Epithelial-Mesenchymal Transition: its impact on lymph node metastasis in oral squamous cell carcinoma

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Abstract

Background: Epithelial-mesenchymal transition (EMT) enables tumor cell invasion and metastasis. Many studies have demonstrated EMT's critical role in lymph node metastasis in oral squamous cell carcinoma (OSCC). During EMT, epithelial cancer cells lose intercellular adhesion and apical-basal polarity and acquire mesenchymal properties like motility and invasiveness. A major feature of EMT is cadherin switching, involving downregulation of E-cadherin and upregulation of N-cadherin. The TGF-β/SMAD pathway can also induce EMT. We aimed to evaluate EMT markers as predictors of lymph node metastasis in OSCC.

Methods: We performed genetic profiling of 159 primary OSCCs from TCGA, analyzing expression of EMT markers including cadherin switch genes (CDH1, CDH2), TGF-β/SMAD pathway genes, SNAIL, and keratins. Samples were divided into high (stage III-IV) and low (stage I-II) grade groups. Differential expression analysis was performed.

Results: TGF-β/SMAD pathway genes like SMAD6 were upregulated in high-grade tumors. N-cadherin and SNAIL2 were overexpressed in node-positive tumors. Keratins were downregulated in these groups.

Conclusion: Our findings demonstrate EMT marker expression correlates with lymph node metastasis in OSCC. Developing therapies targeting regulators like N-cadherin may prevent metastasis and improve outcomes. Further research is warranted to elucidate EMT signaling in OSCC progression.

Introduction

Oral squamous cell carcinoma (OSCC) is a malignant epithelial tumor arising from the mucosal surfaces of the oral cavity. Despite advances in treatment, OSCC continues to have poor survival rates, with 5-year survival around 50% [1]. The presence of cervical lymph node metastasis is the most significant prognostic factor, mandating treatment intensification and poorer outcomes [2, 3].

The development of lymph node metastasis requires that tumor cells detach from the primary site, migrate through the lymphatic system, and establish new tumors in the lymph nodes [4]. This process involves a phenotypic transition known as epithelial-mesenchymal transition (EMT). During EMT, epithelial tumor cells lose intercellular adhesion properties and apico-basal polarity, and acquire increased motility and invasiveness [5, 6].

A key molecular alteration in EMT is cadherin switching: the downregulation of E-cadherin and upregulation of N-cadherin. E-cadherin is an adhesion molecule that maintains intercellular contacts in epithelial tissues. The loss of E-cadherin disrupts adherens junctions, releasing β-catenin into the cytoplasm. This leads to activation of signaling pathways involved in EMT, such as TGF-β/SMAD [7, 8]. The concomitant increase in N-cadherin further augments cell motility and invasion [9].

Other EMT-related pathways include upregulation of transcriptional factors like SNAIL, ZEB1, and TWIST, which repress E-cadherin expression [10]. Transforming growth factor beta (TGF-β) signaling can also induce EMT through both SMAD-dependent and independent mechanisms [11].

Overall, the induction of EMT allows OSCC cells to acquire a mesenchymal phenotype permissive for migration and metastasis [12, 13]. Elucidating the molecular underpinnings of EMT may reveal therapeutic targets to halt lymph node spread.

In this study, we aimed to evaluate the significance of EMT markers, particularly cadherin switching and TGF-β/SMAD signaling, in predicting lymph node metastasis in OSCC. We performed genetic profiling of primary OSCCs to examine the expression of EMT-related genes in relation to pathological lymph node status.

Material and Methods

Molecular data sets of 528 head and neck carcinoma patients were obtained from TCGA data portal (https://cancergenome.nih.gov/) and the Genome Data Analysis Center (GDCA). Genomic processing of the molecular data sets was done using cBioPortal for Cancer Genomic analysis (http://www.cbioportal.org/).

The molecular datasets included genetic analysis based on whole-genome sequencing. The HPV status was defined using an empiric definition of > 1,000 mapped RNA-seq reads, primarily aligning to viral genes E6 and E7[16]. The HPV status by mapping of RNA-seq reads was concordant with the genomic, sequencing and molecular data, and indicated that 36 tumors were HPV(+) and 243 were HPV-negative (Supplementary data: Table 1.1). To eliminate unnecessary molecular or genetic diversity, only HPV negative and pathologically-proven oral cavity tumors were included in this study (n = 159) (Supplementary data: Table 1.1).

The selected genes that were analyzed were: “VIM”, “SNAI1”, “SNAI2”, “TWIST”, Karatins genes (51 genes), SMAD genes, MET pathway genes (by mSIGdb: http://www.gsea-msigdb.org/gsea/msigdb/); Cadherin genes (CDH1, CDH2); and TGF pathway genes (84 genes). Samples were then divided into two main groups: low-grade, which included samples that were staged pathologically as stage I and II according to the AJCC 8th edition, and high-grade tumors, which included tumors that were pathologically staged as stage III and IV.

Main packages used for technical analysis and filtering: Deseq2 for gene normalization and analysis of differentially expressed genes, while the P value for filtering was < 0.1.

For gene clustering, the principal Component Analysis (PCA) technique was used, and Uniform Manifold Approximation and Projection technique was used for visualizing the clustering patterns.

For group comparison, the Bonferroni correction was used, the P value was adjusted to < 0.1, positive fold indicated differentially expressed genes in the high-stage tumors (Stage III and IV), while negative fold

changes indicated genes differentially expressed in the low-stage tumors (Stage I and II).

Clinical features versus comparison: To evaluate significant differences found in the selected gene in different clinical groups, gene expression was statistically evaluated using Kruskal-Wallis or Wilcox test; based on the number of groups in each feature, significantly expressed genes were set at P value < 0.05.

Overall survival (OS) and recurrence-free survival (RFS) were estimated from the clinically available data using Kaplan-Meier analysis. Follow-up time was defined as the time that passed from the date of the initial diagnosis as seen on the pathological report of the biopsy until either the date of death or the last clinical follow-up as recorded in the files.

Results

Data on gene expression were available on TCGA for 159 patients (OSCC patients, HPV negative).

Clinical and pathological data of the entire cohort are summarized in Table 1. The mean age at diagnosis was 62 ± 13 years with a male-to-female ratio of ~ 2:1. Eighty-two (51%) patients had a history of tobacco exposure (with an average of 47 pack-years), and 101 (63%) patients reported alcohol consumption. The mean follow-up for the entire cohort was 26 months.

Table 1
clinical and disease-related data of the study cohort.

Characteristic	Study cohort	"Low Stage" group	"High Stage" group	P value
Num.	159	47	112	
Mean age (± STD)	62 ± 13 years	64 ± 2.0 years	61 ± 2years	0.3
Male / Female	105/54	26/21	78/34	0.06
Tobacco exposure	82 (51%)	5 (10%)	77 (69%)	0.001
Num. (%)	47	16.13	27.91	
Av. Pack/year				
Alcohol consumption (%)	101 (63%)	24 (51%)	77 (68%)	0.2
Primary Tumor Site (%)	70 (44%)	24 (51%)	46 (41%)	0.04
Oral tongue	25 (15%)	4 (8%)	21 (19%)	
Floor of mouth	8 (4%)	2 (4%)	6 (5%)	
Buccal Mucosa	5 (2%)	0	5 (4%)	
Alveolar ridge	4 (2%)	0	4 (3%)	
Hard Palate	1 (0.6%)	0	1 (0.8%)	
Lip	46 (28%)	17 (36%)	29 (26%)	
Oral Cavity*				
p N staging (by H&E)	81 (50%)	47 (100%)	34 (30%)	-
N0 (%)	35 (22%)	0	35 (31%)	
N1 (%)	2 (1%)	0	2 (2%)	
N2A (%)	20 (12%)	0	20 (18%)	
N2B (%)	12 (7%)	0	12 (10%)	
N2C (%)	1 (0.6%)	0	1 (0.8%)	
N3 (%)				

*Unspecified site in oral cavity

** Pathological staging according to the American Joint Committee on Cancer (AJCC).

¥ for statistical analysis, close margins were considered positive.

Characteristic	Study cohort	“Low Stage” group	“High Stage” group	P value
pT staging	9 (5%)	7 (15%)	2 (1.7%)	-
T1 (%)	54 (33%)	40 (85%)	14 (13%)	
T2 (%)	45 (28%)	0	45 (40%)	
T3 (%)	50 (31%)	0	50 (44%)	
T4a (%)	1 (0.6%)	0	1 (0.8%)	
T4b (%)				
TNM staging **	7 (4%)	7 (15%)	0	
Stage 1 (%)	40 (25%)	40 (85%)	0	
Stage 2 (%)	41 (26%)	0	41 (36%)	
Srage 3 (%)	69 (43%)	0	69 (61%)	
Stage 4a (%)	2 (1%)	0	2 (2%)	
Stage 4b (%)				
Surgical Margins status	103 (64%)	35 (74%)	69 (61%)	0.06
Negative margins (%)	19 (12%)	5 (10%)	14 (12%)	
Positive margins¥ (%)	37 (23%)	7 (16%)	30 (27%)	
Close margins¥ (%)				
Overall survival (months)	32 months	36 months	19 months	0.01
*Unspecified site in oral cavity				
** Pathological staging according to the American Joint Committee on Cancer (AJCC).				
¥ for statistical analysis, close margins were considered positive.				

The most common primary tumor site was the oral tongue (44%), followed by the floor of the mouth (15%), with the remainder distributed between the buccal mucosa, hard palate, and alveolar ridges.

Lymph node dissection (selective and radical) was performed in 137 (86%) patients. 70 (44%) patients had lymph node metastasis, as seen on histopathology, with an average of 2 positive lymph nodes for each patient.

the study cohort was divided into two groups based on the pathological staging. High-stage samples included patients who were pathologically staged stage III and IV (112 patients), and Low-stage samples included stage I and II (47 patients).

Cross-tab analysis: recurrent group versus the cohort (Table 1)

According to the TNM staging (8th edition), the study population was divided into two subgroups. The high-stage group included 112 patients. 41 patients were diagnosed with stage III disease, while the remaining 71 with stage IV disease. The male-to-female ratio in this group was 78/34, and the mean age at diagnosis was 61 ± 2 years. Seventy-seven (69%) patients of this group also reported substantial tobacco exposure (average 28 packs/year); this finding was significantly higher than the Low-stage group (*P value .001*).

The majority of the index primary tumors in the High-stage group, similar to the study cohort, was the oral tongue (46 patients, 41%) followed by the floor of the mouth (21 patients, 19%). In the low-stage group, the oral tongue was also the primary tumor site (24 patients, 51%), while the floor of mouth composed only 5% of the primary tumors (*P-value = 0.04*).

The patients' survival status was studied as a function of the recurrence status. The overall survival was significantly lower among patients in the High-stage group (mean: 19 months versus 36 months, *P value = .01*).

Genetic profiling analysis

The genetic profiling of 159 primary tumors for the plotting of the selected genes "VIM", "SNAI1", "SNAI2", and "TWIST1", "Karatins genes which included 51 genes ("KRT1" "KRT10" "KRT13" "KRT14" "KRT15"….); the "cadherin switching" pathway genes, i.e., down-regulation of E-cadherin and up-regulation of N-cadherin in each group (Comparative RNA analysis). We also analyzed TGF pathway genes such as "BAMBI," "CREBBP", "EP300", and "E2F4" and SMAD genes expression.

For Pathological TNM staging: genetic analysis identified a set of TGF signaling genes overexpressed in the High-stage tumor group *(P value < 0.1).* As demonstrated in Fig. 1, the genes "CREBBP", "SMAD6", "CHRD", "EP300", "BMP4", and "PITX2" were found to be upregulated as the tumor stage advanced. Those genes are known for their transcriptional and co-activator role in several diseases and tumors.

For the pathological N staging: all the investigated gene groups were found to have a role in determining the N stage. In the TGF group, the genes "ACVR1", "PPP2R1B", "GDF7", "RBX1", and "MAPK3" were upregulated in positive N pathological staging, "SNAIL2" which is known for promoting migration in specific cells during the development process was also significantly upregulated in the N positive group, especially in the N2 group. On the other hand, Keratin genes were upregulated in the pathological N-negative patients. The gene "KRT80" was significantly upregulated in the N-negative patients, while KRT78 was upregulated in both N-negative and pN1 patients. "CDH2" gene is a neuronal cadherin, also known as N-Cadherin, associated with the cadherin switching" pathway; was significantly upregulated in the pathological positive N stage. This gene is the critical gene in the formerly mentioned pathway, and its upregulation indicates the presence of the EMT process (Fig. 2).

Keratin and TGF genes were also significantly overexpressed in advanced diseases in terms of pathological T staging. The genes KRT18 and RPS6KB1 were upregulated in stage T2, T3, and T4 disease and downregulated in T1 disease. On the other hand, the genes KRT76 and EP300 were overexpressed in T1 disease (Fig. 3).

Discussion

The Epithelial Mesenchymal transition (EMT) is a well-known mechanism in tumor advancement and progression. In this biological process the epithelial cells which are characterized by a tight cell-to-cell adhesion and basal polarity, lose those properties, and acquire weak cell-to-cell interactions, thus enabling them more migration and transition ability [17]. The presence of EMT markers has been known to promote metastasis and invasion in various types of cancers, such as breast cancer, lung cancer, colorectal cancer, and oral cancer [18].

In this study, we investigated the significance of EMT markers in predicting lymph node metastasis in OSCC.

Oral squamous cell carcinoma is composed of cancerous epithelial cells that are connected to one another by calcium-dependent bonds, those bonds regulated by a transmembrane protein called E-cadherin [19].

The biological switch of E-cadherin to N-cadherin enables the epithelial OSCC cells to assume a mesenchymal phenotype, which, among others, enables the epithelial cancer cells to gain the potential to invade the lymphatic cervical system by increasing their motility [2]. The switch from E-cadherin to N-cadherin initiates several processes: it mediates cell adhesion weakness by degrading the intracellular contacts and cell-to-cell adhesions, degrades the intracellular contacts, it promotes cancer cells to detach from their primary environment and enable them to invade the adjacent lymphatic system. Concurrently, the upregulation of N-cadherin promotes cell motility and increases resistance to apoptosis, facilitating lymphatic colonization and metastasis [2, 15].

In cancer progression, EMT type 3 is demonstrated, and specific biological and genetic markers characterize it. In this study, we analyzed the genetic expression of those markers in oral squamous cell carcinoma and its effect on the overall staging of the tumor, specifically on the presence of lymph node metastasis.

In oral cancer, lymph node metastasis is the most important prognostic factor in determining patient survival and tumor recurrence [20]. The loss of proper function of E-cadherin results from genetic germline or somatic mutation, or more commonly due to epigenetic alteration such as DNA hypermethylation in the promoter region of the E-cadherin gene in the cancerous cells [21]. E-cadherin in normal epithelial cells co-localizes with β-catenin and p120-catenin in the cytoplasmic membrane. This connection is important for the intercellular and intracellular junctions and to the adherence with the actin filaments [22]. Consequently, the loss of the proper function of E-cadherin will cause a detachment

between β-catenin and p120-catenin and their accumulation in the cytoplasm. Free excessive β-catenin in the cytoplasm can migrate to the nucleus, where it activates several transcriptional factors such as T-cell factor (TCF) and SMAD genes, this will eventually trigger TGF-β signaling pathways which are critical for tumor cell proliferation and migration [22]. In our study, SMAD6 along with other TGF-β genes expression were significantly correlated with TNM staging; it overexpressed as the disease advanced. This finding indicates that the TGF-β signaling pathways have been activated, leading to upregulating of several target genes (including SMAD genes); thus, the tumor cells can mimic critical mesenchymal characteristics such as invasion and prefoliation. In terms of loco-regional recurrence, SMAD4 and other TGF-β genes (TGF-β2) were also significantly upregulated. SMAD4 is a major transcription factor in the TGF-β2 pathway expression and activating [23]. In several studies, TGF-β genes were found to induce autophagy through the SMAD4 pathway, which had a critical effect on tumor growth and invasion [24]. However, SMAD4 is associated with a favorable prognosis and plays a significant role as a tumor suppressor gene in several solid tumors, such as breast and rectal cancer [25]. On the other hand, several *in vitro* studies demonstrated that SMAD4 is a major key player in the EMT process, and it is strongly related to the TGF-β signaling process [26]. The exact role of SMAD4 in the EMT process and its effect on the TGF-β pathways is yet to be completely understood. Pre-clinical studies regarding SMAD3 and its role in the EMT process indicate that intracellular levels of SMAD3 navigate the TGF-β signaling process [27]; in like manner, the intracellular levels of SMAD4 determines the response of the TGF-β pathway, high levels of SMAD4 may cause an effective inhibition of cells proliferation. In contrast, low levels may not be sufficient for proper cell proliferation inhibition and allow the initiation of the EMT process and cell motility and invasion [28].

As the main interest of our study was to evaluate the significance of EMT markers in the development of lymph node metastasis, we found that all the studied genes had a significant effect in determining the presence of lymph node metastasis. TGF genes, such as “ACVR1”, “PPP2R1B”, “GDF7”, “RBX1”, and “MAPK3” were upregulated in positive N pathological staging. Several whole-transcriptome chip and subsequent tissue microarray analysis have demonstrated that MAPK3 singling pathway is associated with lymph node metastasis in OSCC. High levels of MAPK-related proteins were associated with advanced tumor stage and lymph node metastasis [29]. The expression of TGF-β results in an affluence of metabolic dysfunction and promotes EMT which may lead to fibrosis and cancer [30]. Also, activated TGF-β stimulates different downstream signaling pathways, such as SMAD pathway. As a result, different kinase and signaling pathways, which generally act as an expression-regulating protein become dysfunctional; thus, uncontrolled proliferation and cell migration occur [31, 32]. SNAIL2 which is known for promoting migration in specific cells in the development process was also significantly upregulated in the N-positive group. SNAIL1,2 are master genes in regulating the expression and action of E-cadherin, which as mentioned before, is a key player in the EMT process. Several OSCC models showed that the expression of SANIL can successfully transfer the epithelial cells into a fibroblast-like appearance, which included vimentin filaments, E-cadherin/N-cadherin switching and almost complete lack of cell-to-cell adherence, and hemidesmosomes [33].

SNAIL2, which is also known as Slug, is a zinc-finger transcription factor and acts as a repressor of E-Cadherin transcription since it binds to the E-box in the promoter region of E-cadherin gene and inhibits its transcription; it also induces the activity of histone deacetylase genes which removes the acetyl group from the histone protein, consequently resulting in further preventing of E-cadherin gene [34]. Slug also binds to the cis-elements in the promoter region of the E-Cadherin gene, which acts as a further repressor for the transcription of E-Cadherin by recruiting chromatin-modifying proteins through the N-terminal domain [34]; this dual suppression ability resulted in tumor metastasis in several *in vivo* studies [35]. The resulting suppression of E-cadherin induces EMT in several ways: activation of SMAD pathways; mediating TGF-β pathway; repressing the expression of multiple encoding junctional proteins, such as desmosomes, desmoplakins, and tight junction protein claudin; and upregulating the expression of mesenchymal cell proteins including N-cadherin [36, 37]. Several clinical studies have shown that the upregulation of N-cadherin is significantly correlated with the aggressive and malignant behavior of OSCC. Also, it is strongly correlated with poor histological differentiation and lymph node metastasis [38].

These findings highlight the importance of developing targeted therapies to block N-Cadherin and other EMT markers to prevent or mitigate lymph node metastasis.

To conclude, understanding the intricate interplay between EMT, N-cadherin switching, SMADs, SNAILs, and lymph node metastasis is crucial in unraveling the molecular mechanisms underlying oral cancer progression. Further research is warranted to fully elucidate the signaling pathways and molecular players involved in these processes. Targeting N-cadherin switching holds excellent potential for developing effective therapeutic interventions to prevent or treat lymph node metastasis, ultimately improving the survival rates and quality of life for oral cancer patients.

Declarations

Data Availability: All data generated or analyzed during this study are included in this published article [and its supplementary information file: Supplementary Table 1.1.].

Competing interests. The authors declare no competing interests.

Ethic approval. No ethical approval is needed for online data sets.

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Figures

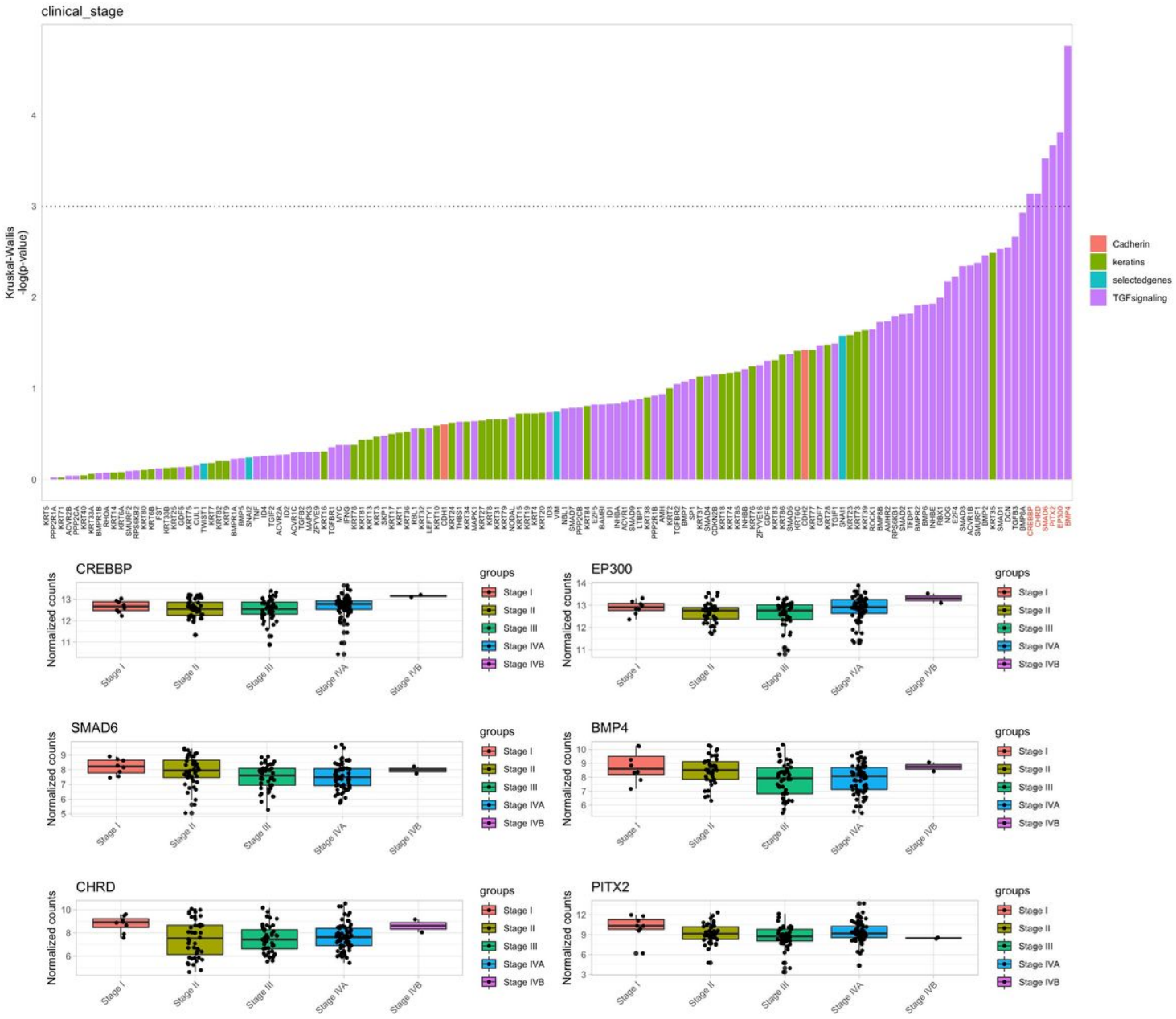

Figure 1

TNM staging and genetic analysis comparison using the Kruskal-Wallis test. The top bar-plot shows the statistical significance of differences in the selected group per gene. Gene over the dash-line presents a significant difference in expression between the groups (p-value < 0.05). Regarding TNM staging, TGF genes were mainly upregulated in the high-stage group.

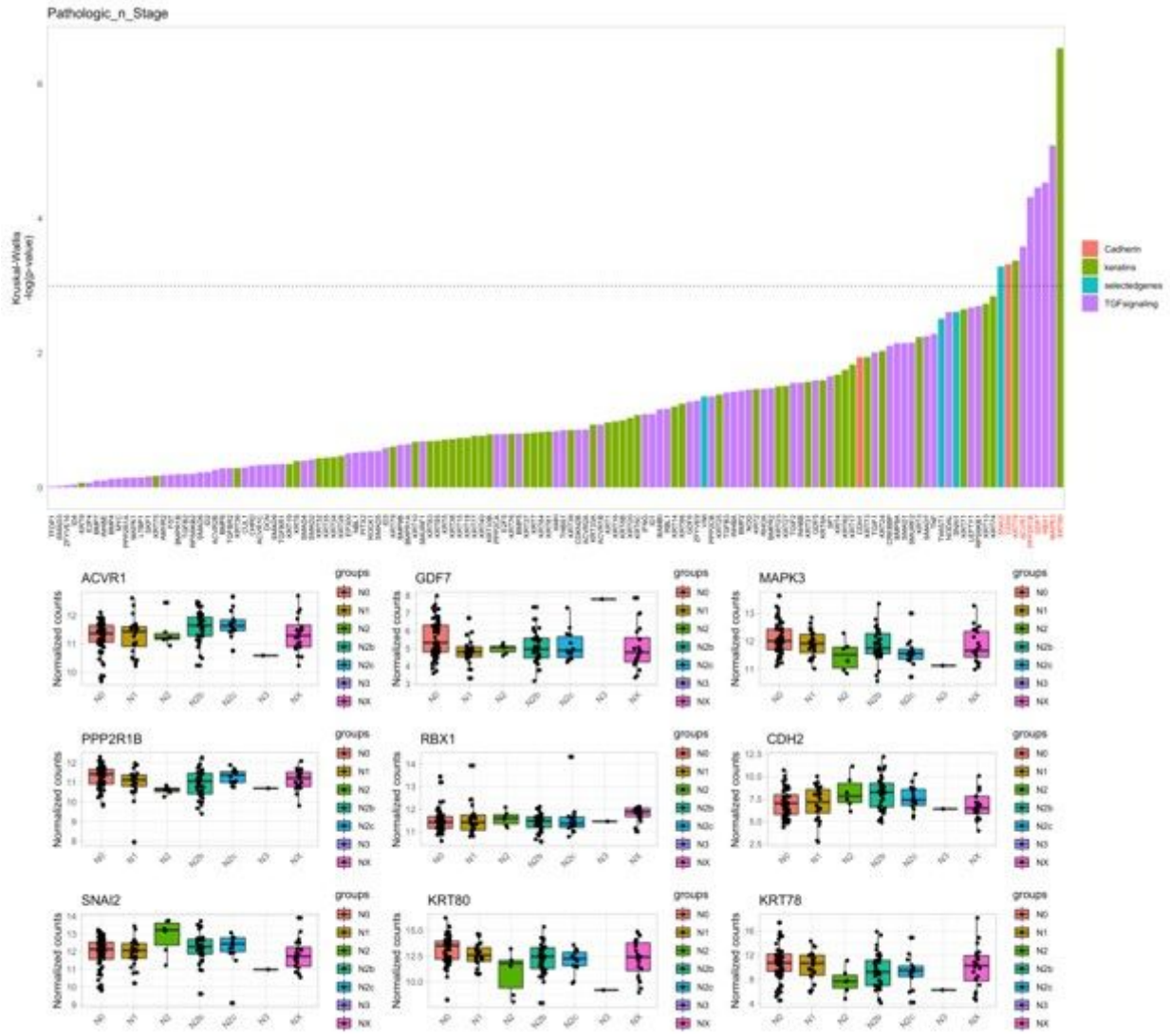

Figure 2

Pathological N stage and genetic analysis comparison using the Kruskal-Wallis test. The top bar-plot shows the statistical significance of differences in the selected group per gene. Gene over the dash-line presents a significant difference in expression between the groups (p-value < 0.05). In terms of pathological N staging, SNAI2 gene, CDH2 gene, and several TGF genes were upregulated in the high-stage group. On the other hand, Keratin genes were upregulated in N0 and N1 groups.

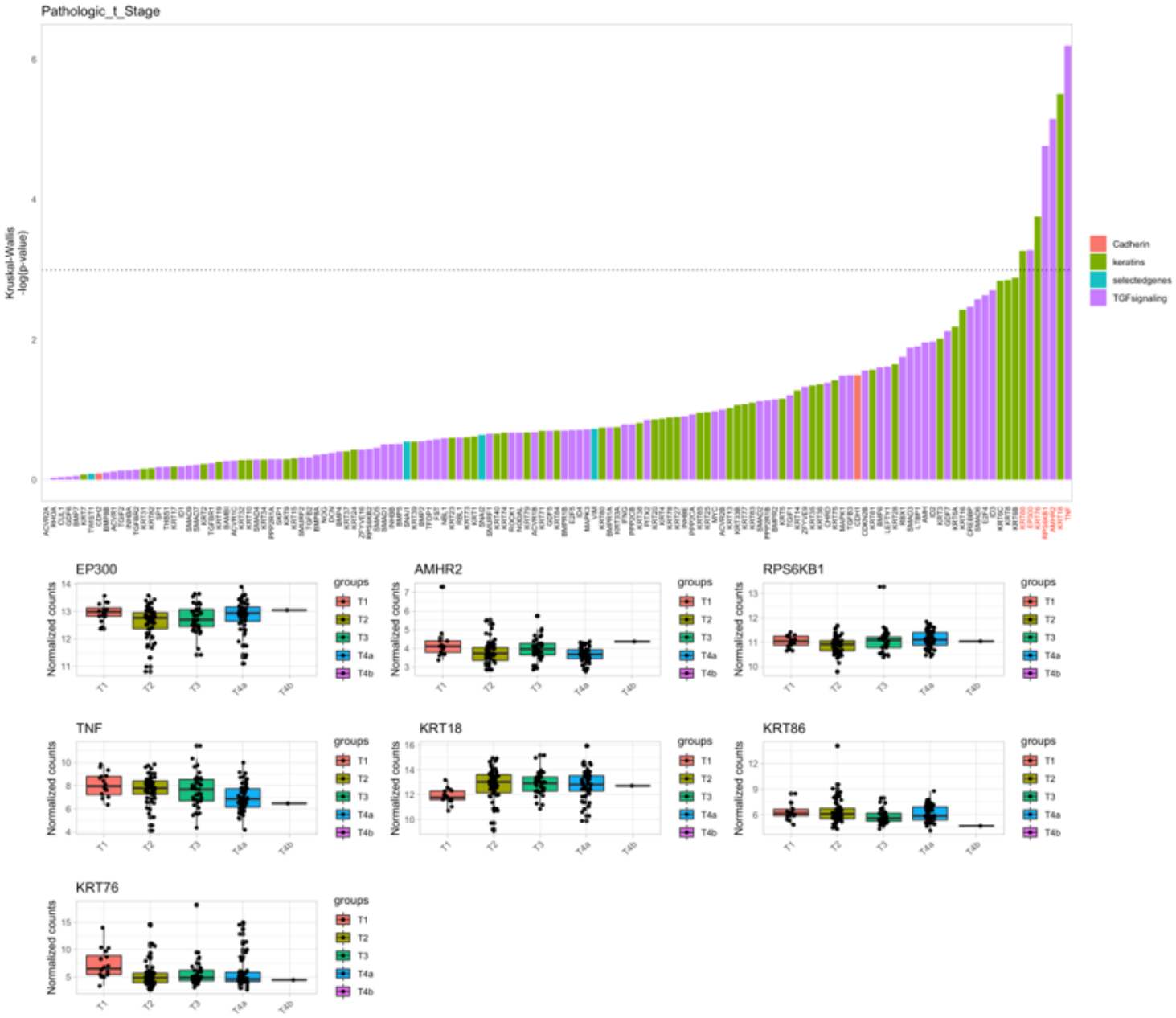

Figure 3

Pathological T staging versus genetic analysis using the Kruskal-Wallis test. The top bar-plot shows the statistical significance of differences in the selected group per gene. Gene over the dash-line presents a significant difference in expression between the groups (p-value < 0.05). Keratin and TGF genes are significantly overexpressed in advanced diseases.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- table1.1.xlsx