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Toxicological Effects of Tobacco Compounds on the Expression of Genes Involved in Actinic Cheilitis

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ABSTRACT

Background
The molecular effects of substances present in tobacco and cancer development have been well described. Morphological studies have demonstrated tissue changes in patients with these lesions and tobacco substances. However, the effects of the tobacco components and the development of potentially malignant lesions remain unknown.

Materials and Methods
Thus, by chemical-biological analysis, we investigated compounds present in tobacco and the expression of genes involved in the etiopathogenesis of actinic cheilitis.

Results and Discussion
Analysis showed a ratio of 51 harmful substances present in tobacco that are involved in several biological processes that can cause abnormal epithelial-mesenchymal transition pathway. Also, describe how cadmium can adversely affect signaling and cell differentiation through the inhibition of specific proteins.

Conclusion
This study provides the first approach that describes how different tobacco constituents affect a vast network of biological processes in the development of actinic cheilitis and possible progression of the lip carcinoma.

Keywords
Lip carcinoma; Oral cancer; Epithelial-mesenchymal transition.

Abbreviations
HUGO: Human Genome Organisation; MCODE: Molecular Complex Detection; CPI: Chemical-Protein Interaction; PPI: Protein-Protein Interaction; HBs: hubs-bottlenecks; EGF: Epidermal Growth Factor; EMT: Epithelial-Mesenchymal Transition; HDACs: Histones Deacetylases Proteins; ANOVA: Analysis of variance.

INTRODUCTION

Actinic cheilitis is the name given to inflammation of the lips, having great clinical importance because it is a potentially malignant disease that affects the lower lip. Patients are most often elderly adults, with males being affected more often than females. Clinical signs of Actinic cheilitis are subtle but can cause discomfort and inconvenience and the diagnosis preceding the injury, important to reduce the risk of developing cancer.[1]

The injury presents clinically in three forms: acute, subacute and chronic. The acute form occurs to more rare and episodic,

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can occur in a mild, moderate or severe form, been characterized by white erythema, swelling, cracks and severe ulcers, and occurs when there is excessive exposure to sunlight within a short period of time, leading the individual to discomfort when feeding or speech.[2]

Among the carcinogens that promote oral cancer use tobacco products stands out as one of the main etiological factors involved in the pathogenesis of the disease. However, several other risk factors cannot be disregarded, such as a systemic condition of the individual, sun exposure, age, heredity, etc.[3,4] There are more than 4,800 compounds present in the particulate and vapor phases of cigarette smoke and several of these compounds are considered to represent a human health risk. The vast majority belong to three groups: 1) polycyclic aromatic hydrocarbons, 2) aromatic amines and 3) nitrosamine, the latter related to nicotine.[5] In smokers, a form in which the tobacco triggers lesions, such as actinic cheilitis, with the interaction of nicotine and its receptor on epithelial cells. However, the molecular mechanisms associated with actinic cheilitis and cigarette smoking remains unknown.[6,7]

Taking these data together, by systems chemo-biology tools, we investigated various compounds present in tobacco and the expression of genes involved in the etiopathogenesis of actinic cheilitis.

MATERIALS AND METHODS

Bioinformatics and Interaction Networks Analysis

The leader gene approach was described previously by Feltes et al.[5] To determine a primary set of genes associated with actinic cheilitis, a search considering only human genes was performed on the following databases: PubMed, Gene-Bank, Geneatlas, and Genecards. The gene nomenclature adopted was defined by the Human Genome Organization (HUGO). In this process, new genes directly linked to actinic cheilitis could be identified. Literature data from PubMed, Gene-Bank, Geneatlas, Genecards was performed using the STRING software of pertinent keywords chosen by experts as well as Medical Subject Headings were used to carefully check the terms and all their possible Boolean logic based combinations to avoid false positive data. After this step, a list of genes related to actinic cheilitis was generated. In order to evaluate the interaction between the genes selected in the previous step, a network of interaction was built. The construction of the network of interactions was performed using web-available STRING software (version 10) Topological analysis was carried out with Cytoscape and FANMOD, while the ontological analysis was performed with BinGO.

Interactome Data Mining and Design of the Chemobiology Network

The general methods used to develop this study were based on previously used by Feltes et al[5]. To design chemobiology interactome networks and to elucidate the interplay between Actinic cheilitis and tobacco compounds, the meta search engines STITCH 4.0 and STRING 10.0, were used. In this sense, we selected a previous list of 51 commonly found TCs[5] and used as an initial seed for network prospection in STITCH. Whereas STRING software shows protein-protein interactions, the STITCH software allows visualization of the physical connections among different chemical compounds and proteins. The results gathered using these search engines were analyzed with Cytoscape 3.3.0.

Module Analysis of Major Tobacco Component associated Networks

The plugin Molecular Complex Detection (MCODE) was used to analyze the large chemical-protein interaction (CPI) and protein-protein interaction (PPI) network obtained from the initial search.

Centrality and Gene Ontology Analyses of the Major Tobacco Component associated CPI-PPI Networks

Centrality analysis was performed using the program CentiScaPe version 1.2. The CentiScaPe algorithm evaluates each node from the network, according to the node degree, betweenness and closeness to establish the most "central" nodes within the network. The CPI and PPI modules generated by MCODE were further analyzed by focusing on biology associated processes using the Biological Network Gene Ontology version 2.44 Cytoscape plugin (BiNGO). The degree of functional enrichment for a given cluster and category was quantitatively assessed (p-value) using a hypergeometric distribution. Multiple test correction was also assessed by applying the false discovery rate algorithm, which was fully implemented in BiNGO software plugin at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Many cases of oral cancer are preceded by potentially malignant lesions, and the most important is the presence of epithelial dysplasia in the epithelial area. Early diagnosis and appropriate treatment of epithelial dysplasia can significantly prevent morbidity and mortality, increasing patient survival time. Before becoming cancer, the majority of malignant lesions of the oral cavity clinics suffer changes in dysplasic changes that may take years before the occurrence of the lamina propria invasion, through carcinogenic initiation and promotion stages during some time.[8-10]

Although it's considered a common injury, Actinic cheilitis most of the time it can result in the development of malignancies. Some risk factors should be considered as the probability of becoming malignant, they are tobacco and alcohol, that associated with carcinogenic factors.[11-14]

As for risk factors, exposure, the age of onset, time and the frequency of cigarette consumption are factors that appear to influence the incidence of cancer of the oral cavity. The two principal mechanisms by which tobacco contributes to oncogenesis mouth include direct deoxyribonucleic acid (DNA) nicotine and cotinine exposures, and also to metabolic products such as polycyclic aromatic hydrocarbons and aromatic amines, cigarette smoke components.[6,7,9]

The systems chemobiology tools allow prospecting of

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new drug targets and interactome networks of high-through put data to be designed for CPI and PPI networks. In this respect, these tools have been employed in various areas of research and exploration of new anti-cancer drugs, in order to assess the interaction of different proteins and molecules and major biological pathways potentially affected by the action of these compounds.[5,15]

Initially, the analysis of this study was based on a list of 51 compounds present in tobacco, extracted from Feltes et al.[5] We examined the relationship between the 51 compounds and their possible interactions with proteins involved in the etiopathogenesis of actinic cheilitis.

Preliminary analysis considering the keywords actinic cheilitis, research has linked 17 genes associated with this injury. Then, the analysis of the interaction between genes was carried out in software STRING (Figure 1). The difference in a number of links was confirmed by analysis of variance (ANOVA) with post hoc Tukey test (p<0.001). The interaction network showed a strong correlation, in accordance with the biological interaction network scale (data not shown).

We used the CentiScaPe 1.2 software to identify proteins and/or further relevant compounds in the network. In a biological network of free-range, the most important, we called hubs-bottlenecks (HBs) because they combine the control function of information flowing in a given network and a number of above-average value network connections. So HBs are key points in a biological network.[16-19] In our analysis, we observed 48 HBs nodes, of which a compound has been identified as working in the molecular pathway (Figure 2).

The cell interactions as well as remodeling of extracellular matrix proteins, mediated by specific receptors, are the basis of many biological processes, including invasion of the underlying tissue by the neoplastic cells. The invasive procedure is governed by coordinating cell-extracellular matrix interactions, with the participation of dynamic promoted by the cytoskeleton and pericellular proteolysis. Integrins mediate these events in neoplasms. The more invasive cells have increased expression of integrins,[20] enhancing cell motility, with the participation of many molecules, including extracellular matrix proteins and growth factors such as epidermal growth factor (EGF).[21] In the present study, we observed that after the analysis carried out by MCODE software, cluster 1 interaction network is associated directly with the molecular signals induced by integrins, demonstrating a possible malignant potential for actinic cheilitis injury which may lead to lip cancer (Figure 3A, Table 1).

Epithelial-mesenchymal transition (EMT) is characterized by the change in the epithelial phenotype in mesenchymal leading to the reduced loss or expression of epithelial markers, such as E-cadherin and claudin, and increased expression of mesenchymal markers, such as N-caderina and vimentin, as well as increased expression of the transcription factor twist. EMT may be induced by extracellular matrix components and growth factors, such as beta transforming growth factor, responsible for the regulation of cellular differentiation and proliferation, migration and apoptosis.[22-26] In this study, we showed association with cell

Figure 1. STRING Results for Actinic Cheilitis

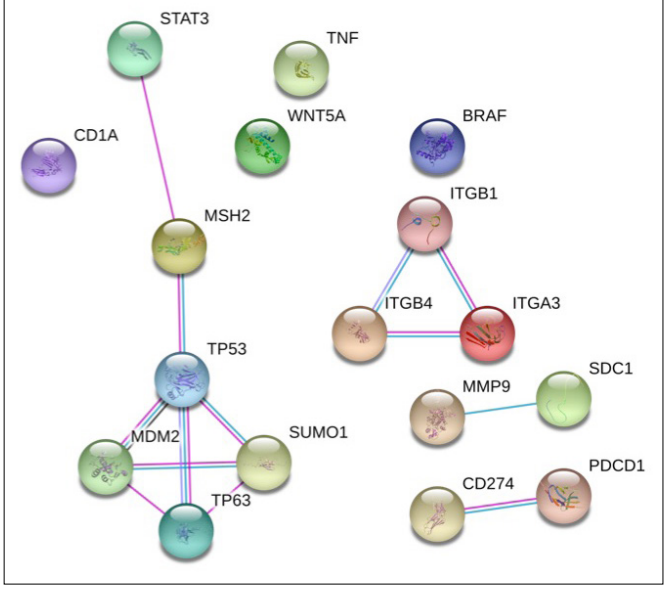

Figure 2. HBs Found in the Major CPI-PPI Network. Betweenness and Node Degrees were Assessed using the Program CentiScaPe

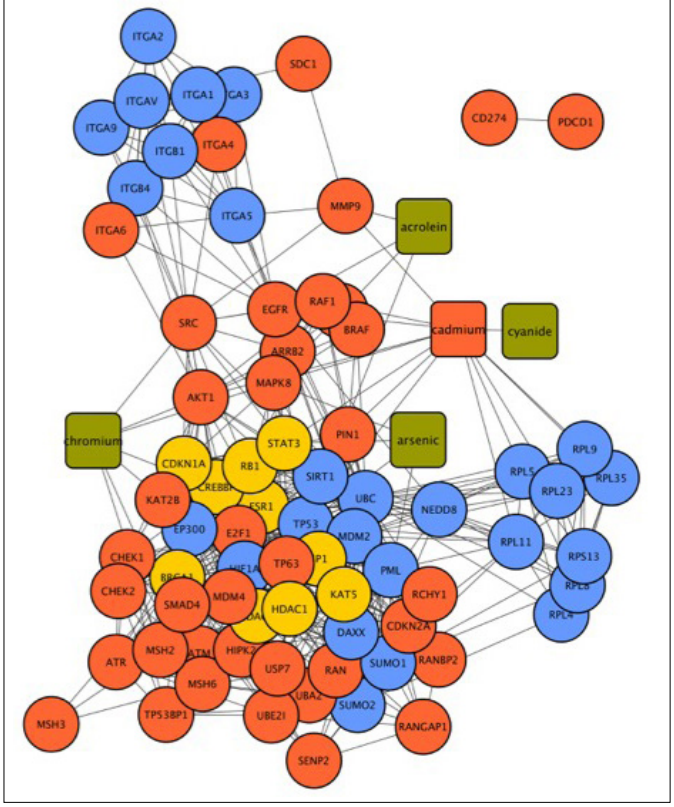

Table 1. Major Bioprocesses in Cluster 1 Associated with the Hub-Bottleneck Subnetwork

GO-ID	*p*-value	corr *p*-value	X*	N#	Description	Genes in test set
7229	5,63E-10	5,28E-07	8	57	Integrin-mediated signaling pathway	ITGB1, ITGB4, ITGA3, ITGA2, ITGA1, ITGAV, ITGA5, ITGA9
6414	4,33E-08	2,03E-05	8	96	Translational elongation	RPL4, RPL5, RPL23, RPL11, RPL35, RPL8, RPL9, RPS13
44419	1,31E-06	4,08E-04	10	327	Interspecies interaction between organisms	ITGB1, DAXX, ITGA2, MDM2, EP300, ITGAV, ITGA5, SIRT1, TP53, PML
7160	7,71E-05	1,46E-02	6	85	Cell-matrix adhesion	ITGB1, ITGB4, ITGA3, ITGA2, ITGA1, ITGAV
44267	7,77E-05	1,46E-02	17	2151	Cellular protein metabolic process	RPL4, RPL5, DAXX, RPL23, RPL11, ITGA1, NEDD8, RPL8, RPL9, SIRT1, PML, SUMO1, SUMO2, MDM2, EP300, RPL35, RPS13
31589	1,94E-04	3,03E-02	6	99	Cell-substrate adhesion	ITGB1, ITGB4, ITGA3, ITGA2, ITGA1, ITGAV
32268	2,31E-04	3,07E-02	10	559	Regulation of cellular protein metabolic process	DAXX, SUMO1, ITGA2, SUMO2, MDM2, EP300, ITGAV, ITGA5, TP53, PML
6412	2,87E-04	3,07E-02	8	289	Translation	RPL4, RPL5, RPL23, RPL11, RPL35, RPL8, RPL9, RPS13
32270	2,95E-04	3,07E-02	8	290	Positive regulation of cellular protein metabolic process	SUMO1, ITGA2, SUMO2, MDM2, EP300, ITGA5, TP53, PML
51247	4,59E-04	4,30E-02	8	307	Positive regulation of protein metabolic process	SUMO1, ITGA2, SUMO2, MDM2, EP300, ITGA5, TP53, PML
51246	7,72E-04	6,58E-02	10	635	Regulation of protein metabolic process	DAXX, SUMO1, ITGA2, SUMO2, MDM2, EP300, ITGAV, ITGA5, TP53, PML

*Number of nodes for a given GO in the network;
#Total number of proteins for a given GO annotation.

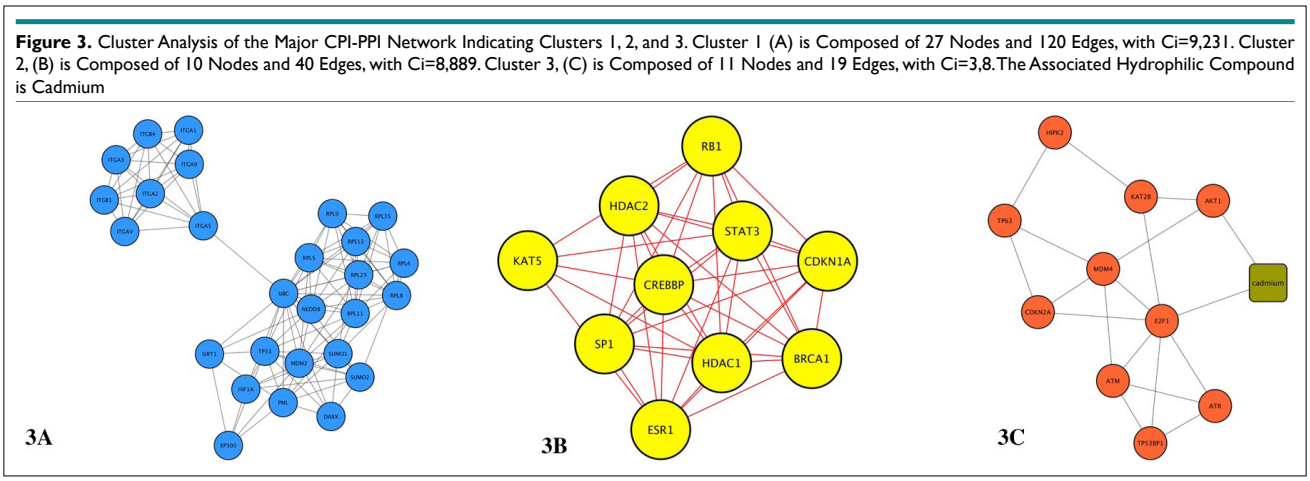

Figure 3. Cluster Analysis of the Major CPI-PPI Network Indicating Clusters 1, 2, and 3. Cluster 1 (A) is Composed of 27 Nodes and 120 Edges, with Ci=9,231. Cluster 2, (B) is Composed of 10 Nodes and 40 Edges, with Ci=8,889. Cluster 3, (C) is Composed of 11 Nodes and 19 Edges, with Ci=3,8. The Associated Hydrophilic Compound is Cadmium

adhesion molecules as well as related metabolism, which may indicate that the group of genes analyzed operate in the EMT process (Figure 3B, Table 2).

Histones deacetylases proteins (HDACs) have been associated with regulating the expression and activity of many proteins involved in neoplastic initiation and progression. Moreover, several studies have shown that certain families of HDACs are aberrantly expressed in tumors and has no specific function in cancer development.[27-31] Increased HDAC2 expression of protein has been associated with neoplastic progression of various cancers.[30,32,33] This protein is associated with response to damage to the DNA molecule, which may be observed in actinic cheilitis. Similarly, Table 2 demonstrates increased cellular proteins involved in the biosynthesis highlighting HDAC-2. Thus, the results suggest that activation of the HDAC-2 protein, as well as other cell involved in the biosynthesis, contributes to the development and progression of actinic cheilitis to lip cancer.

Then, by the cluster 3 analysis shows that the cellular events associated with events related to cellular response to injury, which is mediated by p53 (Figure 3C and Table 3). The TP53 gene is not a classical tumor suppressor gene, is essential for the maintenance of a population of precursor cells (stem cells) in various epithelial tissues. This gene in the basal epithelial cells of various organs such as skin, and prostate, may be considered a cell differentiation marker. The gene is activated in response to cellular damage signals. Its transcription factor interacts with at least six other genes.[34-37] For example, it binds to the promoter p21 gene whose protein product is a kinase inhibitor that blocks cyclin-dependent inactivation of Rb by CDK4. This activity promotes cell cycle arrest in G1 phase, so before there is a doubling of DNA (S phase), allowing the repair of damaged DNA. A p53 activity alternative unrepaired damage if the route with the pRb protein is not intact, is the induction of apoptosis (programmed cell death). In addition, p53 also promotes S G2 checkpoint, which depends on the integrity of the C-terminal domain of the gene.[37-40]

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Table 2. Major Bioprocesses in Cluster 2 Associated with the Hub-Bottleneck Subnetwork

GO-ID	*p*-value	corr *p*-value	X*	N#	Description	Genes in test set
10628	3,82E-08	2,34E-05	9	603	positive regulation of gene expression	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1, ESR1
45893	8,93E-07	2,01E-04	8	500	positive regulation of transcription, DNA-dependent	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1
51254	9,83E-07	2,01E-04	8	506	positive regulation of RNA metabolic process	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1
10604	2,10E-06	3,21E-04	9	941	positive regulation of macromolecule metabolic process	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1, ESR1
45941	2,73E-06	3,34E-04	8	575	positive regulation of transcription	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1
9893	4,25E-06	4,34E-04	9	1018	positive regulation of metabolic process	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1, ESR1
45935	7,79E-06	6,82E-04	8	656	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1
10557	9,78E-06	6,82E-04	8	675	positive regulation of macromolecule biosynthetic process	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1
51173	1,00E-05	6,82E-04	8	677	positive regulation of nitrogen compound metabolic process	RB1, HDAC2, CREBBP, KAT5, SP1, HDAC1, STAT3, BRCA1
45944	1,15E-05	6,92E-04	7	388	positive regulation of transcription from RNA polymerase II promoter	RB1, HDAC2, CREBBP, SP1, HDAC1, STAT3, BRCA1

*Number of nodes for a given GO in the network;
#Total number of proteins for a given GO annotation.

Table 3. Major Bioprocesses in Cluster 3 Associated with the Hub-Bottleneck Subnetwork

GO-ID	*p*-value	corr *p*-value	X*	N#	Description	Genes in test
43517	9,84E-06	8,02E-03	3	4	positive regulation of DNA damage response, signal transduction by p53 class mediator	CDKN2A, ATM, ATR
80135	1,45E-04	5,40E-02	5	131	regulation of cellular response to stress	CDKN2A, AKT1, ATM, ATR, HIPK2
51716	1,99E-04	5,40E-02	8	987	cellular response to stimulus	KAT2B, E2F1, AKT1, ATM, TP53BP1, TP63, ATR, HIPK2
43516	4,05E-04	8,25E-02	3	11	regulation of DNA damage response, signal transduction by p53 class mediator	CDKN2A, ATM, ATR
8219	6,75E-04	9,63E-02	7	698	cell death	CDKN2A, E2F1, AKT1, ATM, MDM4, TP63, HIPK2
16265	7,09E-04	9,63E-02	7	703	death	CDKN2A, E2F1, AKT1, ATM, MDM4, TP63, HIPK2
48523	8,55E-04	9,96E-02	9	1844	negative regulation of cellular process	KAT2B, CDKN2A, E2F1, AKT1, ATM, MDM4, TP63, ATR, HIPK2

*Number of nodes for a given GO in the network;
#Total number of proteins for a given GO annotation.

In this sense, it can be inferred that mutation of the p53 gene in lesions caused by direct effects of ultraviolet light, is an independent mechanism, unlike oral leukoplakia that smoking and alcohol consumption are considered determinants. Once mutated TP53 gene undergoes conformational and structural changes that cause a disabling and make it impossible to carry out its function.[41] This deactivation leads to a further stabilization considerably increases the half-life, allowing identification of proteins by immunohistochemistry. Although rare events considered, the presence of mutations "meaningless" or the deletion of both alleles of the gene TP53 prevents its translation due to the production of unstable proteins that interfere directly with its expression and detection.[34,35,37,40]

Cluster 3 analysis also revealed that the cadmium has an intimate association in this direction (Figure 2 and Figure 3C). Experimental studies clearly show that cadmium is active in several cellular pathways by joining to induce the formation of benign and malignant tumors in several organs.[42-47] Early studies show that cadmium as a carcinogen bioassays was in the 1960s These findings demonstrating the carcinogenicity of cadmium preceded the first epidemiological study in humans.[48] Similar to other toxic metals, cadmium can act mimicking other metals and/or essential nutrients; i.e., Competing for binding sites on sites that are important in gene regulation, the enzymatic activity or the maintenance of genomic stability.[49-53] Unfortunately, for most of the compounds present in tobacco analyzed in this study, data on their meta-

bolism and detoxification process are virtually unknown. The use of systems chemobiology tools should enhance the understanding of how these compounds affect the etiopathogenesis of actinic cheilitis.

CONCLUSION

We performed systems chemobiology analyses to elucidate nature and number of proteins and modules that are associated with actinic cheilitis tobacco smoke association. Different protein-protein interaction and chemical-protein interaction networks derived from interactome projects were described. In a first analysis, we prospected and analyzed a network using a list of 51 commonly found harmful tobacco constituents, to elucidate how these substances could act together to influence actinic cheilitis development. Furthermore, we conducted gene ontology (GO) analyses of the major biological processes derived from the PPI and CPI networks. In the present study, we demonstrated, using systems chemo-biology tools, how tobacco may interact with specific biological processes and affect them. Our cluster analysis of the results shows that these compounds participate in many biological processes, including epithelial-mesenchymal transition. Thus, the present compounds in tobacco, in particular cadmium, act on cellular processes which can mean that there is the development of actinic cheilitis and possible progression to the lip carcinoma. In particular, further studies are needed to consider which tobacco-related genes play significant roles in actinic cheilitis and the molecular mechanisms that might explain a possible association between the two pathological conditions. Our analysis suggests that multiple tobacco compounds may play important roles in actinic cheilitis pathogenesis, generating hypotheses that should be further studied and validated.

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The authors deny any conflicts of interest related to this study.

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