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Original article

Cell adhesion genes expression level in oropharyngeal squamous cell carcinoma and laryngeal squamous cell carcinoma: a preliminary study

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Abstract

Head and neck cancers (HNCs) are tumors developed in the upper aerodigestive tracts, more than 90% originating in the squamous cells lining the mucosa of the upper airways. There are between 650,000 and 900,000 new cases each year, most of them advanced, resulting in high mortality (five-year survival about 50%). HNCs do not metastasize to distant sites, but often affect local lymph nodes. Their etiology is multifactorial, with smoking, alcohol consumption and HPV infection being the most common. HNCs rapidly enter hypoxia and synthesize angiogenic factors, which contribute to further tumor development and recurrence. Although there is a relatively wide variety of therapeutic strategies, their effectiveness is reduced because of resistance development. Cell adhesion mediated by integrins and the extracellular matrix is an important mechanism of tumor progression. In this study, expression levels of genes involved in cell adhesion were determined in two patients with oropharyngeal squamous cell carcinoma and three patients with laryngeal squamous cell carcinoma. The obtained data showed that there is a differentiation in the expression of genes for collagens (COL15A1, COL18A1, COL4A1, COL4A2 and COL4A3), integrins (ITGA4, ITGAV, ITGB3) and two molecules involved in their interactions (CD44 and MMP2) for the two tumor types, the results obtained need validation on a statistically significant sample.

Keywords

collagens; integrins; oropharyngeal squamous cell carcinoma; laryngeal squamous cell carcinoma; cell adhesion

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Introduction

Although the head region is very complex, encompassing all the organs and bony and muscular structures of the upper airways, the cranial vault, eyes, ears, their appendages, endocrine glands, the cervical spine, cranial and cervical musculature, blood vessels, nerves and the covering integument, the term head and neck cancers refers only to tumors arising in the upper airways [1]. Because the structures of the head and neck are complex, upper airway cancers are highly heterogeneous [2], with more than 90% of their origin in the squamous cells lining the mucosa of the upper airways. Head and neck cancers account for an estimated 650,000 to 900,000 new cases each year [1; 3] and are some of the most common cancers, ranking sixth [3] or seventh [1] worldwide [4; 5]. Head and neck cancers are diagnosed at advanced stages and have a five-year survival of about 50% and high mortality [6]. Head and neck cancers rarely metastasize to distant sites (in less than 10% of cases [7]), but they affect regional lymph nodes with high frequency, greatly complicating the patients' situation [8]. Among the most implicated factors in the etiology of head and neck cancers are: smoking, alcohol consumption, most frequently combined [9-11] and HPV (human papillomavirus) infections [9; 12-19], followed by other factors with variable participation in the occurrence of head and neck cancers: EBV (Epstein-Barr virus) infections [20; 21], gastroesophageal reflux [22], chewing betel quid (Areca nuts) [23], poor oral hygiene [24], oral dysbiosis [25], proinflammatory diet [26; 27], inhalation of air pollutants [8], genetic aberrations [3; 28-31] and epigenetic factors [32].

Therapeutic strategies for head and neck cancers are varied and include cytoreductive surgery, radiotherapy and chemotherapy (when necessary) and, in recent years, innovative therapies such as immunotherapy and targeted therapies, but the results are modest [33]. The low efficacy of treatments may be due to the fact that head and neck tumors rapidly enter hypoxia, synthesizing numerous pro-angiogenic factors that amplify the angiogenic process [34], and to treatment resistance [35]. The association between integrins and components of the extracellular matrix plays an essential role in cell-cell interactions [36]. The extracellular matrix has the structure of a three-dimensional network of fibers, which serves as a physical support and participates in the regulation of cellular processes [37]. The structure of the extracellular matrix in the vicinity of the tumor is remodeled by head and neck cancers, while constantly interacting with the stromal components of the tumor microenvironment [38; 39]. Thus, integrins in the tumor cell membrane support the biosynthesis of extracellular matrix elements, promote the formation of collagen fiber networks, contribute to chemotaxis-driven tumor invasiveness [40] and, by interacting with extracellular matrix elements, integrins A2, A2B, A3, A6, A6, AV and B1 serve to activate PTK2/FAK-PI3K–AKT–mTOR/IKK signaling pathways involved in angiogenesis and evading apoptosis [41]. In the present study we investigated the expression of genes for collagens 15A1, 18A1, 4A1, 4A1, 4A2 and 4A3 and genes for AV and A4 integrins in five patients with head and neck cancers and the relationships between them by constructing a network using String software, available at https://string-db.org/ [42].

Collagens (COLs) are found in the extracellular matrix, being a major component of the tumor microenvironment and participate in tumor fibrosis [43]. They belong to a family of 28 protein types classified into four subfamilies based on the molecular assemblies they form: (i) *fibril-forming collagens*, i.e., collagens I, II, III, V, XI, XXVI, XXVII; (ii) *collagens associated with fibrils with interrupted triple helix*, i.e., collagens IX, XII, XIV, XVI, XIX, XX, XXI, XXII, XXIV, which do not form fibrils but are characteristically associated with the surface of collagen fibrils; (iii) *network-forming collagens*, i.e., collagens IV, VIII, X; and (iv) *membrane-anchored collagens*, i.e., collagens XIII, XVII, XXIII, XXV [44], with collagens I, III and V being produced mainly by cancer-associated fibroblasts (CAFs), and collagen IV mainly by epithelial and endothelial cells, but also some tumors, such as oral squamous cell carcinoma [45], in which COL4A1 appears to promote cell proliferation and migration [46]. Under certain circumstances, tumor cells and tumor microenvironment macrophages (TAMs) can produce significant amounts of collagen [47; 48]. Collagens and elastin, the second most abundant components in the extracellular matrix, form a supramolecular structure resistant to proteolysis, but which is susceptible to matrix metalloproteinases. Proteolysis is facilitated by the binding of MMP2 and MMP9 to collagen and by the activity of remote exosites of the catalytic domain of MMP12 [49].

Integrins (ITGs) are a family of heterodimeric cell adhesion molecules composed of α and β subunits that mediate junctions between cells and between cells and the extracellular matrix [50]. Regulating cell proliferation, survival and migration [51], certain combinations of integrins possess specific roles in carcinogenesis, particularly in metastatic processes and in interactions between tumor cells and the extracellular matrix [52]. Among them, integrin A4 (ITGA4), a receptor for fibronectin [53], promotes cell myogenesis, and the five-membered αV integrins (ITGAV) (αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8) function as receptors for fibronectin, vitronectin and fibrinogen and promote the progression of head and neck squamous cell carcinoma [54].

Patients and methods

Patients and tumor samples. Fragments of tumor tissue and normal-appearing tissue, harvested from the resection border and used as control, preserved in RNA later solution at -20°C were collected from five adult patients with oropharyngeal and laryngeal neoplasms, operated at Ilfov County Emergency Hospital. All procedures carried out were in accordance with the ethical standards of the institutional research committee and the 1964 Declaration of Helsinki and its subsequent amendments, informed consent was obtained from the patients for the processing of the samples, and the study was approved by the ethics committee.

RNA extraction and reverse transcription. Total RNA was extracted with the NucleoSpin RNA II-Total RNA Isolation Kit (ref.740955.50, MACHEREY-NAGEL GmbH & Co. KG, 52355 Düren, Germany) following the protocol in the kit. RNA spectrophotometric analysis was performed on a Perkin-Elmer UV-VIS Lambda 40 spectrophotometer and RNA molecule integrity was analyzed with the Lab on Chip system (AGILENT): kit ul 6000 RNA Nano, on the 2100 bioanalyzer (AGILENT). Reverse transcription reaction was performed with the High-Capacity cDNA Reverse Transcription Kit (cat.no.4368814, APPLIED BIOSYSTEMS, Foster City, CA 94404, USA).

Real-Time PCR. Gene expression analysis was performed in TaqMan Array 96-Well Plates Gene Signature for Human Angiogenesis (92 genes for analysis and 4 housekeeping genes: 18S (for 18S RNA), GAPDH (for glyceraldehyde-3-phosphate dehydrogenase), HPRT1 (for hypoxanthine phosphoribosyltransferase 1) and GUSB (for β-glucuronidase), using TaqMan® Gene Expression Master Mix (2x) (cat. no. 4369016, Amplification reaction was performed on a Real-Time PCR 7500 (Applied Biosystems), and data were analyzed using Data AssistTM v. 3.01 software (Applied Biosystems). The housekeeping genes considered as endogenous controls were 18S and GUSB, which had values closest to 1.

Results

Baseline patient characteristics. Samples were taken from five male patients, aged 53 to 73 years, whose clinical data and anatomopathological diagnosis are shown in Table 1.

Gene expression in head and neck cancers. The expression levels of each gene in the tumor tissue samples from the five patients are shown in Table 2, by comparison with the expression levels in peritumoral tissues with normal histopathological appearance, which have a value of 1. For a gene to be overexpressed, values equal to or greater than 2, i.e. with expression at least double the value in normal tissue, were considered. To form an overview of the grouping of samples and genes with similar expression behavior, the heat map shown in Figure 1 was generated using the Data AssistTM Software v. 3.01 (Applied Biosystems). The distances between samples are calculated in hierarchical clustering based on ΔCT values using Pearson's correlation, and the clustering method is average linkage.

Table 2. Gene expression levels in the five patients, expressing the ratio between the amount of mRNA in the tumor tissue sample and the control sample, Fold Change (RQ).

Gene	P1	P2	P3	P4	P5
CD44	3,55	0,04	5,72	14,19	18,21
COL15A1	0,11	1,02	0,39	0,59	0,66
COL18A1	0,79	0,18	1,64	6,30	7,34
COL4A1	0,72	0,28	0,69	4,36	7,07
COL4A2	0,80	0,76	1,28	8,23	9,49
COL4A3	0,75	17,17	0,17	0,64	0,01
ITGA4	0,79	0,26	35,50	10,63	12,07
ITGAV	2,93	0,45	2,93	6,75	19,81
ITGB3	0,05	1,64	0,55	0,49	4,30
MMP2	0,43	2,49	1,78	0,89	15,35

Table 1. Clinical data and anatomopathological diagnosis of the patients.

Patients			TNM	Anatomopathological diagnosis	Associated diagnoses	5-year following status
Code	Age	Sex				
P1	55 y	M	T3N2bM1	Well differentiated, keratinized oropharyngeal squamous cell carcinoma; latero-cervical lymph node with metastasis.	Obesity, chronic tonsillitis, weight loss	Deceased
P2	62 y	M	T3N2bM0	Moderately differentiated oropharyngeal squamous cell carcinoma, invasive in the tonsil tissue; no lymph node metastasis.	Type 2 diabetes, obesity, chronic tonsillitis, chronic bronchitis	Relapse
P3	71 y	M	T3N2bM1	Keratinized laryngeal squamous cell carcinoma; latero-cervical lymph node with metastasis.	Chronic tonsillitis, weight loss	Stable
P4	53 y	M	T3N3M1	Moderately to poorly differentiated, nonkeratinized laryngeal squamous cell carcinoma, invasive with necrotic areas; latero-cervical lymph node with metastasis.	Obesity, chronic tonsillitis, chronic sinusitis, gastroesophageal reflux	Deceased
P5	73 y	M	T3N2cM0	Moderately differentiated, invasive laryngeal squamous cell carcinoma; no lymph node metastasis.	Chronic tonsillitis, weight loss	Deceased

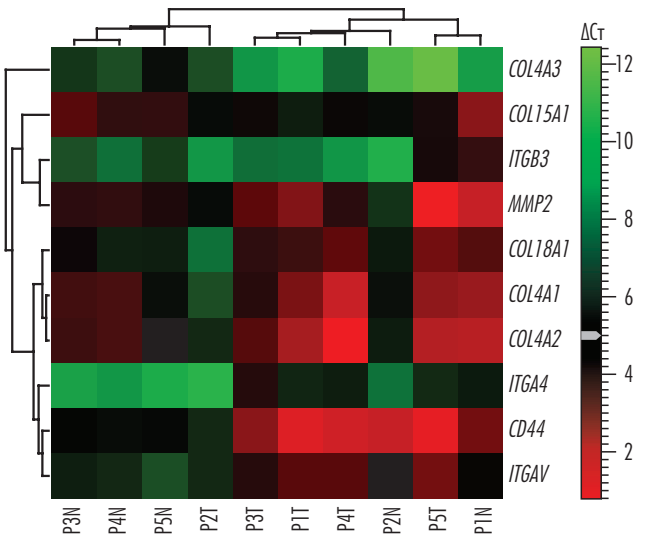

Fig. 1. Heat map displaying gene expression levels.

Gene expression for collagens 15A1, 18A1, 4A1, 4A1, 4A2 and 4A3, AV, A4 and B3 integrins, CD44 and MMP2 is variable, being maintained at low levels in oropharyngeal squamous cell carcinoma, with definite overexpression (values above 2) of *CD44* and *ITGAV* (in one patient) and *COL4A3* and *MMP2*, in the second patient, and at somewhat higher levels in laryngeal squamous cell carcinoma, where *COL15A1* and *COL4A3* are the least expressed genes, all others having expression peaks exceeding 2 in at least one patient (Figure 2). Thus, for the *CD44* gene, one sample of oropharyngeal squamous cell carcinoma and all three samples of laryngeal squamous cell carcinoma were positive; for the *COL15A1* gene all samples were negative, for the *COL18A1*, *COL4A1* and *COL4A2* genes two samples of laryngeal squamous cell carcinoma were positive each; for the *COL4A3* gene, only one sample of oropharyngeal squamous cell carcinoma was positive; for the *ITGA4* gene all laryngeal squamous cell carcinoma samples were positive, for the *ITGAV* gene one oropharyngeal squamous cell carcinoma sample and all laryngeal squamous cell carcinoma samples were positive, for the *ITGB3* gene one oropharyngeal squamous cell carcinoma sample was positive and for the *MMP2* gene one sample from both groups was positive.

Discussions

In the present study, we aimed to identify the expression levels of genes for collagen and integrin, with two additional genes, *CD44* and *MMP2*, which are not part of these protein families, to observe possible particularities between the two types of cancers. As illustrated in Figure 2, there is a clear differentiation between the two types of tumors, in particular with respect to the overall level of gene expression. Thus, in oropharyngeal squamous cell carcinoma, expression of selected genes appears poorer than in laryngeal squamous cell carcinoma. On the other hand, *COL15A1*, which, according to GeneCards [55], is expressed in a wide variety of tissues, functioning predominantly in basement membrane adhesion complexes to the underlying connective tissue stroma. At the same time, the C-terminal fragment resulting from proteolysis of type XV collagen is restin, a protein with antiangiogenic potential, closely related to endostatin, and reduced *COL15A1* gene expression is probably associated with tumor angiogenesis, a trait that may characterize many head and neck cancers.

The CD44 protein is a cellular receptor involved in cell-cell interactions, cell adhesion and migration, participating in the cell's response to changes in the tumor microenvironment and probably in tumor metastasis [56]. In oropharyn-

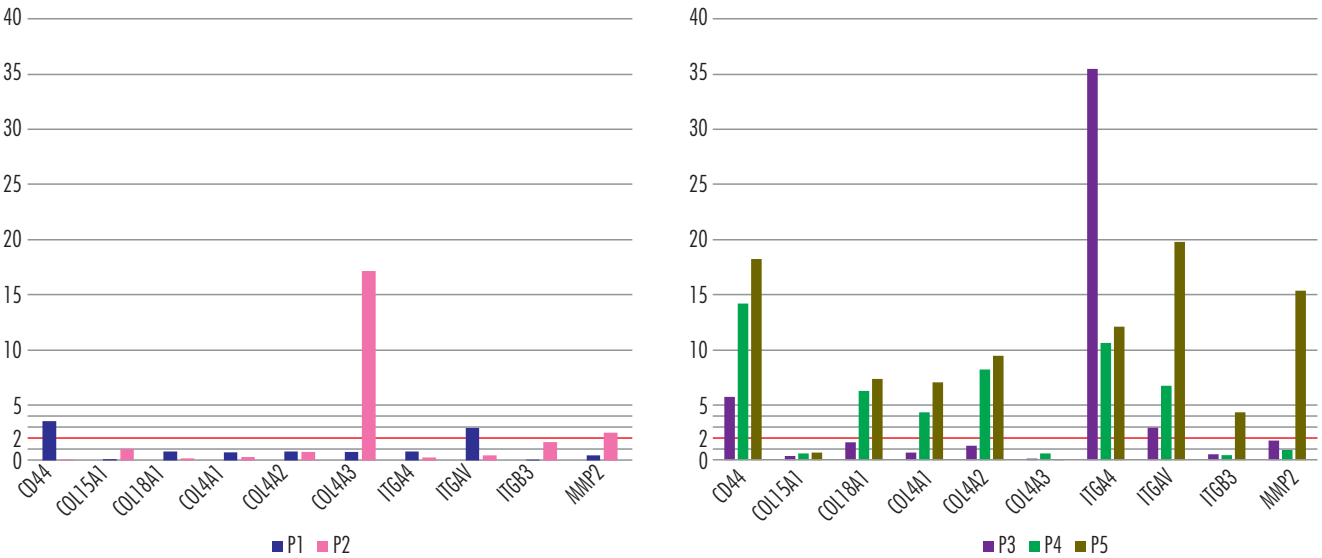

Fig. 2. Gene expression levels in the five patients. On the left, oropharyngeal squamous cell carcinoma cases and on the right, laryngeal squamous cell carcinoma cases. Values of 2 or greater are considered to reflect gene overexpression, and 1 characterizes normal tissue.

geal squamous cell carcinoma, the gene is present in one sample from a patient with keratinized and metastasized cancer, and in laryngeal squamous cell carcinoma in all samples, with the highest level observed in the patient with metastasis, so that no correlation can be made between *CD44* gene expression and metastasis.

Collagen IV is the major component of basement membranes and is composed of three subunits, A1, A2 and A3, the first two subunits being overexpressed in two laryngeal squamous cell carcinoma samples each. Subunit 3 (COL4A3) can be cleaved in the NC1 domain, resulting in an antiangiogenic and antitumor fragment called tumstatin [57], and in head and neck cancers, overexpression of *COL4A3* gene is associated with favorable prognosis [58]. In our cohort, patients 1, 4, and 5 progressed to death, patient 2's tumor, in which the highest *COL4A3* gene expression was reported, relapsed, and patient 3's condition remained stable during the 5-year follow-up period. In this context, the correlation between reduced mRNA levels of *COL4A3* and poor prognosis of patients with oropharyngeal and laryngeal squamous cell carcinoma is confirmed, the exception in patient 2 may be related to the lack of lymph node metastases.

The A1 subunit of collagen XVIII, which can inhibit angiogenesis by binding to heparan sulfate proteoglycans involved in growth factor signaling [59], follows the expression trend of collagen IV A1 and A2 subunits and is overexpressed in patients with laryngeal squamous cell carcinoma who subsequently died.

Integrin A forms heterodimers with integrin B and, anchored in the membrane, interacts with laminins and collagens in the extracellular matrix, establishing the links between the extracellular matrix and the cell, but at the same time participating in the alternative activation of the PI3K–AKT–mTOR signaling pathway, one of the pillars of tumor progression by circumventing apoptosis [41]. In oropharyngeal squamous cell carcinoma, *ITGAV* is overexpressed in one sample, and ITGA4 and ITGB3 are well expressed in laryngeal squamous cell carcinoma samples. Coupled with overexpression of its indirect interactors COL4A1, COL4A2 (via CD44, overexpressed, and SPP1, whose expression was not quantified), overexpression of integrins A4 and AV may indicate activation of the PI3K–AKT–mTOR signaling pathway, unaffected by integrin B3 underexpression.

The expression of the matrix metalloproteinase 2, MMP2, is higher in samples of invasive tumors, but which have not produced lymph node metastases. MMP2 is one of the few enzymes of the MMP family that can be activated at the cell membrane as well as inside or outside the cell. By binding denatured collagens IV and V and elastin and cleaving the extracellular matrix, MMP2 is involved in vascularization, cell migration and metastasis [60].

Further, we present the interaction of selected genes by constructing the protein-protein interaction network using the STRING online database and shown in Figure 3. The variables introduced in the construction of this network were: highest confidence (0.900), to have the certainty of selecting the most likely interactions between the selected proteins, active interaction sources (texmining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence), to ensure a bird's eye view of the interactions, and the maximum number of 20 nodes in the first layer added, to ensure the introduction of missing links between the selected proteins, on which occasion CD44 and MMP2 genes were added to the study. Finally, three groups of proteins were differentiated based on functional affinities: Group 1, colored in red in Figure 3, which comprises 25 proteins, mainly integrins (CD44, COL18A1, DMP1, GP6, IBSP, IBSP, ITGA1, ITGA10, ITGA11, ITGA2, ITGA3, ITGA4, ITGA5, ITGAL, ITGAV, ITGB3, ITGB5, ITGB6, ITGB7, ITGB8, MMP2, NANOGP8, SPP1, TNC, TNN and TNR), the light green colored group 2, which comprises 3 members of the large collagen family (COL15A1, COL4A3 and COL4A4), and group 3, with 2 members (COL4A1 and COL4A2). Interactions between proteins in different groups are marked by dashed lines. In the network, most interactions are realized between integrins, transmembrane proteins, which enter the structure of cell-cell and cell-extracellular matrix junctions, and between collagens, which form the largest proportion of the extracellular matrix. The construction of this network made it possible to map the general interactions between the selected genes and to identify the proteins interposed between some of the proteins encoded by the selected genes. Thus, between COL4A1 and COL4A3 or between COL4A2 and COL4A3 is interposed the protein GP6 (platelet glycoprotein VI), a collagen receptor that plays an important role in thrombus formation, blood platelet aggregation [61] and, in some cancers, promotes metastasis, through interaction with cancer cell-derived galectin-3 [62], making it a putative target for anticancer therapies. Between the B3, B5 and A4 integrins and CD44, which interact with type IV collagens, intervenes SPP1 (secreted phosphoprotein 1 gene), present mainly in mineralized matrices. In head and neck squamous cell carcinoma, high SPP1 mRNA levels were correlated with poor overall survival, being a tumor biomarker [63] and may also be targeted in personalized therapies.

Next, to clarify the interactions between the overexpressed genes, a network was constructed for each sample having at least three overexpressed genes (Figure 4), with a confidence level of 0.900, and including only the over-

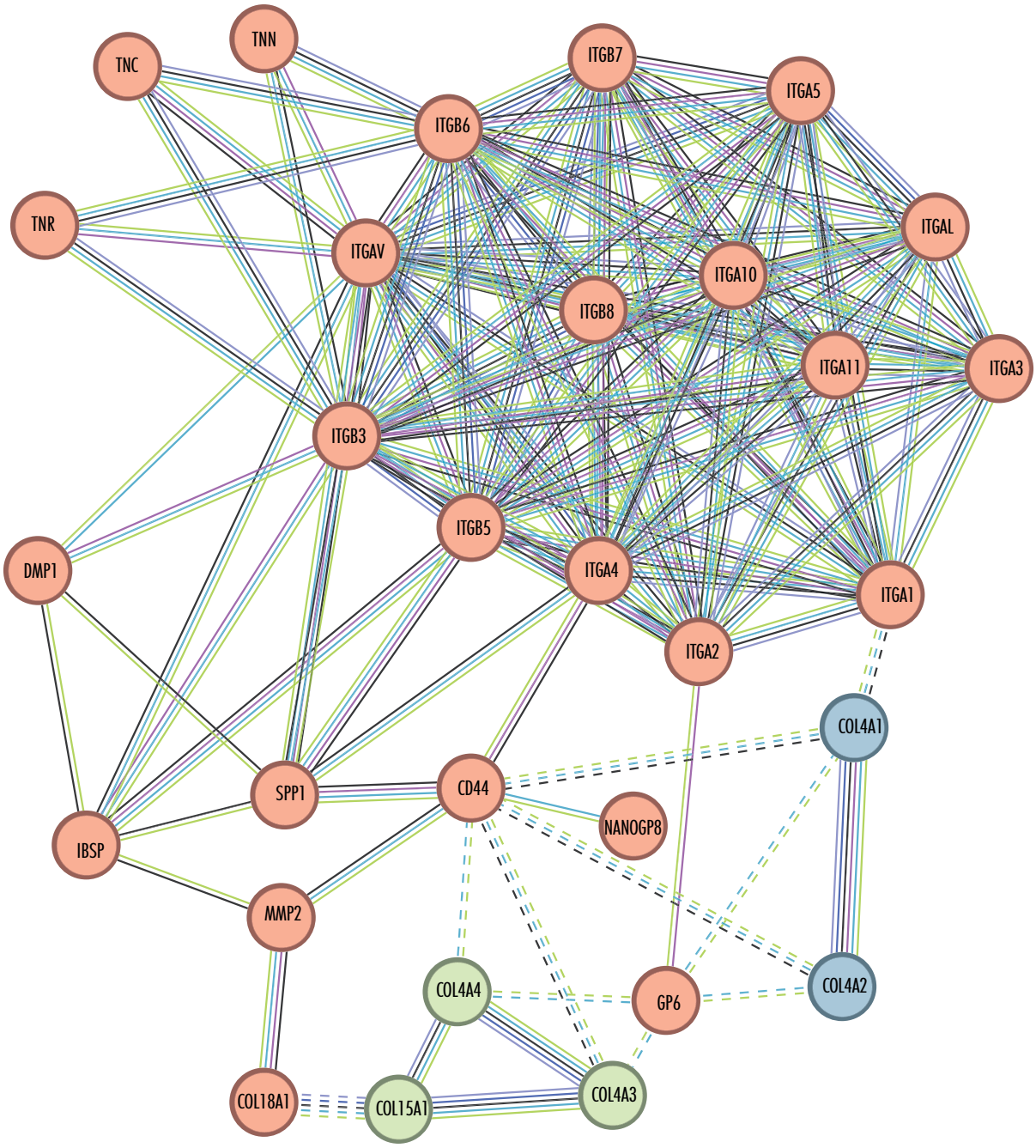

Fig. 3. The network of interactions between selected genes constructed with STRING (https://string-db.org/), with a confidence of 0.900 and including 20 additional interactors, to bind all the selected genes in the network and to identify the linking proteins between them. Using k-means clustering, the genes were grouped into three clusters between which the interactions are highlighted with dashed lines.

expressed genes, without any additional interactors. In the sample from patient 3 (Figure 4A), where CD44, ITGA4 and ITGAV genes are overexpressed, integrin A4 occupies a central place, interacting strongly with integrin AV and less with CD44. Increasing the number of overexpressed genes in the sample from patient 4 to six (CD44, COL18A1, COL4A1, COL4A1, COL4A2, ITGA4 and ITGAV) allowed the realization of a more complex network (Figure 4B), where one pole is occupied by collagens IV (A1 and A2) and the other by integrins AV and A4, linked to the CD44 receptor. Outside the network is collagen XVIII, which needs MMP2 to be integrated into it. In the sample from patient 5 most of the genes are expressed (CD44, COL18A1, COL4A2, COL4A2, ITGA4, ITGAV, ITGB3 and MMP2), allowing for an increase in the complexity of the interactions between them (Figure 4C). AV, A4 and B3 integrins are clustered in the left pole, with complex interactions between them, and the collagens IV and XVIII are localized on the right side. Interactions between collagen IV A2 and integrins are mediated

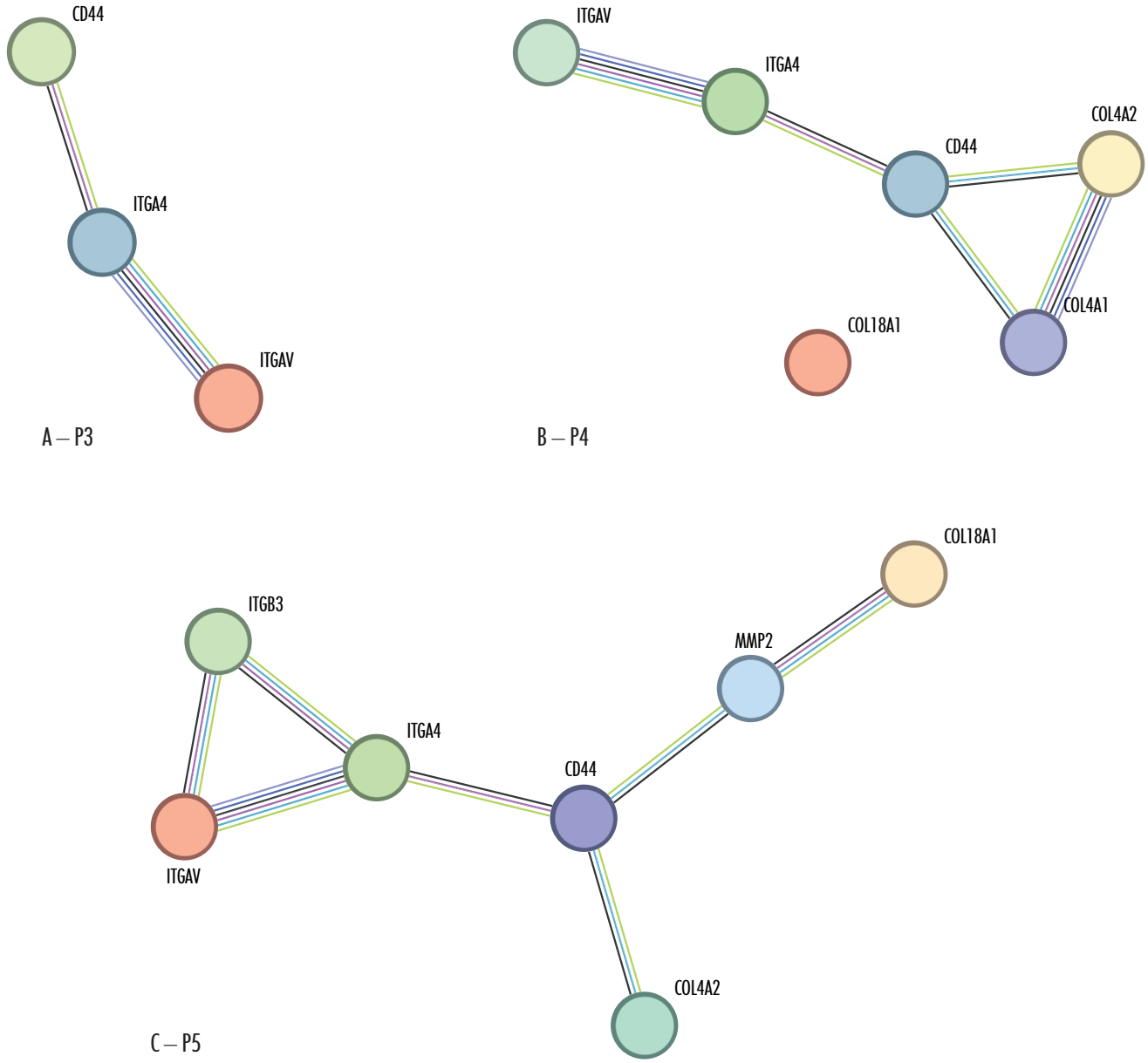

Fig. 4. Protein-protein interaction network constructed with STRING (https://string-db. org/), for samples with at least 3 overexpressed laryngeal squamous cell carcinoma genes, for patients P3 (A), with three proteins (CD44, ITGA4 and ITGAV), P4 (B), with six proteins (CD44, COL18A1, COL4A1, COL4A2, ITGA4 and ITGAV) and P5 (C), with seven proteins (CD44, COL18A1, COL4A2, ITGA4, ITGAV, ITGB3 and MMP2). For the realization of the networks, the confidence level was 0.900.

by CD44, and interactions between collagen XVIII A1 and integrins require two mediators, MMP2 and CD44.

Conclusions and future perspectives

By quantifying the expression levels of CD44, COL15A1, COL18A1, COL4A1, COL4A2, COL4A3, ITGAV, ITGA4, ITGB3 and MMP2 genes in the five patients, a differentiation between oropharyngeal squamous cell carcinoma and laryngeal squamous cell carcinoma was observed, in the sense that the second type of cancer causes the overexpression of a higher number of genes, some of them (CD44, ITGA4 and ITGAV) in all three patients analyzed, and CD44, COL18A1, COL4A1, COL4A2, ITGA4, ITGAV, in patients P4 and P5. After the building of the interaction network using STRING (https://string-db.org/), some proteins, such as COL18A1, were found not to interact directly with any of the proteins in the group, and therefore additional interactors were introduced (e.g. CD44 and MMP9, whose expression levels were quantified, SPP1 and GP6, whose expression levels were not analyzed). Due to the large number and complexity of the interactions between integrins and integrin A1 with the A1 subunit of collagen IV, further studies should increase the number of integrin family members included in the selection of genes analyzed. Integrins

are membrane receptors for extracellular signals that alternatively activate the PI3K–AKT–mTOR signaling pathway. As this pathway is very important for tumor processes, it is necessary to quantify the mRNA expression of some of its members and to establish possible correlations between integrin expression and the expression of these members. Finally, validation of the results and drawing meaningful conclusions in epidemiologic and therapeutic contexts requires an increased number of patients and genes.

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