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Association Between PD-L1 and Histatin1, 3 Expression in Advanced Head and Neck Squamous Cell Carcinoma

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Abstract. *Background/Aim: The prognosis of advanced stage head and neck squamous cell carcinoma (HNSCC) has remained unimproved for the past decades. Therefore, novel diagnostic markers and treatment options are required. Recently, an inhibitor for immune checkpoint program death ligand-1 (PD-L1), was approved by the FDA, and used in HNSCC patients. Histatins (HTNs), one of the common antimicrobial peptides in saliva, have demonstrated wound healing and antifungal capabilities and other functions on the oral epithelium. Dysregulation of HTN1 and HTN3 has also been reported in HNSCC through genomic and proteomic studies. This study aimed to investigate the association between histatins (HTN1 and HTN3) and PD-L1 in advanced HNSCC. Patients and Methods: Data of gene expression in HNSCC were collected from TCGA and analyzed using a data-mining platform website (https://ualcan.path.uab.edu/). Tissue microarrays containing 98 samples of HNSCC patients and non-neoplastic controls were immunolabeled against PD-L1, HTN1, and HTN3. The immunohistochemistry results were quantified using ImageJ. Results: The expression of PD-L1 and HTN1 was significantly higher in tumors than normal tissues ($p<0.001$), but no significant difference was found regarding HTN3. Metastatic HNSCC samples exhibited significantly higher expression of PD-L1 ($p<0.018$), compared to the non-metastatic group. Association between HTN1 and HTN3 was found using Pearson correlation coefficient ($r=0.603$, $p<0.001$). No overall survival difference was evident among our samples. Conclusion: PD-L1 and HTN1 are associated with the progression of HNSCC. PD-L1 expression correlated with that of HTN3.*

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In 2020, head and neck cancers (lip, oral cavity, and pharynx) were estimated to account for over 680,000 new cases and nearly 350,000 deaths annually. The incidence and prevalence vary markedly by geographic location. For the Southeast Asia region, head and neck cancers ranked seventh among all cancer types regarding incidence and mortality rate (1). Head and neck squamous cell carcinoma (HNSCC) is the most frequently histologic diagnosis, present in more than 90 percent of the head and neck cancer cohort. The overall 5-year survival of patients with HNSCC has remained at approximately 50% over the past three decades, without any improvements (2). Molecular mechanisms underlying HNSCC progression have been relentlessly pursued to discover prognostic or diagnostic markers, as well as novel specific targets for early diagnosis and therapeutic purposes (3).

Our view on the inflammatory process and cancer development and progression has been challenged recently by the promising results of cancer immune therapy. For example, it has been shown that programmed cell death 1 and programmed death ligand 1 (PD-1/PD-L1) immune checkpoint proteins play an essential role in the T-cell exhaustion mechanism. This leads to immune evasion in several cancers, including HNSCC (4). After FDA approval of PD-L1 inhibitors, many clinical studies have reported the prognostic efficacy and significant role of PD-L1 (5). For instance, the releasing of IL-10 and PD-L1 from the $CD163^+CD204^+$ tumor-associated macrophages (TAMs) subset can promote invasion and metastasis in HNSCC, as well as associated with 5-year progression-free survival (6). Conversely, PD-L1 positive and infiltrated $CD8^+$ or $CD4^+$ T cells have been associated with better survival rate of patients with oral squamous cell carcinomas (5, 7). These results highlighted the complex role of PD-L1 in HNSCC progression when its evaluation combined with other markers.

Besides their role in the first line of innate immune defense against invading microbes, recently, the roles of antimicrobial peptides (AMPs) in cancer have been highlighted (8). In HNSCC, upregulation of the human β-defensin family was related to poor prognosis due to increased vessel formation in lymph nodes (9). LL-37, the only member of human cathelicidin AMPs, demonstrated antitumor efficacy in nude mice by inhibiting the growth and proliferation of breast cancer (10). The over-expression of LL-37 was implicated in HNSCC progression and a clinical study revealed that serum LL-37 is significantly associated with the prognosis of patients with HNSCC following radiotherapy (11).

Histatins (HTNs) are one of the major antimicrobial peptides frequently expressed in normal salivary glands, oral mucosa, and oral fluid. HTNs are low molecular weight proteins rich in histidine. They are well-known for their anti-fungal and maintenance of oral tissue integrity capabilities. In human, only two genes encoding this AMP family exist, *HTN1* and *HTN3*, despite many derivative fragments (HTN2, 4, 6-12) (12). Besides their antimicrobial property, the biological effects of HTNs on various cell types such as their function as wound-healing promoting factors in human saliva by accelerating cell migration in oral keratinocytes, fibroblasts, and endothelial cells have been well established (13). HTN1 and HTN3 also positively affected proliferation of chondrocytes and human gingival fibroblasts (14, 15). Moreover, HTN3 was related to recurrent events of acinic cell carcinoma, a salivary gland malignancy, by fusing with the Myb/SANT DNA Binding Domain Containing 3 (*MSANTD3*) gene (16). In a gene profile analysis of HNSCC tissue samples, *HTN1* was one of the top fifty dysregulated genes (17). High-throughput saliva proteomic analysis of HNSCC samples showed that HTN3 fragments are highly expressed (18). In addition, the gene expression signature of oral squamous cell carcinoma samples based on a GeneChips array study suggested that HTN1 and HTN3 were highly expressed in advanced stage HNSCC (19, 20).

Even though dysregulation of HTN genes and proteins in HNSCC have been previously identified in several high-throughput studies as mentioned above, the evidence has not yet been translated to the clinical benefit, especially regarding the involvement of such genes in cancer inflammation and cancer immune response. Furthermore, the association between PD-L1 and HNSCC malignant transformation is still unclear, despite the fact that the prognostic efficacy of this immune checkpoint is well-studied. Therefore, this study identified the association between PD-L1 and histatins in HNSCC progression using human tissue microarray (TMA). Furthermore, clinicopathological characteristics were analysed with regard to the expression of PD-L1 and histatins. Finally, the correlation between PD-L1 and histatins was determined according to cancer stage and metastasis.

Patients and Methods

Human oral cancer tissue arrays. Head and neck cancer TMAs were purchased from US Biomax (ORC1021, Rockville, MD, USA). The microarray slide contained 98 cases of HNSCC and normal tissues, accompanied with pathological grade and TNM clinical stage data. The tissues were derived from 61 male individuals and 29 female individuals (male:female ratio around 2:1). The samples were divided into 71 cancer and 19 normal samples. Sites of occurrence were the tongue (n=57), buccal mucosa (n=5), palate (n=1), floor (n=3), gingiva (n=9), hypopharynx (n=13), and unidentified (n=10). Because of the random distribution of lesions and incomplete documentation, sites of the lesions were not included in our analysis. The tumor lesions were identified by a trained oral pathologist.

Antibodies. Rabbit polyclonal histatin-1 antibody (ABIN1176447, antibodies-online GmbH, Aachen, Germany; dilution 1:50), histatin-3 antibody (SAB1402234, Sigma Aldrich, Darmstadt, Germany; dilution 1:50), and rabbit monoclonal PD-L1 [28-8] antibody (AB205921, Abcam, Boston, MA, USA; 1:100) were used for immunocytochemistry (IHC).

Immunohistochemistry. The tissue arrays were prepared using a previously described standard method with modifications (21). IHC was performed using a commercial kit following the manufacturer's protocol (Abcam AB64264). Briefly, the tissue array slides were deparaffinized in xylene, rehydrated in graded ethyl alcohol (100%, 95%, 70%), and then washed in running tap water. Antigen retrieval was performed in sodium citrate (pH 6.0) buffer with Tween-20 using microwave at the high heat setting for 1 min, followed by heat at the 50% setting for 2 min to maintain the boiling stage. The slides were then sequentially cooled, rinsed in running water for 10 min, and incubated in 3% H_2O_2 to deplete endogenous peroxidase activity. Next, the slides were washed for 3 min in phosphate-buffered saline (PBS; 2 times) and incubated in blocking solution for one hour. The antibodies directed against HTN-1 (Antibodies-online GmbH; ABIN1176447) and HTN-3 (Sigma Aldrich; SAB1402234) were used as indicated above to label tissues overnight at 4˚C. After washing with PBS, biotinylated secondary antibodies were applied for 30 min and streptavidin peroxidase for 10 min. For the PD-L1 labelling, antibodies directed against PDL-1 (Abcam AB205921) were used to incubate the TMA for one hour at room temperature. After washing with PBS, the amplifier and detector were both individually applied for 10 min according to the manufacturer's protocol for using the IHC detection kit (Abcam AB209101). Immunoreactivity colors were developed in DAB solution and slides were counterstained with Hematoxylin. Images were acquired using a slide scanner (V2000, Olympus, Tokyo, Japan) and Viewpoint software (Precipoint, Freising, Germany).

Image semi-quantification. Expression of HTN1, HTN3 and PD-L1 was semi-quantified using ImageJ Fiji software (Ver. 1.53e6, National Institute of Health, Bethesda, MD, USA) as described previously (22). Briefly, color deconvolution was applied to images, and converted to black and white images. Subsequently, the maximum threshold was adjusted to remove the background signal, and DAB signals were measured as the intensity per area. The result was then interpreted as the mean grayscale of the field.

TCGA data analyses. Gene expression of PD-L1 was retrieved from The Cancer Genome Atlas (TCGA) database. The dataset consisted of the pathological characteristics (tissue type, cancer stage, tumor grade) and clinical data (sex, metastasis level, survival data) of the patients. In total, 520 of HNSCC patients and 44 of normal patients were included in our analyses. Analyses were completed using the integrated data-mining platform website (23).

Statistical analysis. Data were analyzed using the statistical software SPSS 27 (Inc., Chicago, IL, USA). The normality was examined using the Shapiro-Wilk test. Association between cancer progression (tissue type, cancer stage, and metastasis) and gene expression (HTN1, HTN3, and PD-L1) was investigated by using the Student's *t*-test and ANOVA with multiple comparisons. The correlation between histatins and PD-L1 was analyzed with Pearson correlation coefficient; *p*-values <0.05 were considered as statistically significant.

Results

Cancer immune response correlates of HNSCC stage and nodal metastasis. To investigate HNSCC immune evasion, we analyzed PD-L1 cancer immune checkpoint gene expression in 564 cases of head and neck tumor samples and normal individuals from the TCGA database. First, we found that the expression of PD-L1 was significantly higher in tumors than in normal tissues (Figure 1A). Additionally, the cancer tissues were categorized into stages of cancer progression (stage I, II, III, IV). All cancer stages demonstrated significantly higher expression of PD-L1 compared to normal tissues. However, no significantly different expression was observed between cancer stages (Figure 1B). We further investigated the association of PD-L1 and nodal metastasis status to determine the involvement of immune maintenance in metastatic cancer. The findings revealed significantly higher expression in patients with stage 0 and 1 nodal metastasis (N0 and N1) cancer compared to normal tissues (Figure 1C). Sex variation was considered unrelated with HNSCC immune response as no difference in PD-L1 expression between males and females was observed (Figure 1D). Clinical survival records showed that patients in the early stage (High expression + Grade 1) with high PD-L1 expression had a slightly higher survival probability; however, the difference was not statistically significant (Figure 1E, F).

Histatins and PD-L1 were highly expressed in HNSCC samples. Antimicrobial peptides have recently been related to cancer progression especially involved with cancer inflammation and cancer immune response (8). Histatins are one of the major antimicrobial peptides frequently found to be expressed in normal salivary glands, oral mucosa, and oral fluid (13) and their functions in cancer have not been well characterized (8). Therefore, histatins may have a major role in tumor immune response in HNSCC. IHC was performed to investigate the expression of HTN1, HTN3, and PD-L1 in the HNSCC tissue array.

By using antibodies labeling HTN1, HTN3, and PD-L1 on TMA of HNSCC with various degrees of tumor progression, the differential expression of the individual proteins was revealed. HTN1 and HTN3 showed punctate staining in the cytoplasm of tumor cells (Figure 2A, C). Positive cytoplasmic staining of both histatins in human salivary acini and negative immunoreaction in fibroblasts of fibrosis tissues were used as a positive and negative control, respectively (data not shown). PD-L1 immunoreactivity was observed predominantly in the cell membrane of well-differentiated tumor cells, and scarcely observed in poorly differentiated tumor and basal cells. PD-L1 immunoreactivity was also observed in the cytoplasm of some tumor cells (Figure 2E). Weak immunolabeling was also observed in both normal and cancerous lesions (Figure 2B, D, F). Noteworthy, inflammatory cell infiltrations were generally observed in neoplastic tissues in our microtissue array, indicating HNSCC-related tumor inflammation (Figure 2A, C, E). The human tonsillar tissues were used as a positive control for PD-L1 (data not shown).

To analyze the differential expression of histatins and PD-L1, we quantified the positive labeling using a previously described computerized method (22). The digital images of staining of histatins and PD-L1 were scanned and analyzed by comparing normal and tumor groups. The color deconvolution process was performed before the quantification (Figure 2G). The samples distribution and mean expression levels of HTN1, HTN3, and PD-L1 were presented according to sex and tumor stage (Table I). Differential expression of each protein marker in HNSCC was generated by cluster analysis and illustrated using expression heatmap. According to the unbiased clustering method, the expression of HTN1 shared more common pattern with HTN3 than PD-L1 (Figure 2H). The correlation among different markers were also determined by Pearson correlation coefficient (r). A significant correlation between HTN1 and HTN3 (r=0.603, $p<0.001$) was found (Table II).

The clinicopathological characteristics were analyzed to determine factors associated with the expression of histatins and PD-L1. First, sex demonstrated no significant difference in the expression of all three proteins. Normal and cancer tissues were compared. Independent t-test revealed statistically significant differences between HTN1 and PD-L1 expression ($p<0.001$) (Figure 3A, Table III). To further demonstrate the involvement of histatins and PD-L1 in HNSCC clinical progression, the stained tissues were categorized according to their cancer staging. HTN1 expression differed significantly between cancer stages ($p=0.019$) (Table III). Samples in stage I, III, and IV showed significantly higher expression than normal tissue ($p=0.025$, 0.001, 0.046 respectively) (Figure 3B). PD-L1 expression also differed significantly between cancer stages (ANOVA, $p<0.001$) and all cancer samples showed higher expression than normal tissue ($p<0.05$) (Table

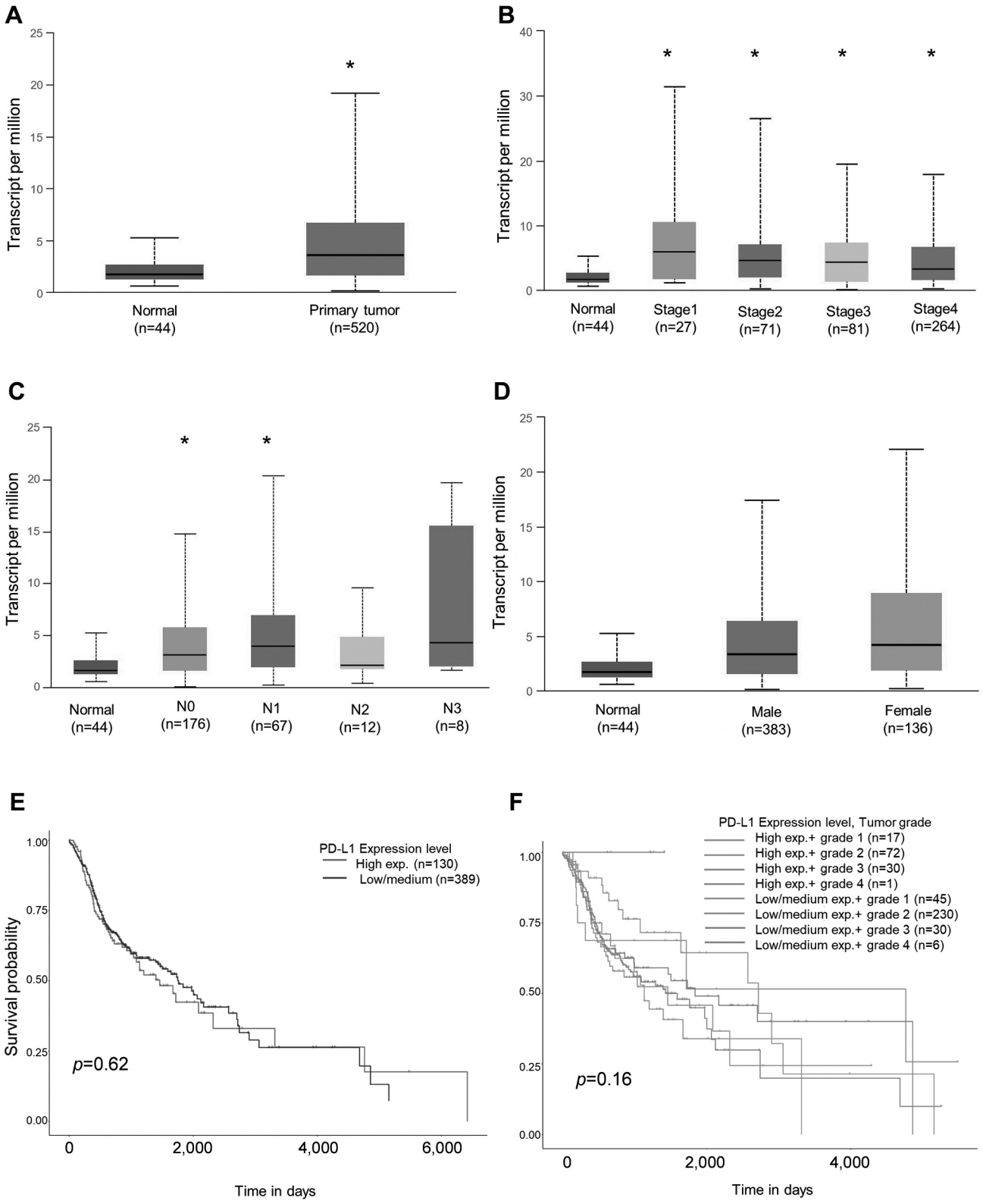

Figure 1. *TCGA database analyses of PD-L1 (CD274) gene expression in HNSCC according to sample types (A), cancer stages (B), nodal metastasis (C) and sex (D). The Kaplan-Meier survival according to the level of expression of PD-L1 were compared (E) and classified by tumor grades (F). Significant differences to normal tissue are symbolized by asterisks (*p<0.05).*

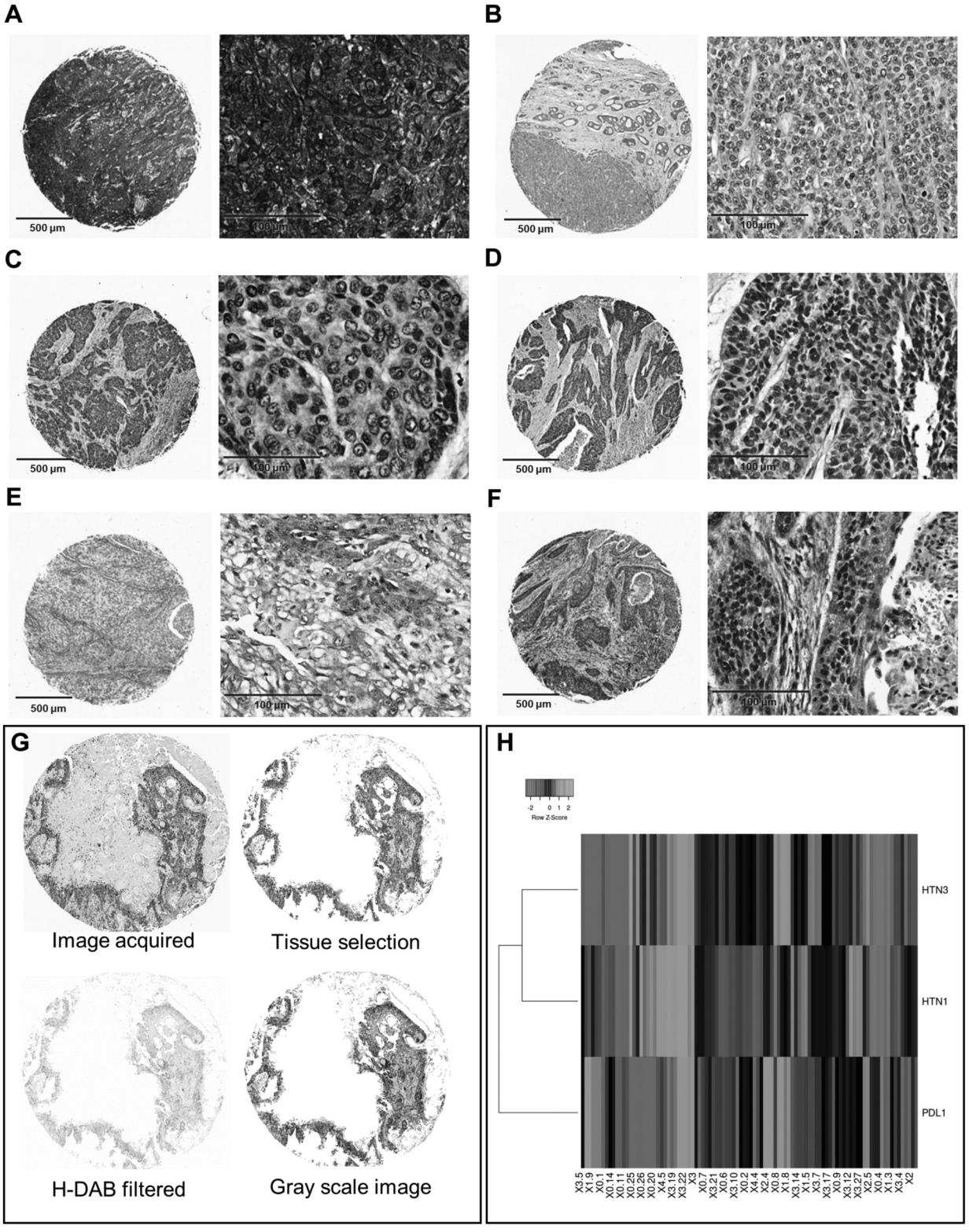

Figure 2. *HNSCC tissue microarray was immunostained for HTN1 (A and B), HTN3 (C and D), and PD-L1 (E and F). Tissue slides were scanned and immunohistochemistry results were acquired. The representative staining in tumor samples is shown: strong staining signal for HTN1, a moderate signal for HTN3, and moderate signal for PD-L1. (G) Extraction of H-DAB intensity and measurement process are demonstrated. (H) Cluster analysis results and expression heat map showing differential expression of HTN-1, HTN-3 and PD-L1 in normal and cancer tissue samples.*

Table I. *Analyses of tissue microarray parameters (sex, sample types, cancer stages) and HTN1, HTN3 and PD-L1 expression data.*

			HTN1		HTN3		PD-L1	
	N	Percentage	Mean	SD	Mean	SD	Mean	SD
Tumor stage								
Normal	19	21.1	75.90	8.01	109.08	9.65	117.49	8.06
1	11	12.2	118.67	10.53	135.46	7.93	125.35	6.83
2	13	14.4	103.75	7.71	132.28	8.49	138.47	5.92
3	35	38.9	114.10	5.85	121.19	5.46	126.13	4.31
4	12	13.3	119.13	15.88	131.53	9.60	138.61	6.44
Sex								
Male	61	67.8	106.66	4.55	121.58	4.01	125.39	3.24
Female	29	32.2	112.24	9.29	131.69	7.27	136.85	4.93
Total	90	100.0	100.96	39.5	124.3	31.24	128.48	24.38

Table II. *Pearson correlation coefficient between HTN1, HTN3 and PD-L1 expression in HNSCC samples. Significant differences to normal tissue control are symbolized with asterisks (*p<0.05, **p<0.001).*

		HTN1	HTN3	PDL1
HTN1	Pearson correlation coeff.	1	0.603	0.145
	p-Value		0.000**	0.154
	N	98	86	98
HTN3	Pearson correlation coeff.	0.603	1	0.284
	p-Value	0.000**		0.008**
	N	86	86	86
PDL1	Pearson correlation coeff.	0.145	0.284	1
	p-Value	0.154	0.008**	
	N	98	86	98

III, Figure 3B). However, there was no significant difference in HTN3 expression between cancer stages (Figure 3B). The cluster analysis of cancer stage between histatins and PD-L1 was demonstrated in a scatter plot. A linear relationship between HTN1 and HTN3 was observed (Figure 4A). However, a linear association between PD-L1 and HTN1, and PDL1 and HTN3 was established when normal tissues were excluded (Figure 4C and E).

PD-L1 was involved in clinical nodal metastasis. To investigate whether these tumor markers were involved in HNSCC metastasis, we excluded normal tissues and compared non-metastatic tumors with the metastatic group. The results revealed that PD-L1 expression was significantly higher in metastatic HNSCC (p=0.021) (Table III). In addition, we evaluated the association between nodal (N) metastasis involvement and each protein expression on the cluster analysis of N value (Figure 4B, D, F). Similar to cancer stage clustering, our findings showed a linear relationship between the expression of PD-L1 and that of both histatins when the normal samples were excluded.

Discussion

Recently, increasing evidence on the roles of inflammation in tumor development and progression has been reported (24). AMPS were described as one of the factors mediating tumor-associated inflammation, including the effects of human β-defensin-3 and LL37 on tumor-associated macrophages (TAMs) (10, 25). PD-L1, one of the crucial regulators for tumor-associated T cells, has been recognized as one of the crucial players in cancer progression (26, 27). In this study, the association between histatins and PD-L1 in HNSCC progression was demonstrated using both TCGA database and IHC to determine protein expression in HNSCC TMA. First, the results showed that PD-L1 was highly expressed in HNSCC tissues, albeit with no significant difference between cancer stages. These results indicate that PD-L1 contributes to an early neoplastic transformation by

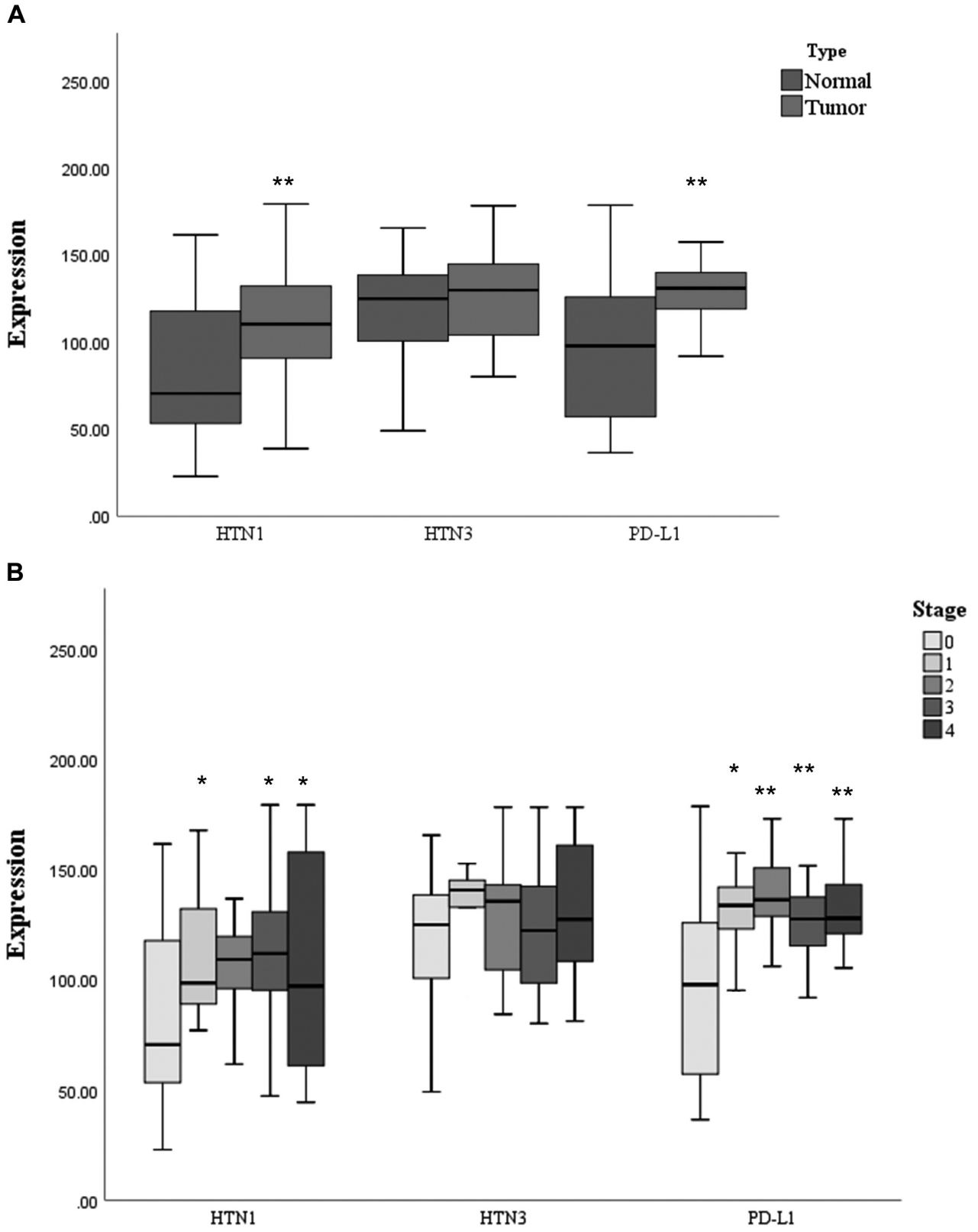

Figure 3. *Expression of HTN1, HTN3, and PD-L1 in HNSCC was classified according to tissue types (A), and cancer stages (B). Significant differences compared to normal tissue control are symbolized by asterisks (*p<0.05, **p<0.001).*

Table III. *Association of HTN1, HTN3 and PD-L1 expression with HNSCC clinicopathological characteristics (Tissue types, sex, cancer stages, metastasis status). Significant differences are symbolized with asterisks (*p<0.05, **p<0.001).*

Subset analysis		HTN1 *p*-Value	HTN3 *p*-Value	PD-L1 *p*-Value
Tissue types	Normal/Tumor	0.001**	0.675	0.001**
Sex	Male/Female	0.427	0.207	0.097
Cancer stages	0/I/II/III/IV	0.019*	0.660	0.000**
Metastasis status	Non-metastasis/Metastasis tumor	0.113	0.930	0.021*

facilitating tumor immune evasion (28), and is related to a lesser extent to HNSCC tumor progression. Further analyses on nodal metastasis showed higher expression of PD-L1 in non-metastasis and early metastasis (N0 and N1) samples. Kaplan–Meier survival using TCGA data showed no statistically significant differences in PD-L1 expression among cancer stages. Previous studies revealed that high expression PD-L1 alone was related to poor prognosis and metastasis in HNSCC (26, 29). However, high PD-L1 expression together with other parameters from immune cell such as $CD4^+$, $CD8^+$ and TAMs subsets were recognized as a good clinical prognostic marker for patients' survival (5, 7, 30). Furthermore, our results revealed no significant differential expression of PD-L1 between sexes, albeit the reports showing that females have a higher incidence of HNSCC with PD-L1 (26). This may be due to several factors, including different base populations, clones of antibodies, and techniques used to acquire the result.

Second, we investigated the relationship of histatins and oral AMPs with the progression of HNSCC. We found that HTN1 was highly expressed in HNSCC without differential expression between cancer stages. In addition, HTN3 was not significantly different between normal and cancer tissues. Previously, HTN1 and HTN3 were shown to play a role in cell migration and proliferation in normal cells, but had not been demonstrated in tumor cells (14). Our study is the first to highlight the potential role of HTN1 as a tumor-promoting factor for early HNSCC development. Finally, we investigated the correlation between HTN1, HTN3, and PD-L1 expression in HNSCC. HTN1 and HTN3, which originate from the same gene family, displayed a strong correlation ($p<0.001$). By excluding the normal tissues, a linear relationship was observed between non-metastasis and nodal metastasis in HTN1 *vs*. HTN3 and PD-L1 *vs*. HTN3. Even though our results demonstrated that HTN3 expression was not significantly associated with HNSCC progression, its correlation with PD-L1 expression indicates the supporting role of AMPs in tumor inflammatory response. These results show possible interaction between histatins and PD-L1 in HNSCC progression.

Due to the retrospective nature of this study, certain limitations need to be addressed. First, the variation in patients' demographic data *e.g.*, ethnicity and other clinical characteristics from our commercial TMA samples. Second, to justify the relationship of histatins and PD-L1 with HNSCC progression described in our study, further studies are required in various populations. In addition, studies should be performed on the signaling pathways and mechanisms involved.

Conflicts of Interest

All Authors declare that they do not have any financial and personal relationships with individuals or organizations that could inappropriately influence their work. All Authors have read and agreed to the published version of this manuscript.

Authors' Contributions

Wassapol Wongpanuwich: Literature review, Methodology, Data acquisition and analysis, Data interpretation, Figure preparation, Manuscript preparation. Somchai Yodsanga: Methodology, Tissue preparation, Manuscript review. Risa Chaisuparat: Methodology, Data interpretation, Manuscript editing, Manuscript review. Panomwat Amornphimoltham: Conceptualization, Methodology, Data interpretation, Quality control of data, Manuscript editing, Manuscript review, Supervision of the manuscript.

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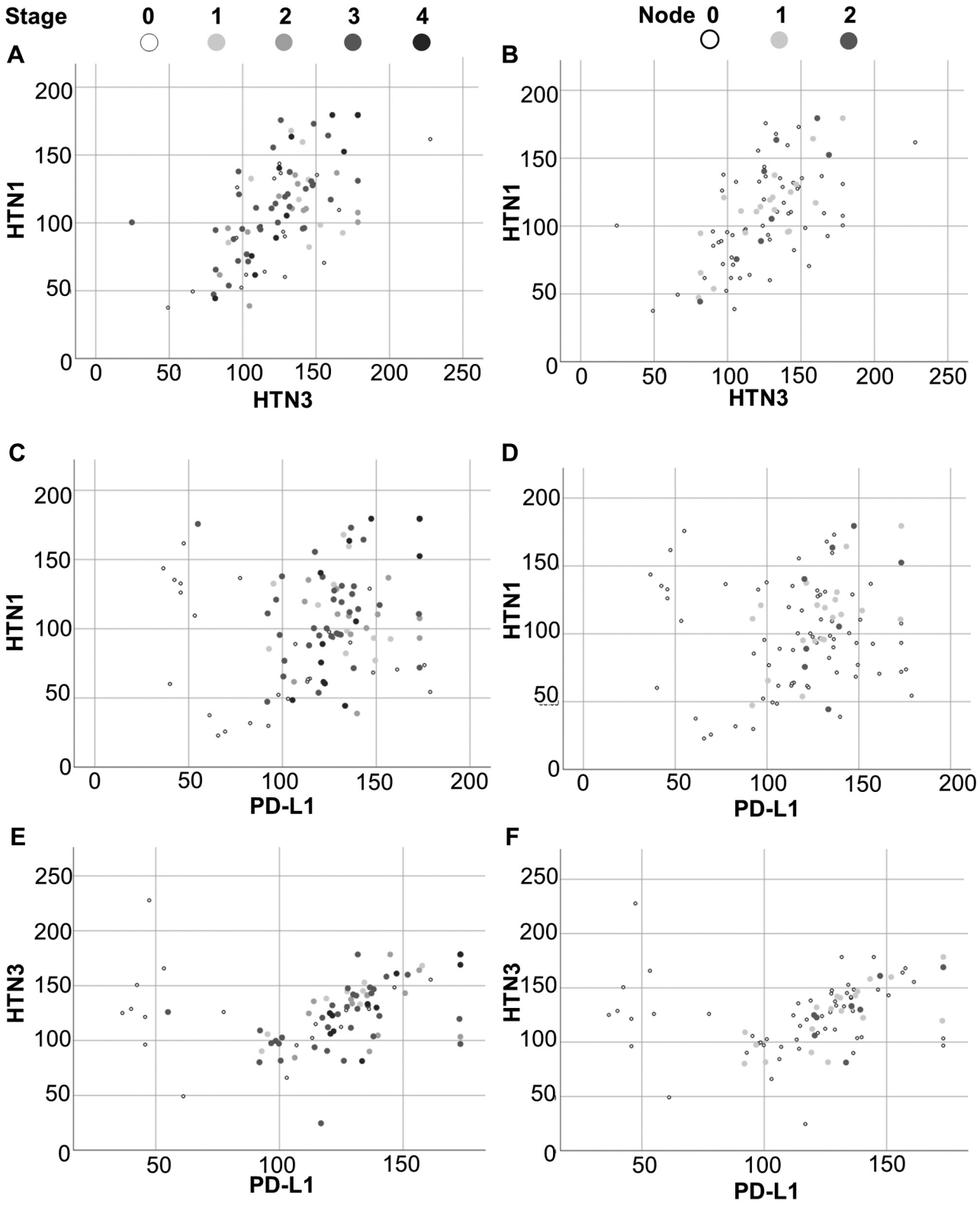

Figure 4. *Scatter plots show a relationship between HTN1 vs. HTN3 (A and B), HTN1 vs. PD-L1 (C and D), and PD-L1 vs. HTN3 (E and F) according to TNM stage (A, C, and E) and nodal involvement stage (B, D, and F).*

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