Pak. J. Chest Med. 2021;27(04)

Original Article

journal homepage: https:/www.pjcm.net/

Pakistan Journal of Chest Medicine

Official journal of Pakistan Chest Society

Salivary Microbiome Dysbiosis is associated with Non-Smoking Male Lung Cancer and correlated with Immunocytochemistry Markers

Atiq Ur Rehman[1], Farzeen Khan[2], Karishma Ali[3], Shazia Naz[4], Saman Hussain[5], Shaista Alam[6] ✉

[1]Department of Pulmonology, Combined Military Hospital, Peshawar - Pakistan [2]Department of Community and Preventive Dentistry, Peshawar Dental College, Peshawar, Riphah International University, Islamabad - Pakistan [3]Department of Pathology, Peshawar Medical and Dental College, Riphah International University, Islamabad - Pakistan [4]Department of Pathology, Kabir Medical College, Gandara University, Peshawar - Pakistan [5]Department of Pathology, Northwest School of Medicine, Peshawar - Pakistan [6]Department of Pathology, Pak International Medical College, Peshawar - Pakistan

Corresponding author:
Shaista Alam
Department of Pathology,
Pak International Medical College,
Peshawar - Pakistan
Email: shaistalam123@gmail.com

Article History:

Received:	Sep 20, 2021
Revised:	Nov 10, 2021
Accepted:	Nov 22, 2021
Available Online:	Dec 02, 2021

Author Contributions:
AUR conceived idea, FK KA drafted the study, KA SH collected data, SA AUR did statistical analysis and interpretation data, SA SN did critical reviewed manuscript. All approved final version to be published.

Declaration of conflicting interests:
All authors declare that they have no conflict of interest.

How to cite this article:
Rehman AU, Khan F, Ali K, Naz S, Hussain S, Alam S. Salivary Microbiome Disbiosis is associated with Non-Smoking male lung cancer and correlated with immunocytochemistry marker. Pak J Chest Med. 2021;27(04): 316-322.

A B S T R A C T

Background: There may be a link between oral micro-biota and a higher risk of cancer of lung, according to many researches. However, there has not been much research done on the possible link between male nonsmokers' salivary microbiota and lung cancer. Furthermore, there is a paucity of research on the connection between immunohistochemical markers and the salivary microbiota.

Objective: To find an association between lung cancer and salivary microbiome in non-smoker males as well as a relationship between immunohistochemical markers and salivary microbiota.

Methodology: This study was conducted at the Pulmonology Department, Combined Miitary Hospital, Peshawar Pakistan for duration of six months from January 2021 to June 2021. A 16S rRNA gene amplicon sequencing was used to evaluate the salivary flora of 120 non-smoker male lung cancer patients and 80 healthy persons. Furthermore, we found a correlation coefficient among the salivary micro-biota and three immunohistochemistry markers likeTTF-1, Napsin A, and CK7 using Spearman's rank.

Results: The lung cancer patients exhibited considerably lower microbial richness and diversity compared to the control group ($p < 0.0001$). The comparison of the similarity analysis between the control group and lung cancer patients showed clear changes in the composition of their flora. ($p=0.001$). Notably, non-smoking lung cancer male patients had comparatively greater levels of the genera of bacterial "Sphingomonas" ($P < 0.05$) and "Blastomonas" ($p = 0.0001$), while the control group had higher levels of the genus "Acinetobacter" ($p = 0.001$) and "Streptococcus" ($P = 0.01$).

Conclusion: The study demonstrated that salivary flora might be a useful resource for identifying non-invasive lung cancer biomarkers. The study additionally identified distinct salivary microbiome patterns in male lung cancer patients who do not smoke. It proposed potential connections between the immune-cyto-chemistry markers and salivary microbiota used in clinical diagnostics.

Keywords: Lung Cancer; Salivary Microbiome; Dysbiosis; Biomarker

Introduction

Globally, the majority of the cancer-related deaths occurs due to lung cancer, responsible for about 300,000 fatalities.[1] While cancer of lung is commonly associated with smoking, according to worldwide data, tobacco smoking cannot be linked to 15% of instances of lung cancer in male and 53% in women.[2] People who do not smoke who have been confirmed to have lung cancer are frequently seen as a different group.[3] In comparison with non-small cell lung cancer (NSCLC) in smokers, non-small cell lung cancer (NSCLC) in non-smokers is clinically described by an high frequency in men and a greater prevalence of adenocarcinoma.[4]

Oral disorders, systemic diseases, and potentially systemic tumors have been the focus of recent advances in the study of the oral flora.[5] It has been suggested that the host's immune-inflammatory response and the oral microbiome are in a state of dynamic balance.[6] The respiratory tract of human serves as the principal and reliable entry route for many types of bacteria, mostly airborne but also those spread by saliva. Oral bacterial communities most likely seed the lungs with oral bacteria and can arise from the environment through inhalation or from the oropharynx and tracheobronchial.[7]

This association reaches into the systemic circulation and is essential to understanding illnesses and creating non-invasive treatment methods.[8] But smoking cigarettes may upset the oral microbiome's delicate equilibrium, which could change functioning pathways and have an impact on illnesses linked to smoking.[9] Prior research on the oral microbiome in lung cancer did not adequately account for confounding variables like smoking in the detection of bacterial biomarkers, nor did it routinely analyse clinical samples depending on smoking status.[10] Furthermore, there are not many studies describing the oral microbiota of male nonsmokers who have lung cancer. It is thought that genetic predisposition and environmental variables increase non-smokers' chance of developing lung cancer.[11]

The primary objective of the current study is to present diverse experimental and methodological perspectives to enhance our understanding of the impact of changes in the oral flora on onset of disease or progression through different stages.

In this pilot investigation, we used 16S rRNA gene sequencing to thoroughly compare the salivary flora of male nonsmoking lung cancer patients and normal control people. The purpose of our investigation was to find out dysbiosis with respect to of structure, composition, and function within the salivary microbiome and to clarify the differences in the balance of the microbiome in male lung cancer patients who do not smoke.

Furthermore, we identified a link between the distinct microorganisms associated with lung cancer and the salivary microbiota, as well as immunocytochemical markers such as napsin A, thyroid transcription factor (TTF-1),and cytokeratin (CK7).[12] To improve evaluations for people and communities at risk, community-level salivary microbial indicators might be enhanced by evaluating the effect of oral flora composition. This is especially relevant in developing non-invasive diagnostic procedures.

Objective

To find an association between lung cancer and salivary microbiome in non-smoker males as well as a relationship between immunohistochemical markers and salivary microbiota.

Methodology

This study was conducted at the Department of Pulmonology, Combined Miitary Hospital, Peshawar – Pakistan for duration of six months from January 2021 to June 2021. PRoBE design was the foundation upon which the study was built.[13] Prior to determining the outcome, a crucial component of the PRoBE design is the prospective collection of clinical samples from a study appropriate to the therapeutic application. In order to avoid potential biases that are frequently observed during the discovery stage, PRoBE concepts must be incorporated early in the development of biomarker tests proposed for approval from FDA and clinical usage.[13]

Male smokers who had recently been diagnosed with lung cancer but had not yet received treatment, as well as healthy controls were gathered from Combined Miitary Hospital, Peshawar. The patients were only eligible if they were male with non-small-cell lung cancer diagnosis. The criteria for exclusion were a recorded history of alcohol or tobacco use, signs of metastatic, prior use of radiation or chemotherapy previous to saliva collection, and a diagnosis of other cancers during the five years before to collection of saliva. To match the research group, healthy controls were chosen based on factors such as age, gender, and alcohol and tobacco use. Written informed consents and data sheets for questionnaire were collected from all individuals who willingly participated to serve as sample donors. This study's experimental protocols and methodologies were all carried out in compliance with the relevant guidelines and standard operating procedures.

Before the samples were collected, the participants were asked to refrain from eating, drinking, and using mouthwash for at least two hours. Whole saliva that had not been stimulated was routinely obtained, handled, and preserved in accordance with previously established guidelines.[14] After that, the centrifugation of the whole saliva samples was done for 15 minutes at 4°C at 2,600 × g. then the pellet was frozen promptly and then kept at -

Table 1. Characteristics of the study cohorts

Variables	Lung cancer patients n= 120	Healthy patients n=80
Age (years) (mean ± SD)	60.51 ± 6.35	58.03 ± 6.65
Living environment		
City	80	50
Village	40	30
Smoking status		
Smokers	0	15
Non-smokers	120	65
Drinking status		
Non-drinker	120	65
Rare drinker (once/2 weeks)	0	6
Medium drinker (once/week)	0	9
Diet		
Vegetarians	20	10
Non- vegetarians	100	70

80°C until the test was conducted.

The 5-µm thick, formalin-fixed, paraffin-embedded tissue sections of each case were used for the immunohistochemical investigation of the resected tumours, and all slides were stained using a Bench Mark Autostainer equipped with an EnVision detection system.[15] Following the manufacturer's instructions, the extraction of DNA was done through UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, California, USA).

STAMP was used for graphical representations of the data.[16] Without a specific indication, the presentation of data was done in the form of Mean and ± SD. The unpaired-sample t-tests and Mann-Whitney test and were used to evaluate continuous variables comparing independent samples. Results with P-values less than 0.05 were deemed statistically significant. Using Spearman's rank correlation, the statistical dependency among continuous variables was assessed. Specifically, the ANOSIM test was utilized to assess differences in microbial community composition. All analyses were conducted using the SPSS statistical package, version 24.0 (SPSS).

Results

For this study, a total of 200 participants were included. There were 120 males patients with non smoking lung cancer while 80 were healthy controls. There was an age match between the group of lung cancer and the healthy control group (60.51 ± 6.35 vs. 58.03 ± 6.65, correspondingly, P = 0.104). Table 1 displays the clinical characteristics and demographics of all participants (80 healthy controls and 120 cancer patients).

Male lung cancer patients who do not smoke had a less diverse salivary microbiome than healthy controls. The structure of the salivary flora was shown to change significantly between the control and lung cancer groups, and there is a significant difference between the two groups.

We assessed a series of Immunohistochemical markers for cytokeratin (CK7), napsin A, and thyroid transcription factor (TTF-1), and we compared the outcomes with the matching salivary microbiota. Between the three immunohistochemical markers and the two bacterial biomarkers, there were significant associations ($p< 0.05$).

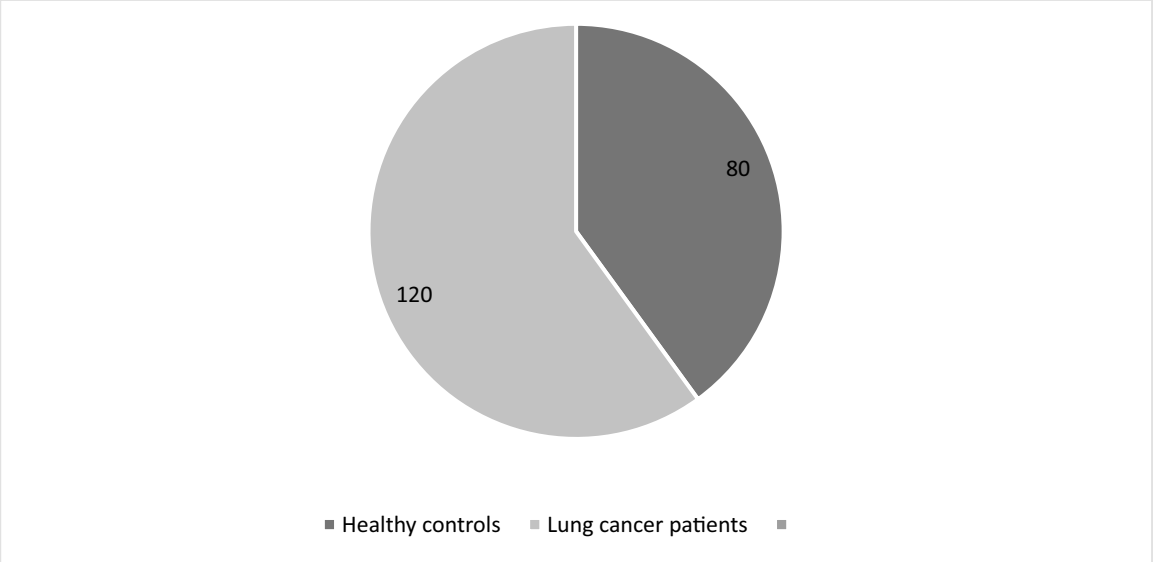

Figure 1. Comparison of healthy controls with male's non-smoker lung cancer patients

CK7 and Enterobacteriaceae had a strong positive connection ($p < 0.05$). Moreover, there is a positive correlation between Napsin A and the Blastomonas genera ($p < 0.05$). Between TTF-1 and Enterobacteriaceae, there is a statistically significant positive connection ($p < 0.05$). The study of 16S rRNA genes may reveal the existence of bacteria in a sample. It has been shown that non-smoking male lung cancer patients showed an enrichment of functional pathways associated with cancer, apoptosis, the p53 signaling pathway, and TB.

Discussion

According to our research, males who do not smoke and their salivary microbiome had a significant association with lung cancer. The study employed a high throughput approach to analyze the bacterial communities present in the samples of saliva of male patients with lung cancer who did not smoke and their matched controls. The salivary flora of male smokers with lung cancer was shown to have a dysbiosis and decreased microbial richness and diversity.

The structure of the flora was also found to be different from that of the healthy controls. Additionally, there is data that suggests a correlation between the variance in the salivary microbiome and specific clinical immunohistochemical indicators. According to recent study, high throughput examinations of the salivary microbiome in males who do not smoke may indicate bacterial dysbiosis associated with lung cancer. The oral environment, including smoking, age,[17] ethnic origins, and food intake habits all affect the salivary bacteria.[18]

Numerous investigations have shown how smoking affects mouth microorganisms.[9] Lung cancer is often associated with smoking; yet, research on lung cancer in nonsmokers has not received much attention.[19] Lung cancer may not be the same illness in smokers and non-smokers, since significant gender differences as well as genomic and clinical-pathological variances are currently found.[2] There is a suggestion that gender-specific hormones may have a possible influence on the lung cancer development. This suggestion is on the basis of observation that non-smoking males had a larger percentage of lung cancer cases compared to non-smoking females.[20]

Regarding the higher risk of carcinogenesis in male patients who do not smoke, it is currently unknown if risk factors other than smoking contribute more.[21] One of our study's main strengths is the distinct group of male non-smoking lung cancer patients.[22] This group allows for a more appropriate examination of the relationship between oral flora and lung cancer because smoking was taken out of the equation.

The main findings of the current study show that the genera Sphingomonas and Blastomonas exhibited a significant increase in the oral flora of lung cancer patients, whereas Acinetobacter and Streptococcus were found to be higher in the control group. Recent studies have also noted the presence of Streptococcus in COPD [23] and cystic fibrosis (CF) lung flora.[24] Under some circumstances, Streptococcus has the capacity to invade the host fibronectin. This, in turn, could trigger a cytokine response, promoting inflammation and potentially leading to carcinogenesis.[25] Blastomonas and Sphingomonas have been shown to be present in large quantities in individuals with pneumonia.[26] These genera also have a significant impact on systemic immunological responses,

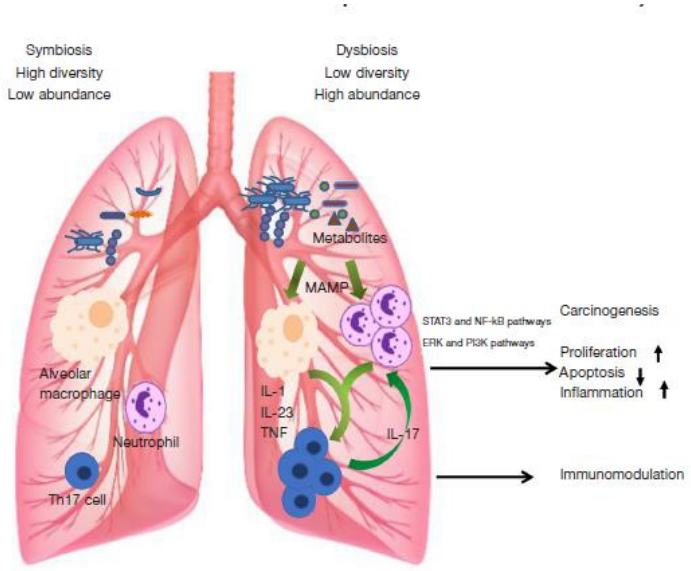

Figure 2. Potential pathways balancing lung floraand lung cancer. By reducing lung inflammation and the recruitment of dendritic cells, the commensal microbiome supports immunological tolerance. Responding to microbial colonisation, macrophages and T lymphocytes stop the overabundance of pathogens or metabolites (left panel). Pathogens and metabolites operate on MAMP to up-regulate the expression of inflammatory mediators and cytokines (e.g., IL-1, IL-23, TNF, and IL-17) when the balance is upset. These inflammatory mediators or cytokines set off important signalling cascades downstream, such as the NF-kB and STAT3 pathways and the ERK and PI3K pathways that further the carcinogenesis of the host cells.

which in turn affects the treatment of COPD and cancer of lung.[27]

The complex interaction between the oral microbiome and the body's defense system is still not extensively studied. In a healthy oral cavity, the body's immune system not only interacts with beneficial bacteria but also has the task of protecting against harmful microorganisms.[28] Immune deregulation can, in turn, alter the composition of the oral microbiota. Various human diseases have the potential to disrupt the delicate balance between the host's oral flora and the immune system. Any disturbance in the state of balance of the immune system might cause a change in the mutually beneficial connection, leading to the widespread colonization and proliferation of opportunistic infections. These disease-causing microorganisms may trigger pathogenic mechanisms, eventually resulting in the development of different symptomatic malignancies.[29]

Consequently, the systemic immune system is affected by the dysbiosis of oral microbiota, potentially exacerbating immunological diseases. As a result, oral bacteria may have a role in the etiology and progression of lung cancer.[30] A number of contributing factors cause the microbial balance, or "symbiosis," to become imbalanced, or "dysbiossis," which may potentially put in to the etiology of diseases by inducing inflammatory responses.[25]

Our study showed that the mouth flora was linked with immunohistochemical markers in cancer of lung. Enterobacteriaceae and CK7 and TTF-1 showed a statistically significant positive association, while the genus Blastomonas and Napsin A showed a positive correlation. The main benefit of this research is the new theory and the first documentation of the connection between immune markers and salivary flora in male non-smokers with lung cancer. These findings offer fresh perspectives that are crucial for research on the salivary micro-biota linked to lung cancer in men who do not smoke and that correlate with immunohistochemical indicators. TTF-1 is present in lung and thyroid tumor epithelial cells. Research has indicated that TTF-1 may be essential to the pathophysiology of primary lung adenocarcinoma.[31] Clinically, CK7 is used to identify specific subtypes of glandular epithelia, both benign and malignant, that may be positive in the lungs.[32]

According to our research, there was a substantial positive association between Enterobacteriaceae and TTF-1 and CK7. Under normal circumstances, Enterobacteriaceae, a highly opportunistic pathogen, be able to coexist in the human gut without producing symptoms or illnesses.[23] Thus, immunity of the host and some environmental factors may contribute to significant variation in Enterobacteriaceae, which in the case of lung floradysbiosis may result in immunological responses to the

development of disease.[21] Recent research has begun to concentrate on the gut-lung axis and gut flora in lung illness.[35] Collectively, these new discoveries have helped us in understanding the associations between exposure of microbes and allergies and autoimmunities.[21]

Furthermore, our research found a favourable correlation between the genus Blastomonas and napsin A. It is possible to isolate the Sphingomonadaceae family's species Blastomonas from fresh, lake, sea, or even hospital water.[29] Water consumption may affect the variety and makeup of oral and gut bacteria in humans, changing the immunological response and risk of lung illnesses.[11]

There exist a unique association between oral flora and the host, but our understanding to them is still quite restricted.[23] It is not important that changes in these associations can have an impact on human health and lead to disease.[23] The host may be impacted by these bacteria's release of various microbial bioactive compounds.[32] It is well known that apoptosis affects both cancer and the immune response.[31] Lung cancer development and survival are known to be influenced by the p53 signaling pathway.[24] The potential harmful roles of salivary flora are suggested by the up-regulation of p53 signaling pathways. According to these results, the p53 pathway is not involved alone. It may be used by oral flora to control the apoptotic process of lung cancer cells.

More researches are required in order to identify the variations in microbiome as causative factor for carcinogenesis or cancer initiation.[34] Salivary flora may contribute to immunity and the host's ability to withstand stress in addition to influencing secondary metabolism.[31] Oral flora may contribute significantly to inflammatory reactions even if it is not viable.[23] On the other hand, individuals with lung disease are more likely to have an overgrowth and colonization of oral bacteria in their respiratory tracts. Numerous bacterial species can contribute to host-microbe interactions in different ways, necessitating more research to understand the processes behind each of these relationships.[34]

Conclusion

According to this study, lung cancer in men who do not smoke may be caused by specific bacterial species and flora dysbiosis may play a significant role in this disease. These findings show that male non-smokers with lung cancer have different salivary microbiome profiles and they also show that salivary flora can be a useful tool for identifying non-invasive lung cancer indicators.

References

1. I H, Cho JY. Lung cancer biomarkers. Adv Clin Chem. 2015;72:107–70. DOI: 10.1016/bs.acc.2015.07.003
2. Sun S, Schiller JH, Gazdar AF. Lung cancer in never smokers–a different disease. Nat Rev Cancer. 2007;7:778–90. DOI: 10.1038/nrc2190
3. Kim JH, Park K, Yim SH, Choi JE, Sung JS, Park JY, et al. Genome-wide association study of lung cancer in Korean non smoking women. J Korean Med Sci. 2013;28:840–7. DOI: 10.3346/jkms.2013.28.6.840.
4. Govindan R, Ding L, Griffith M, Subramanian J, Dees ND, Kanchi KL, et al. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. Cell. 2012;150:1121–34. DOI: 10.1016/j.cell. 2012.08.024.
5. Lan Q, Hsiung CA, Matsuo K, Hong YC, Seow A, Wang Z, et al. Genome-wide association analysis identifies new lung cancer susceptibility loci in never smoking women in Asia. Nat Genet. 2012; 44:1330–5. DOI: 10.1038/ng.2456.
6. Chen H, Jiang W. Application of high-throughput sequencing in understanding human oral microbiome related with health and disease. Front Microbiol. 2014;5:508. DOI: 10.3389/fmicb.2014.00508.
7. Shoemark DK, Allen SJ. The microbiome and disease: reviewing the links between the oral microbiome, aging, and Alzheimer's disease. J Alzheimers Dis. 2015;43:725–38. DOI: 10.3233/JAD-141170.
8. Zhang X, Zhang D, Jia H, Feng Q, Wang D, Liang D, et al. The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. Nat Med. 2015;21:895–905. DOI: 10.1038/nm.3914.
9. Li J, Hao C, Ren L, Xiao Y, Wang J, Qin X. Data mining of lung florain cystic fibrosis patients. PLoS One. 2016;11:e0164510. DOI: 10.1371/journal.pone. 0164510.
10. Long J, Cai Q, Steinwandel M, Hargreaves MK, Bordenstein SR, Blot WJ, et al. Association of oral microbiome with type 2 diabetes risk. J Periodontal Res. 2017;52:636–43. DOI: 10.1111/jre.12432.
11. Shukla SD, Budden KF, Neal R, Hansbro PM. Microbiome effects on immunity, health and disease in the lung. Clin Transl Immunol. 2017; 6:e133. DOI: 10.1038/cti.2017.6.
12. Sun J. Mechanisms Underlying Host-Microbiome Interactions in Pathophysiology of Human Diseases. New York, NY: Springer Science Business Media. 2018. DOI: 10.1007/978-1-4939-7534-1.
13. Marsland BJ, Gollwitzer ES. Host-microorganism interactions in lung diseases. Nat Rev Immunol. 2014;14:827–35. doi: 10.1038/nri3769
14. Krishnan K, Chen T, Paster BJ. A practical guide to

the oral microbiome and its relation to health and disease. Oral Dis. 2017;23:276–86. DOI: 10.1111 /odi.12509.

15. Segal LN, Alekseyenko AV, Clemente JC, Kulkarni R, Wu B, Gao Z, et al. Enrichment of lung microbiome with supraglottic taxa is associated with increased pulmonary inflammation. Microbiome. 2013;1:19. DOI: 10.1186/2049-2618-1-19.
16. Segal LN, Clemente JC, Tsay JC, Koralov SB, Keller BC, Wu BG, et al. Enrichment of the lung microbiome with oral taxa is associated with lung inflammation of a Th17 phenotype. Nat Microbiol. 2016;1:16031. DOI: 10.1038/nmicrobiol.2016.31.
17. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, YuWH, et al. The human oral microbiome. J Bacteriol. 2010;192:5002–17. DOI: 10.1128/JB.00542-10.
18. Yan X, Yang M, Liu J, Gao R, Hu J, Li J, et al. Discovery and validation of potential bacterial biomarkers for lung cancer. Am J Cancer Res. 2015;5:3111–22.
19. Gomez A, Nelson KE. The oral microbiome of children: development, disease, and implications beyond oral health. Microb Ecol. 2017;73:492–503. DOI: 10.1007/s00248-016-0854-1.
20. Kumar PS. From focal sepsis to periodontal medicine: a century of exploring the role of the oral microbiome in systemic disease. J Physiol. 2017;595:465– 76. DOI: 10.1113/JP272427.
21. Wu J, Peters BA, Dominianni C, Zhang Y, Pei Z, Yang L, et al. Cigarette smoking and the oral microbiome in a large study of American adults. ISME J. 2016;10:2435–46. DOI: 10.1038/ismej.2016.37.
22. Huber RM. Is lung cancer in never-smokers a different disease?–Back to the figures. J Thorac Oncol. 2007;2:787–8. DOI: 10.1097/JTO. 0b013e318153f3c5.
23. Choi JR, Park SY, Noh OK, Koh YW, Kang DR. Gene mutation discovery research of non-smoking lung cancer patients due to indoor radon exposure. Ann Occup Environ Med. 2016;28:13. DOI: 10.1186 /s40557-016-0095-2.
24. Ao MH, Zhang H, Sakowski L, Sharma R, Illei PB, Gabrielson E, et al. The utility of a novel triple marker (combination of TTF1, napsin A, and p40) in the subclassification of non-small cell lung cancer. Hum Pathol. 2014;45:926–34. DOI: 10.1016/j.humpath. 2014.01.005.
25. Kawai T, Tominaga S, Hiroi S, Kameda K, Ogata S, Nakashima H, et al. Expressions of thyroid transcription factor-1, Napsin A, p40, p63, CK5/6 and Desmocollin-3 in non-small cell lung cancer, as revealed by imprint cytology using a malinol-based cell-transfer technique. Acta Cytol. 2015;59:457–64. DOI: 10.1159/000442659.
26. Pepe MS, Feng Z, Janes H, Bossuyt PM, Potter JD. Pivotal evaluation of the accuracy of a biomarker used for classification or prediction: standards for study design. J Natl Cancer Inst. 2008;100:1432–8. DOI: 10.1093/jnci/djn326.
27. Zhang L, Farrell JJ, Zhou H, Elashoff D, Akin D, Park NH, et al. Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. Gastroenterology. 2010;138:949–57. DOI: 10.1053/j.gastro. 2009.11.010.
28. Farrell JJ, Zhang L, Zhou H, Chia D, Elashoff D, Akin D, et al. Variations of oral floraare associated with pancreatic diseases including pancreatic cancer. Gut. 2012;61:582–8. DOI: 10.1136/gutjnl-2011-300784.
29. Zhao W, Wang H, Peng Y, Tian B, Peng L, Zhang DC. Delta Np63, CK5/6, TTF-1 and napsin A, a reliable panel to subtype non-small cell lung cancer in biopsy specimens. Int J Clin Exp Pathol. 2014; 7:4247–53.
30. Chen R, Ding Z, Zhu L, Lu S, Yu Y. Correlation of clinicopathologic features and lung squamous cell carcinoma subtypes according to the 2015 WHO classification. Eur J Surg Oncol. 2017;43:2308–14. DOI: 10.1016/j.ejso. 2017.09.011.
31. Woese CR, Fox GE. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci.1977;74:5088–90. DOI: 10.1073 /pnas.74.11.5088.
32. Woese CR, Kandler O, Wheelis ML. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci. 1990;87:4576–9. DOI: 10.1073/pnas.87.12.4576.
33. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011;27:2957–63. DOI: 10.1093/bioinformatics/btr507.
34. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high throughput community sequencing data. Nat Methods. 2010;7:335–6. DOI: 10.1038 /nmeth.f.303 .