RESEARCH ARTICLE

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Evaluation of Salivary Carcinogenic *microR-21* and *miR-125a* Expression Associated with Alcohol Consumption and Smoking

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Abstract

Objective: The concept of "lifestyle" encompasses various factors, including nutrition, behavior, stress, physical activity, work habits, smoking, and alcohol consumption. Increasing evidence suggests that environmental and lifestyle factors can influence epigenetic mechanisms, such as DNA methylation, histone acetylation, and microRNA expression. Given that microRNAs (miRNAs) are an emerging focus in cancer research, there is growing interest in understanding how lifestyle choices affect miRNA responses. MiR-21 is well-established as an oncogenic miRNA, while *miR-125a* is reported as a tumor-suppressive miRNA in different cancers. This study aimed to analyze whether cigarette smoking and alcohol consumption are associated with altered levels of these salivary miRNAs in healthy individuals.**Methods:** Saliva supernatant samples from 50 healthy individuals (10% smokers and 34% alcohol drinkers) were analyzed alongside non-smokers and non-alcohol drinkers using real-time polymerase chain reaction (PCR). The expression levels of *miR-21* and *miR-125a* were compared across samples based on demographic characteristics, social status, and smoking and drinking habits. **Result:** The data showed overexpression of salivary *miR-21* in individuals who regularly consumed alcohol and smoked, while *miR-125a* expression was not significantly affected in either group. **Conclusion:** The differential expression of salivary *miR-21* in healthy individuals from a localized population suggests a correlation with common lifestyle risk factors.

Keywords: Supernatant saliva- *miR-21- miR-125a*- alcohol- smoking

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Introduction

Lifestyle is the way of living of a society or individual [1]. Epigenetics of an individual could be influenced by different factors such as environmental, genetics, lifestyle, psychological state and many other factors. It has been reported that those factors could impact the epigenetic process, for instance DNA methylation and miRNA expression. Not only could genome function be influenced by epigenetic mechanisms, but further stable propagation of gene activity could be impacted as well [2]. Moreover, P53 gene has been stated to be hypomethylated in blood cells of smoker lung cancer patients [3]. Starkman and colleagues have stated that interested studies in the influence of alcohol on epigenetic expression have been grown [4].

Tobacco and alcohol have been considered to be major risk factors for different types of cancer, especially head and neck cancer, in addition to human papillomavirus (HPV) infection which is considered to be an independent risk factor [5]. On the other hand, alcohol consumption has been suggested to be associated with LINE-1 hypomethylation in gastric cancer patients [6]. Many different human diseases have also been demonstrated to be associated with epigenetic mark alterations [7].

Alcohol can be metabolized by an oxidative pathway that occurs in the liver or non-oxidative pathway that takes place in the other tissues in the body. The oxidative pathway of ethanol metabolism is considered the major pathway in the liver which results in reduced of NAD+ to NADH and produces acetaldehyde, a highly reactive and toxic molecule by cytosolic alcohol dehydrogenase (ADH). Any change in ethanol metabolism could influence the ratio of NADH to NAD+. Hepatic NADH/ NAD+ ratio in the cytoplasm and mitochondria of hepatocytes is signicantly increased in response to the alcohol metabolism with other changes in different molecules in the cell such as upregulation in the ratio of lactate/ pyruvate in the cytoplasm and ratio of γ-hydroxybutyrate/ acetoacetate in the mitochondria [8]. Krebs and Veech have stated that significant alteration in different reversible metabolic pathways of the hepatocytes could be revealed by alteration of a redox state [9]. Consequently, availability of oxidizable NADH to the electron transport

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chain will be severely increased by alcohol oxidation. Cellular reactions are influenced by NAD+. For instance, energy metabolism, histone deacetylation, and cell death could be revealed by enzymes that use NAD+ and NADH. Several studies have reported that the cytosolic free NAD+ to NADH ratio is 700:1, while it has been stated to be 7–8 to 1 in the mitochondria under normal conditions [10, 11]. Several metabolic reactions in the liver such as lipid, carbohydrate, protein, lactate, and uric acid could be changed in response to several hepatic reversible reactions that result from an increase of NADH/NAD+ ratios in both cytosol and mitochondria of the liver cells. As a result, modulation of gene expression could be revealed from any of these metabolic alterations.

MiRNA is a small non-coding RNA molecule containing about 22 nucleotides. miRNA targets mRNA via base-pairing of complementary sequence molecules. As a result, these mRNA molecules are silenced, which could inhibit the expression of the tumour suppressor gene or proto-oncogene [12]. It has been reported that human tumours could be caused by miRNAs [13]. However, other studies have stated that gastric cancer could be correlated with aberrant expression of miRNAs. [14, 15]. Moreover, several studies have reported that *miR-21* was upregulated in plasma samples of patients with large B-cell lymphoma, gastric cancer and pancreatic ductal adenocarcinoma [16-18]. In addition, *miR-21* has been stated to be upregulated in saliva samples of patients with head and neck cancer and pancreatic cancer [19, 20]. Meanwhile, *miR-125a* was reported to be up- or down-regulated depending on the type of cancer. For instance, in non-small cell lung cancer, it was down regulated but up regulated in invasive lung cancer cells [21]. Based on a recent study, correlation between deregulation of miRNAs expression and lifestyle manner such as smoking, alcohol consumption, stress, infection and radiation has been concluded [22]. These evidences of association between miRNAs expression and lifestyle factors have resulted in a clear understanding of molecular mechanisms and cancer disease which has helped to create a developed preventative method.

The aim of this study was to investigate the influence of lifestyle and social habits especially alcohol consumption and smoking on the *miR-21* and *miR-125a* expressions, which were diagnosed as significant dysregulated miRNAs in oral cancer patients in our previous study.

Materials and Methods

Subjects

Samples was collected from different ages and genders of 50 healthy individuals who work or study at Griffith University in Gold Cost / Australia. 50 healthy adults also comprised 25 males and 25 females with a mean age of 56.6 ± 8.2 years (range 38-75 years), Consent was obtained from all participants. All participants were recruited from February 1, 2022, to July 30, 2022.

Saliva Collection

A whole saliva sample was collected without stimulation from healthy people as previously described [23, 24]. Prior, participants were prevented from eating, drinking, and using oral hygiene prior to sample collection. To keep samples clear and uncontaminated, participants were asked to wash their mouths before collecting the sample. A round 8 ml of saliva sample was spat into sterile free falcon tube size 50 ml and kept on ice.

Total Salivary RNA extraction

Total RNAs were isolated from 440 µl saliva sample using RNeasy kit (Qiagen, Valencia, CA, USA). The purification of RNAs was 1.5- 2.0 while the quantification was 500-900 ng/µ using NanoDrop spectrophotometer (ND2000; Wilmington, DE, USA).

Reverse transcription

Total RNA (10-50 ng) was reverse transcribed to cDNA via the miRNA reverse transcription kit (Origene, HP). The10-µl RT reaction mixture contained 1 to 2µg of total RNA, 1 µl of poly A tailing buffer , 1 µl of mM ATP, 1 µl of Poly (A) polymerase and was adjusted to 10 µl by nuclease-free water. The polyadenylation reaction continued at 37˚C for two hours. Subsequently, 1µl oligo dT primer was added to the reaction and then the tube was incubated at 70˚C for five minutes and on ice for two minutes. For final cDNA synthesis, 4 µl of 5X MMLV buffer was added, then incubated at 42˚C for 1 hour, 95˚ for 5 minutes and then placed on ice. The reaction mixture was diluted with 200 µl molecular water and stored at -20˚C prior to real-time RT-qPCR assay.

Real-time Quantitative Polymerase Chain Reaction

To validate salivary *miR-21* and *miR-125a* expressions of healthy individuals in different conditions, RT-qPCR was performed. RT-qPCR was performed with SYBR green assay (Bio-RAD, HP 172-5017). RT-qPCR detection for the miRNAs was performed using specific forward primers and a universal reverse primer according to the manufacturer's protocol (Origene). The primers used in this study are listed in Table 1. PCR amplicons were detected by the level of fluorescence emitted by SYBR green. Each reaction was performed in triplicate and cycling conditions were as follows: 95˚C for 30 seconds then 95˚C for 15 seconds, 55˚C for 10 seconds, and 72˚C for 30 seconds repeated 42 times. At the end of the PCR cycles, melt curve analysis was performed to validate generation of the expected PCR product. The setting of the melting curve was 60.0 to 95.0˚C at increments of 0.5˚C for 0.05 minutes. All Ct values were < 35. A negative

Table 1. Primers and Probes used for miRNA-specific RT-qPCR

Name	Forward primer	Reverse primer
miRNA-21	GCTTATCAGACTGATGTTG	GAACATGTCTGCGTATCTC
miRNA-125a	CCTGAGACCCTTTAACC	GAACATGTCTGCGTATCTC
miRNA-16	AGCAGCACGTAAATATTGG	GAACATGTCTGCGTATCTC

template control (NTC) was used as a negative control. The RT-qPCR reactions were carried out in a Quant Studio 6 flex (Applied Biosystems); Δct was calculated and normalized against housekeeping miRNA-16.

Statistical analyses

Samples were grouped based on demographic characteristics and lifestyle conditions. The expression level of each *miR-21* and *miR-125a* was quantified by its normalized threshold cycle number ΔCt, where ΔCt = [Ct (Target miRNA)]- [Ct (miR-16)], and the relative expression level was calculated as $2^{-(\Delta Ct)}$ which is commonly used in genome-wide profiling studies of miRNAs. Data analyses were performed using Prism software, version 6. The p-value less than 0.05 was considered significant. Data expressed as mean ±SEM.

Results

In total, 50 saliva samples were collected from healthy individuals. Demographic properties were obtained from individuals. The mean age of the individuals who consumed alcohol was 60.5 ± 3.96 years, while the mean age of non-alcohol consuming individuals was 57.3 ± 2.124. The mean age of individuals who smoked was 62.4 ± 4.578 years and 68 ± 2.017 for individuals who were non-smokers. The percentage of males in the smoker group and non-smoker group was (10, 52 %) respectively, with non-significant differences. The alcohol consuming group consisted of 11 males and 6 females and the non-alcohol consuming group consisted of 20 males and 13 females which represented (22, 12, 40, 26 %) respectively.

Salivary *miR-21* was selected for analysis of the influence of lifestyle (alcohol and smoking) exposure on the miRNA expression, based on the results from our previous study identifying this salivary miRNA (*miR-21*) as differentially expressed between OSCC patients and normal head and neck epithelia.

The expression levels of *miR-21* significantly differed in saliva samples of healthy people with different habits. Upregulation of *miR-21* (p=0.04) was found in alcohol consuming individuals compared with non-alcoholic people (Figure 1). Interestingly, there was statistically significant difference between the two groups in terms of smoker and non-smoker (P <0.05) (Figure 2). In addition, drinking and smoking individuals revealed significant difference in *miR-21* expression when compared with non-drinking and smoking group (P=0.009).

Unlike *miR-21*, *miR-125a* was found to be non-significantly influenced by smoking and alcohol consumption where p>0.05.

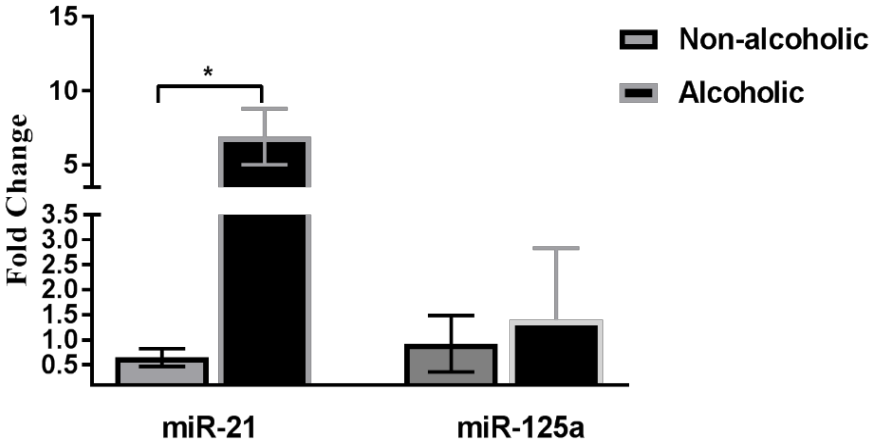

Figure 1. *miR-21* and *miR125a* Expression of Samples According to the Individual's Drinking Habit, * p<0.05

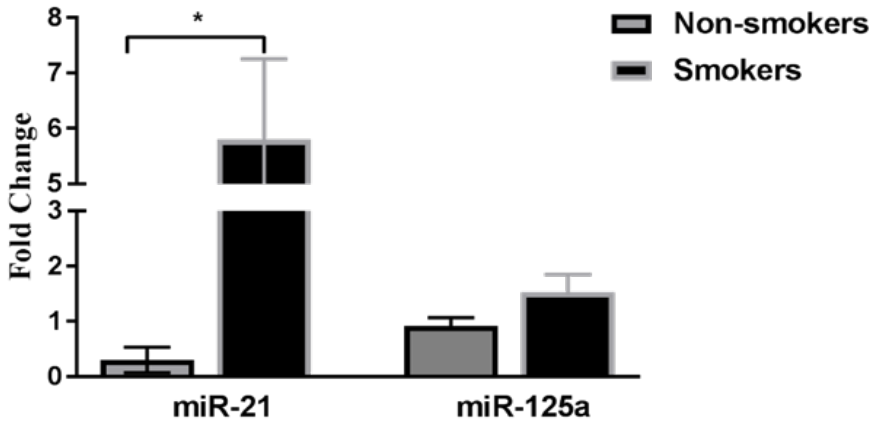

Figure 2. *miR-21* and *miR125a* Expression of Samples According to the Individual's Smoking Habit,*p< 0.05

Discussion

This study aimed to evaluate the correlation between panel of miRNAs (*miR-21* and *miR-125a*) expression and lifestyle condition particularly alcohol consumption and smoking. The expression of *miR-21* and *miR-125a*, in normal saliva samples was investigated as a first step of the analysis, and then we focused on the correlation between *miR-21* expression in various demographic characteristics of the subjects and lifestyle behaviour such as alcohol and smoking. The results have revealed that *miR-21* was significantly increased with alcohol consumption and smoking while non-significant alteration was shown in the expression level of *miR-125a*.

Oral tissue which has direct contact and involved with saliva fluid, can be collected by non-invasive methods. As such saliva samples could be a good source to search for potential biomarkers. Saliva contains different enzymes some of them which degrade RNA, such as ribonucleases, but many types of RNA have been revealed in saliva samples by different studies, the results pointed to the potential presence of some mechanisms that protect salivary RNAs from degradation [25, 26]. Saliva fluid has been described to be directly contact oral tissue and could be easily obtained from individuals by non-invasive method. Several studies have stated that salivary RNAs can be protected from distraction by different processes.

It has been reported that miRNAs have a great influence on the carcinogenesis by targeting the tumor suppressor gene and oncogenic genes [27]. Furthermore, *miR-21* has been reported to be upregulated in different types of cancer, for instance pancreas, breast, lung, kidney, bladder and prostate [28]. Inhibition of expressions for several genes could be revealed by *miR-21* which is known as oncomiRNA, and associated with the pathogenesis of several types of human cancer [29, 30]. This study has described various factors that influence the epigenetics of humans (Figure 1 and 2). It revealed that expression level of *miR-21* was increased and positively correlated with the amount of alcohol consumed and smoking in healthy subjects. An imbalance in the many gene regulation processes could be the result of any kinds of epigenetic factors influencing genes or gene expression networks during life stages. These results concur with previous studies pointing to the influence of alcohol on the different epigenetic factors. For instance, up-regulation of *miR-21* expression level was reported in the tissue samples of the smoker and low social status gastric cancer patients [31]. Moreover, hepatic and neuronal tissue were stated to be influenced by the epigenetic of alcohol consumption [32]. It has been stated that methanol could lead to site-selective methylation, acetylation and phosphorylation of histones and DNA hypomethylation and result in reduction of S-adenosyl methionine (SAM) in tissue [32, 33]. Kruman and Fowler have reported that alcohol's interference with one carbon metabolism (OCM) result in reduced availability of methyl groups and increased aberrant methylation of DNA and gene expression in alcoholic individuals [34].

Another study has also revealed that global DNA hydroxyl methylation in the liver of young mice was reduced by chronic alcohol exposure [35]. Furthermore, epigenetic alteration results from alcohol consumption have led to gastrointestinal- hepatic levels like steatosis, carcinogenesis and endotexemia [36]. Neuro-adaptations like tolerance and dependence induction have been stated to be associated with alcoholic epigenetic expression [37]. Moreover, it has been stated that reduced expression of *GAD1,HDAC2,HDAC11* correlated with decreased histone acetylation at *GAD1, HDAC2* promoters and enhanced expression of *MT1, MT2, EGR1*, and associated with upregulation of H3K4me3 at MT2 promoter and a decreased level of H3K27me3 at the MT1 promoter in the cerebral cortex [37]. Other side effects of alcohol have been shown such as an increase in the anxiolytic and motor effects and decrease in alcohol consumption especially in the male progeny of mice [38]. In addition, the same study has found upregulated BDNF expression with DNA hypomethylation in the both genders of mice offspring [38]. Therefore, epigenetic modification could be directly influenced by alcohol or indirect by these epigenetic expressions that result from alcohol consumption.

Based on our results, there are significant differences in *miR-21* expression patterns in healthy individuals depending on lifestyle behaviour. This finding can demonstrate better understanding of the role of alcohol consumption and smoking on epigenetic expression to contribute in carcinogenesis. In addition, it suggests examining salivary *miR-21* as a potential therapeutic target for diseases and as a new biomarker and stress free sample to evaluate, diagnose and prognosis of human health conditions and diseases.

In conclusion, the relation between imRNAs expression and lifestyle factors such as alcohol consumption and smoking has been investigated by several studies over the past few years. This study has presented the influence of lifestyle on *miR-21* expression.

These results revealed that *miR-21*expression is associated with risk factor exposure. Moreover, high expression of *miR-21* is associated with alcohol consumption and smoking in healthy people. Consequently, *miR-21* can be considered a significant marker for health indication. Additionally, this study suggests that *miR-21* could be used as a significant indicator for disease diagnosis.

Author Contribution Statement

Rushdi Fadhil participated in devising the search strategy, data extraction, quality assessment, and analytical strategy for this study. All authors participated in data interpretation, critically revised the manuscript, and have read and approved the final manuscript.

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Ethical Declaration

This study was approved by the human research ethics committees of Griffith University in Australia (GU Ref No: 2015/766) and Queensland Government (HREC/15/QGC/223).Authors Contribution: Lorem ipsum dolor sit amet, consectetur adipiscing elit, sed do eiusmod tempor incididunt ut labore et dolore magna aliqua

Conflict of interest

No conflict of interest.

References

1. Thesaurus of psychological index terms. 11th ed, ed. L.G. Tuleya. 2007, Washington, DC, US: American Psychological Association. xxvi, 463.
2. Probst AV, Dunleavy E, Almouzni G. Epigenetic inheritance during the cell cycle. Nat Rev Mol Cell Biol. 2009;10(3):192-206. https://doi.org/10.1038/nrm2640.
3. Woodson K, Mason J, Choi SW, Hartman T, Tangrea J, Virtamo J, et al. Hypomethylation of p53 in peripheral blood DNA is associated with the development of lung cancer. Cancer Epidemiol Biomarkers Prev. 2001;10(1):69-74.
4. Starkman BG, Sakharkar AJ, Pandey SC. Epigenetics-beyond the genome in alcoholism. Alcohol Res. 2012;34(3):293-305.
5. Ragin CC, Modugno F, Gollin SM. The epidemiology and risk factors of head and neck cancer: A focus on human papillomavirus. J Dent Res. 2007;86(2):104-14. https://doi.org/10.1177/154405910708600202.
6. Hou L, Wang H, Sartori S, Gawron A, Lissowska J, Bollati V, et al. Blood leukocyte DNA hypomethylation and gastric cancer risk in a high-risk polish population. Int J Cancer. 2010;127(8):1866-74. https://doi.org/10.1002/ijc.25190.
7. Santos-Rebouças CB, Pimentel MM. Implication of abnormal epigenetic patterns for human diseases. Eur J Hum Genet. 2007;15(1):10-7. https://doi.org/10.1038/sj.ejhg.5201727.
8. Cunningham CC, Bailey SM. Ethanol consumption and liver mitochondria function. Biol Signals Recept. 2001;10(3-4):271-82. https://doi.org/10.1159/000046892.
9. Krebs HA, Veech RL. Regulation of the redox state of the pyridine nucleotides in rat liver. InPyridine Nucleotide-Dependent Dehydrogenases: Proceedings of an Advanced Study Institute held at the University of Konstanz, Germany, September 15–20, 1969 1970 (pp. 413-438). Berlin, Heidelberg: Springer Berlin Heidelberg.p.413-438.
10. Stubbs M, Veech RL, Krebs HA. Control of the redox state of the nicotinamide-adenine dinucleotide couple in rat liver cytoplasm. Biochem J. 1972;126(1):59-65. https://doi.org/10.1042/bj1260059.
11. Veech RL, Eggleston LV, Krebs HA. The redox state of free nicotinamide-adenine dinucleotide phosphate in the cytoplasm of rat liver. Biochem J. 1969;115(4):609-19. https://doi.org/10.1042/bj1150609a.
12. Zhang B, Wang Q, Pan X. Micrornas and their regulatory roles in animals and plants. J Cell Physiol. 2007;210(2):279-89. https://doi.org/10.1002/jcp.20869.
13. Croce CM. Causes and consequences of microrna dysregulation in cancer. Nat Rev Genet. 2009;10(10):704-14. https://doi.org/10.1038/nrg2634.
14. Pan HW, Li SC, Tsai KW. Microrna dysregulation in gastric cancer. Curr Pharm Des. 2013;19(7):1273-84. https://doi.org/10.2174/138161213804805621.
15. Saito Y, Suzuki H, Hibi T. The role of micrornas in gastrointestinal cancers. J Gastroenterol. 2009;44 Suppl 19:18-22. https://doi.org/10.1007/s00535-008-2285-3.
16. Tsujiura M, Ichikawa D, Komatsu S, Shiozaki A, Takeshita H, Kosuga T, et al. Circulating micrornas in plasma of patients with gastric cancers. Br J Cancer. 2010;102(7):1174-9. https://doi.org/10.1038/sj.bjc.6605608.
17. Wang J, Chen J, Chang P, LeBlanc A, Li D, Abbruzzesse JL, et al. Micrornas in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. Cancer Prev Res (Phila). 2009;2(9):807-13. https://doi.org/10.1158/1940-6207.Capr-09-0094.
18. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated micrornas in serum of patients with diffuse large b-cell lymphoma. Br J Haematol. 2008;141(5):672-5. https://doi.org/10.1111/j.1365-2141.2008.07077.x.
19. Xie Z, Chen G, Zhang X, Li D, Huang J, Yang C, et al. Salivary micrornas as promising biomarkers for detection of esophageal cancer. PLoS One. 2013;8(4):e57502. https://doi.org/10.1371/journal.pone.0057502.
20. Humeau M, Vignolle-Vidoni A, Sicard F, Martins F, Bournet B, Buscail L, et al. Salivary microrna in pancreatic cancer patients. PLoS One. 2015;10(6):e0130996. https://doi.org/10.1371/journal.pone.0130996.
21. Zhao YM, Zhou JM, Wang LR, He HW, Wang XL, Tao ZH, et al. Hiwi is associated with prognosis in patients with hepatocellular carcinoma after curative resection. Cancer. 2012;118(10):2708-17. https://doi.org/10.1002/cncr.26524.
22. Wang J, Cui Q. Specific roles of micrornas in their interactions with environmental factors. J Nucleic Acids. 2012;2012:978384. https://doi.org/10.1155/2012/978384.
23. Gorugantula LM, Rees T, Plemons J, Chen HS, Cheng YS. Salivary basic fibroblast growth factor in patients with oral squamous cell carcinoma or oral lichen planus. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012;114(2):215-22. https://doi.org/10.1016/j.oooo.2012.03.013.
24. Cheng YS, Rees T, Jordan L, Oxford L, O'Brien J, Chen HS, et al. Salivary endothelin-1 potential for detecting oral cancer in patients with oral lichen planus or oral cancer in remission. Oral Oncol. 2011;47(12):1122-6. https://doi.org/10.1016/j.oraloncology.2011.07.032.
25. Nagler RM. Saliva as a tool for oral cancer diagnosis and prognosis. Oral Oncol. 2009;45(12):1006-10. https://doi.org/10.1016/j.oraloncology.2009.07.005.
26. Park NJ, Yu T, Nabili V, Brinkman BM, Henry S, Wang J, et al. Rnaprotect saliva: An optimal room- temperature stabilization reagent for the salivary transcriptome. Clin Chem. 2006;52(12):2303-4. https://doi.org/10.1373/clinchem.2006.075598.
27. Iorio MV, Croce CM. Microrna involvement in human cancer. Carcinogenesis. 2012;33(6):1126-33. https://doi.org/10.1093/carcin/bgs140.
28. Lodygin D, Tarasov V, Epanchintsev A, Berking C, Knyazeva T, Körner H, et al. Inactivation of mir-34a by aberrant cpg methylation in multiple types of cancer. Cell Cycle. 2008;7(16):2591-600. https://doi.org/10.4161/cc.7.16.6533.
29. Buscaglia LE, Li Y. Apoptosis and the target genes of microrna-21. Chin J Cancer. 2011;30(6):371-80. https://doi.org/10.5732/cjc.011.10132.
30. Selcuklu SD, Donoghue MT, Spillane C. Mir-21 as a key regulator of oncogenic processes. Biochem Soc Trans. 2009;37(Pt 4):918-25. https://doi.org/10.1042/bst0370918.
31. Stánitz E, Juhász K, Tóth C, Gombos K, Natali PG, Ember I. Evaluation of microrna expression pattern of gastric adenocarcinoma associated with socioeconomic, environmental and lifestyle factors in northwestern hungary. Anticancer Res. 2013;33(8):3195-200.
32. Shukla SD, Velazquez J, French SW, Lu SC, Ticku MK, Zakhari S. Emerging role of epigenetics in the actions of alcohol. Alcohol Clin Exp Res. 2008;32(9):1525-34. https://

doi.org/10.1111/j.1530-0277.2008.00729.x.
33. Zakhari S. Alcohol metabolism and epigenetics changes. Alcohol Res. 2013;35(1):6-16.
34. Kruman, II, Fowler AK. Impaired one carbon metabolism and DNA methylation in alcohol toxicity. J Neurochem. 2014;129(5):770-80. https://doi.org/10.1111/jnc.12677.
35. Tammen SA, Dolnikowski GG, Ausman LM, Liu Z, Sauer J, Friso S, et al. Aging and alcohol interact to alter hepatic DNA hydroxymethylation. Alcohol Clin Exp Res. 2014;38(8):2178-85. https://doi.org/10.1111/acer.12477.
36. Shukla SD, Lim RW. Epigenetic effects of ethanol on the liver and gastrointestinal system. Alcohol Res. 2013;35(1):47-55.
37. Finegersh A, Homanics GE. Acute ethanol alters multiple histone modifications at model gene promoters in the cerebral cortex. Alcohol Clin Exp Res. 2014;38(7):1865-73. https://doi.org/10.1111/acer.12465.
38. Finegersh A, Homanics GE. Paternal alcohol exposure reduces alcohol drinking and increases behavioral sensitivity to alcohol selectively in male offspring. PLoS One. 2014;9(6):e99078. https://doi.org/10.1371/journal.pone.0099078.