The nicotine applied causes in the cancer pathogenesis by significantly affecting the growth of cancer stem cells, which are the origin of breast cancer [66]. However, nicotine is thought to trigger the proliferation of breast cancer and cancer stem cells, leading to the spread of cancer (mobilization) [67, 68].

Galectin-3 is known to regulate many biological functions such as cell proliferation, tumor progression, and metastasis and apoptosis [23, 24]. The increased expression of galectin-3 in many cancers and its role in promoting tumor growth and metastasis suggests that galectin-3 may be used as an attractive target for cancer therapy [25, 26, 67]. In this study, we have identified galectin-3 and nicotine-binding mechanisms, which describe the linkages between galectin and nicotine, so we can deduce that galectin-3 expression was modulated by nicotine in breast cancer cells such as MCF-7. The most likely binding position between Galectin-3 and Nicotine was determined by hydrogen bonds [22]. In to the active site of galectin, Nicotine was bound with strong hydrogen bond by the residue GLU-72, (1.65 Å) and a salt bridge with the same amino acid residue was established and stability was achieved. At the same time, the $\pi-\pi$interaction with the TRP-69 residue has also increased its stabilization in active site of galectin. We show that the link between nicotine and galectin occurs between the nicotine and the GLU-72 and TRP-69 residues, and as a result, we can say that in breast cancer cells, the expression of the galectin-3 is modulated by nicotine molecule.

In terms of nicotine-induced cell, the most important pathway for MCF-7 cells appears to be replicative [68]. The immediate molecular basis for the senescent behaviour can be attributed to the robust telomerase activity and negligible p21 expression [69, 70]. However, p21 expression is regulated by ATM through Chk2 and p53, and thus implicit in our findings is that reduced Chk2 phosphorylation/activation may play a significant role in the senescence phenotype of MCF-7 mammospheres [71, 72].

Finally, it is to be anticipated that cellular pathways utilized by cancer stem cells to enhance survival will depend on the genetic background of the stem cells [73]. For example, MCF-7 cells are representative of cancer cells that do not readily undergo apoptosis [74], and thus enhanced survival of MCF-7 cancer-

initiating cells is primarily dependent on down-regulation of the senescence pathway [75]. A similar response may be expected in other cancer stem cells, such as those with inactivating mutations in the proline-rich domain of p53, which is required for p53-dependent transactivation of key apoptotic genes but not p21.

On the other hand, avoidance of apoptosis is likely to play a more critical role in the survival response exhibited by cancer stem cells that possess potential functional apoptotic activity [76]. This may explain the differences in DNA repair/cell cycle protein expression and post-translational modification seen between the current study and Bao and colleagues. This study show us smoking and nicotine intake such as through nicotine gums, electronic cigarettes and nicotine pastilles, second handsmoking during the cancer progress, also factor in the DNA damage of the cancer cells and affect the progression and relapse of the disease.

Conclusions

We have carried out a comprehensive comparison of the DNA damage responses of MCF-7 cells. The genome is under numerous different effects, either exogenous or endogenous, that cause DNA damage. All organisms contain DNA repair mechanisms to protect genetic material against damage caused by these environmental factors. In our previous study, we showed that cancer stem cells proliferate and increase the number of cancer stem cells after nicotine administration to breast cancer cells. According to images and analyzes of DNA damage caused by increasing concentrations of nicotine in MCF-7 breast cancer cells, DNA fragments are formed by nicotine application. It is thought that the number of cancer stem cells increases and the cancer cell moves much more actively, which will cause cancer to progress more rapidly in metabolism.

Nicotine-induced up-regulation of galectin-3 might also be involved in the promotion of stem cell property and apoptosis resistance. Because galectin-3 has been shown great interest in cancer research in recent years.In this study, to reveal the relationship the interaction and linkage mechanisms of galectin-3 and nicotine have been determined by using docking calculations for the first time. The other types of cancer cells as well can be examine in the future studies

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Competing interests

The authors declare that they have no competing interests.

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