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The Blood Microenvironment Influences the Molecular Phenotypes of Circulating Tumor Cells in Head and Neck Squamous Cell Carcinoma

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Abstract. *Background/Aim: Circulating tumor cells (CTCs) may be affected by the environment encountered during blood circulation. We aimed to explore the association between the molecular phenotype of CTCs and systemic inflammatory markers. Patients and Methods: CTCs isolated from patients with recurrent/metastatic head and neck squamous cell carcinoma by CD45-negative selection were analyzed for the expression of multiple genes. The correlations between gene expression levels in CTCs and systemic inflammation markers were examined. Results: Thirty-five (83.3%) of the 42 patients were positive for CTCs. No significant differences in systemic inflammatory markers were observed between CTC-positive and CTC-negative patients. Notably, VIM or ZEB2 expression was strongly correlated with that of CD44 or ALDH1. PIK3CA, CD44, ALDH1A1, and PDCD1LG2 expression in CTCs was correlated with lymphocyte- and/or albumin-related systemic inflammatory markers. Conclusion: CTCs acquire a survival advantage through phenotypic alterations in the hostile blood environment, and evade circulatory immune surveillance.*

Similar to primary tumors and metastatic lesions, circulating tumor cells (CTCs) that have detached from a primary tumor into the bloodstream are also heterogeneous tumor cell populations (1, 2). CTC migration to the bloodstream exposes them to a hostile environment; in response, some subpopulations may undergo necrosis, anoikis, or apoptosis by physical forces and/or immune surveillance, while others survive by acquiring resistance mechanisms. Travelling into the blood circulation may affect CTC characteristics through direct and/or indirect interactions with various blood cells and their secreted humoral factors (3). For instance, the association between neutrophils and CTCs drives cell cycle progression within the bloodstream and expands the metastatic potential of CTCs (4). Similarly, platelets can protect CTCs against not only physical stress but also antitumor immune responses (3, 5). Moreover, platelet-CTC interactions induce an epithelial-mesenchymal-like transition and promote metastasis by platelet activation and the release of soluble mediators (6). Thus, in the blood environment, the phenotypic heterogeneity and plasticity of CTCs could be shaped by complex interaction networks between CTCs and circulating blood cells.

Cancer-related inflammation is a hallmark of cancer (7). With disease progression and/or selective pressure due to cancer treatment, the blood environment, specifically the systemic inflammation status, is drastically altered. Tumor cells can activate or inhibit a variety of blood cells and form complicated patient-specific inflammatory states in both the tumor microenvironment and systemic circulation, which are related to clinical outcomes including disease progression, treatment response, and prognosis (8-10).

The clinical significance of systemic inflammation markers based on counts, ratios, or the scores of circulating blood cells or acute-phase proteins, such as neutrophil-to-lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), and a measure based on elevated serum C-reactive protein (CRP) level and decreased serum albumin level, has been extensively investigated (10). Similar to other types of cancers, inflammatory markers may be useful markers for predicting the clinical outcomes of patients with head and neck squamous cell carcinoma (HNSCC). Rassouli *et al.* showed that the platelet-to-lymphocyte ratio (PLR) and NLR are independent predictors of mortality and recurrence, respectively (11). Similarly, LMR is an independent prognostic factor for HNSCC (12).

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Key Words: Circulating tumor cell, head and neck squamous cell carcinoma; blood microenvironment, molecular phenotype, systemic inflammatory marker.

We previously reported that 24 (80.0%) of 30 patients with recurrent/metastatic (R/M) HNSCC were positive for CTCs and measured the expression of immune-regulatory molecules in the 24 CTC-positive patients (13). In the present study, we molecularly characterized the CTCs in 42 patients including those of 12 who were newly diagnosed with R/M HNSCC. Finally, we investigated the association between the CTC molecular phenotype and systemic inflammation in patients with R/M HNSCC.

Patients and Methods

Patients. This study enrolled a total of 42 patients with R/M HNSCC, including 30 patients described previously (13). Their characteristics are shown in Table I. The median age of the patients was 69 years (range=48-86 years). The tumor origins included the oral cavity (n=5), nasopharynx (n=1), oropharynx [n=5 (2 were p16 positive; 3 were p16 negative)], hypopharynx (n=19), larynx (n=5), paranasal sinuses (n=6), and parotid gland (n=1). Recurrences in all patients, including the overlapping cases, were observed in 17 (40.5%), 25 (59.5%), and 23 (54.8%) local, regional, and distant sites, respectively. This study was approved by the Ethical Committee of the Gunma University Hospital (No.12-12), and written informed consent was obtained from each patient.

Circulating tumor cell (CTC) isolation and gene expression analysis. CTC isolation and gene expression analysis were performed as described previously (13). In brief, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation of blood samples (7.5 ml) obtained from patients. The contaminating erythrocytes were further lysed with red blood cell lysis buffer (Roche Diagnostic GmbH, Mannheim, Germany). The cell suspension was incubated with a human CD45 depletion cocktail for 15 min and subsequently with magnetic particles for 10 min (EasySep™ Human CD45 Depletion Kit, Stemcell Technologies). Tubes containing the PBMCs were placed in a magnet for 10 min twice, and the unbound cells (CTCs) were transferred to new tubes.

Total RNA from the CTCs was extracted using a RNeasy Micro kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (QIAGEN) with 14 cycles of pre-amplification using the TaqMan™ PreAmp Master Mix kit (Applied Biosystems). The pre-amplified products were subsequently analyzed by real-time quantitative polymerase chain reaction (Applied Biosystems, Waltham, MA, USA) for the 16 target genes. Sixteen primers for the 15 targets [epithelial cell adhesion molecule (*EPCAM*), *MET,* keratin 19 *(KRT19),* epidermal growth factor receptor (*EGFR*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), cyclin D1 (*CCND1*), snail family transcriptional repressor 1 (*SNAI1*), vimentin (*VIM*), zinc finger E-box binding homeobox 2 (*ZEB2*), *CD44*, nanog homeobox (*NANOG*), aldehyde dehydrogenase 1 family member A1 (*ALDH1A1*), *CD47*, *CD274*, and programmed cell death 1 ligand 2 (*PDCD1LG2*)] and *ACTB* (β-actin) as control were purchased from Applied Biosystems (TaqMan™ Gene Expression Assays). All samples were analyzed in triplicate. The primer sequences for the 16 genes included in this study are shown in Table II. Target gene expression in CTCs was determined using a relative quantification method. Detection of at least one of the four epithelial-related genes (*EPCAM, MET, KRT19,* and *EGFR*) was defined as CTC positivity. Since samples obtained by negative selection are invariably contaminated with leukocytes, the average threshold cycle (Ct) value of the CTC-negative samples was used as the baseline for the control group. The Ct values of the target genes were normalized to a reference gene (*ACTB*), and the expression levels of the target genes in CTCs were estimated as fold changes compared to those in the CTC-negative samples by the relative quantification 2-delta-delta Ct method.

Data acquisition and systemic inflammatory markers. Laboratory data including platelet, neutrophil, lymphocyte, and monocyte counts and serum CRP and albumin levels were collected from

Table I. *Patient characteristics*.

Characteristics	Value
Total patients (Male/Female)	42 (37/5)
Age (yrs): Median (range)	69 (48-86)
Tumor site	
Maxillary sinus	6
Oral cavity	5
Nasopharynx	1
Oropharynx	5
(p16 status)	(p16 positive, 2; p16 negative, 3)
Hypopharynx	19
Larynx	5
Parotid	1
Recurrence/metastasis	
Local recurrence	17
Regional lymph node recurrence	25
Distant metastasis	23
Circulating tumor cells	
Positive	35
Negative	7

Table II. *Polymerase chain reaction primers used in this study.*

Gene signature	Gene symbol	Assay ID
Epithelial	*EPCAM*	Hs00158980_m1
	MET	Hs01565576_m1
	KRT19	Hs00761767_s1
	EGFR	Hs01076090_m1
Cell growth	*PIK3CA*	Hs00907957_m1
	CCND1	Hs00765553_m1
Epithelial-mesenchymal transition	*SNAI1*	Hs00195591_m1
	VIM	Hs00958111_m1
	ZEB2	Hs00207691_m1
Cancer stemness	*CD44*	Hs01075861_m1
	NANOG	Hs04399610_g1
	ALDH1A1	Hs00946916_m1
Immune regulatory	*CD47*	Hs00179953_m1
	CD274	Hs01125301_m1
	PDCD1LG2	Hs01057777_m1
Reference	*ACTB*	Hs01060665_g1

patients' clinical records within 2 weeks of blood collection for CTC isolation. NLR, PLR, and LMR were calculated by dividing the absolute values of the corresponding hematological parameters. The systemic immune-inflammation index (SII), prognostic nutrition index (PNI), and CRP/albumin ratio (CAR) were calculated as follows: SII=platelet count × neutrophil/lymphocyte count (14), PNI=10× serum albumin+ 0.005× lymphocyte count (15), and CAR=CRP/albumin (16).

Statistical analyses. GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analyses. Mann–Whitney *U*-tests were used to examine the differences in continuous variables. The correlations of two continuous variables, gene expression levels in CTC and systemic inflammatory markers, were determined by Spearman's rank tests. The Kaplan–Meier curves were plotted and compared using the log-rank tests to compare survival curves between subgroups. The optimal cut-off values of systemic inflammatory markers for overall survival were determined based on receiver operating characteristics curve analysis. Two-sided p-values <0.05 were considered statistically significant.

Results

Detection of CTCs and systemic inflammatory markers in patients with R/M HNSCC. Thirty-five (83.3%) of the 42 patients with R/M HNSCC, including 30 patients reported in our previous study (13), were positive for at least one epithelial-related gene. The expression levels of epithelial-related markers in 35 CTC-positive patients are summarized in Figure 1. Among the 35 CTC-positive patients, *EPCAM* was detected in 15 (35.7%), *KRT19* in 29 (69.0%), *EGFR* in 14 (33.3%), and *MET* in 21 (50.0%) patients, respectively. Next, we compared systemic inflammatory markers including blood cell counts between CTC-positive and CTC-negative patients and observed no significant differences between the two groups (data not shown).

Molecular characterization of CTCs. The CTCs obtained from 35 R/M HNSCC patients were subsequently investigated for the expression of 11 genes grouped into four gene signatures: cell growth (*PIK3CA* and *CCND1*), epithelial-mesenchymal transition (EMT) (*SNAI1*, *VIM*, and *ZEB2*), cancer stemness (*CD44, NANOG*, and *ALDH1A1*), and immune regulation (*CD47*, *CD274*, and *PDCD1LG2*). The expression of each gene in CTCs varied according to R/M HNSCC patients (Figure 2), confirming our previous report of phenotypic heterogeneity of CTCs among patients (17). Furthermore, we also analyzed the correlations between gene expression levels and observed a significant correlation between the same gene signatures (Figure 3). *VIM* or *ZEB2* expression was strongly correlated with that of *CD44* or *ALDH1*, suggesting a significant role of these CTC subpopulations in the metastatic process (*VIM* and *CD44*, r=0.9328, p<0.001; *VIM* and *ALDH1*, r=0.8602, p<0.001; *ZEB2* and *CD44*, r=0.8098, p<0.001; *ZEB2* and *ALDH1*, r=0.7062, p<0.001).

Prognostic significance of systemic inflammatory markers in patients with R/M HNSCC. The Kaplan–Meier survival analyses were performed to evaluate the prognostic significance of systemic inflammatory markers in patients with R/M HNSCC (Figure 4). R/M HNSCC patients with higher platelet counts, higher CRP levels, lower albumin levels, higher NLR, lower PNI, or higher CAR had significantly shorter survival than those in the pair groups for each systemic inflammatory marker (platelet count, p=0.036; CRP level, p=0.001; albumin level, p=0.041; NLR, p=0.038; PNI, p=0.020; CAR, p=0.002).

Correlation between gene expression levels of CTCs and systemic inflammatory markers. Finally, we examined the correlations between gene expression levels in CTCs and systemic inflammation markers in patients with R/M HNSCC (Figure 5A and B). *PIK3CA* expression in CTCs was correlated with lymphocyte-related systemic inflammatory markers including lymphocyte count, NLR, and LMR, while *CD44* and *ALDH1A1* expression in CTCs was correlated with lymphocyte- and albumin-related markers (lymphocyte count, albumin level, and PNI) and lymphocyte-related markers (lymphocyte count, NLR, PLR, and PNI), respectively. Moreover, *PDCD1LG2* expression in CTCs was correlated with nine of the 12 markers tested (positive correlation: lymphocyte count, albumin level, LMR, and PNI; negative correlation, neutrophil count, NLR, PLR, SII, and CAR).

Discussion

Accumulating evidence has shown an association between the existence or enumeration of CTCs and various clinical factors in HNSCC (18-20); however, CTC heterogeneity and longitudinal changes within the same patient are substantial hurdles to understanding the biology and clinical potential of CTCs. This study aimed to molecularly characterize CTCs and their association with systemic inflammation in HNSCC. Since CTCs are more frequently detected and show extensive heterogeneity in patients with more advanced disease compared to those in patients with early disease (21), we enrolled patients with R/M HNSCC. As expected, CTCs were detected in approximately 80% of patients. We performed molecular characterization of CTCs based on four gene signatures, cell growth, EMT, cancer stemness, and immune regulation. Although the pattern of gene expression levels in CTCs varied among patients, the overall gene expression levels in CTCs revealed a strong positive correlation with those in the same gene signature group, except for genes regulating cell growth. Of note, the expression levels of EMT-related genes were highly correlated with those of cancer stemness. Previous reports have indicated that two CTC phenotypic characteristics,

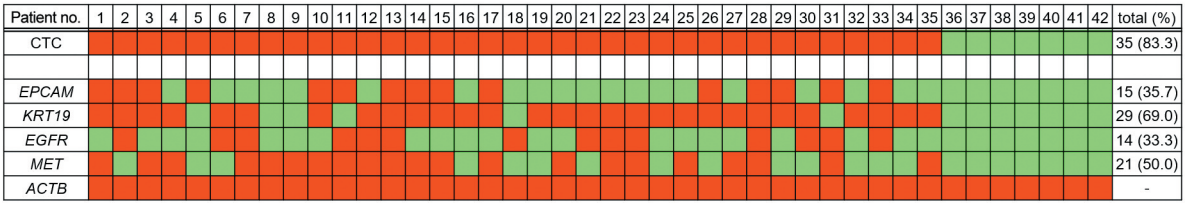

Figure 1. *Heatmap depicting the presence of circulating tumor cells and expression of four epithelial-related genes in patients with recurrent/metastatic head and neck squamous cell carcinoma. The red square indicates positive gene expression.*

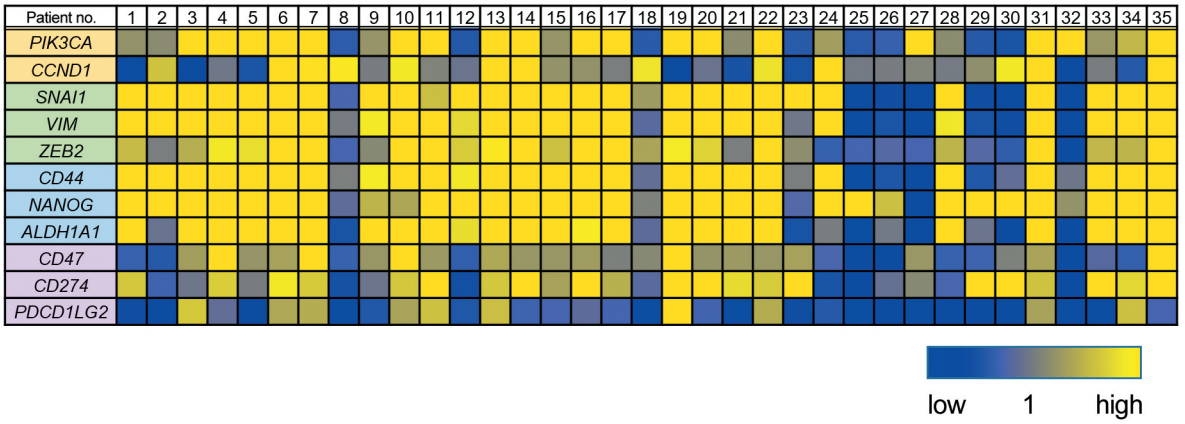

Figure 2. *Gene expression of circulating tumor cells (CTCs) in patients with recurrent/metastatic head and neck squamous cell carcinoma. Heatmap depicting fold changes of expression of 11 genes in CTCs compared to those in CTC-negative samples.*

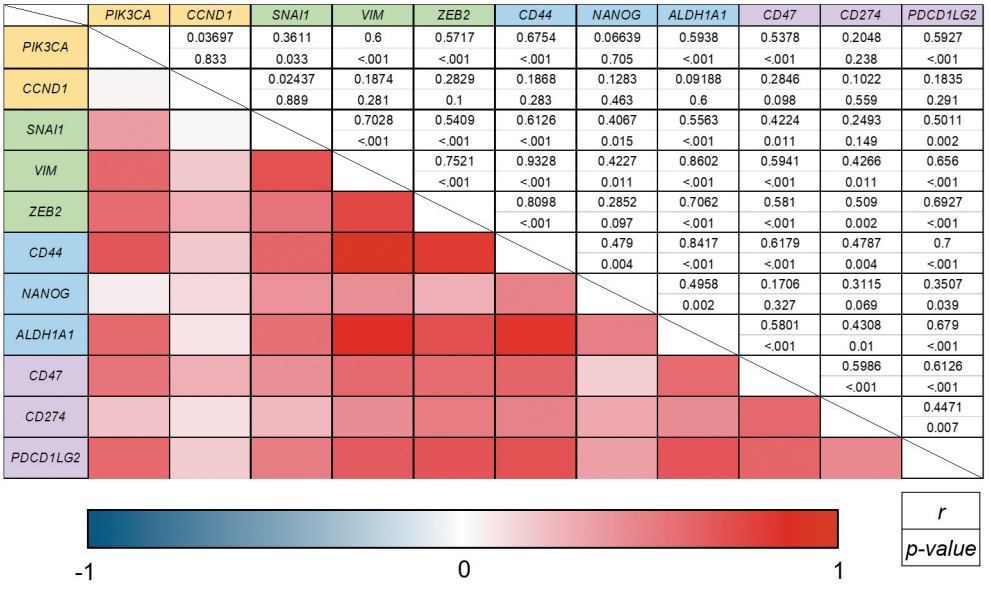

Figure 3. *Correlation heatmap with correlation coefficients based on gene expression levels in circulating tumor cells.*

EMT and cancer stemness, are closely related and essential for metastasis. Most CTCs of metastatic breast cancer possess EMT and stem cell characteristics (22). Similarly, Papadaki *et al*. reported that high CTC expression levels of EMT and stemness markers were more frequently detected in patients with metastatic breast cancer (23). As the subjects in the present study had already developed a locoregional recurrence and/or distant metastasis, they may have had an

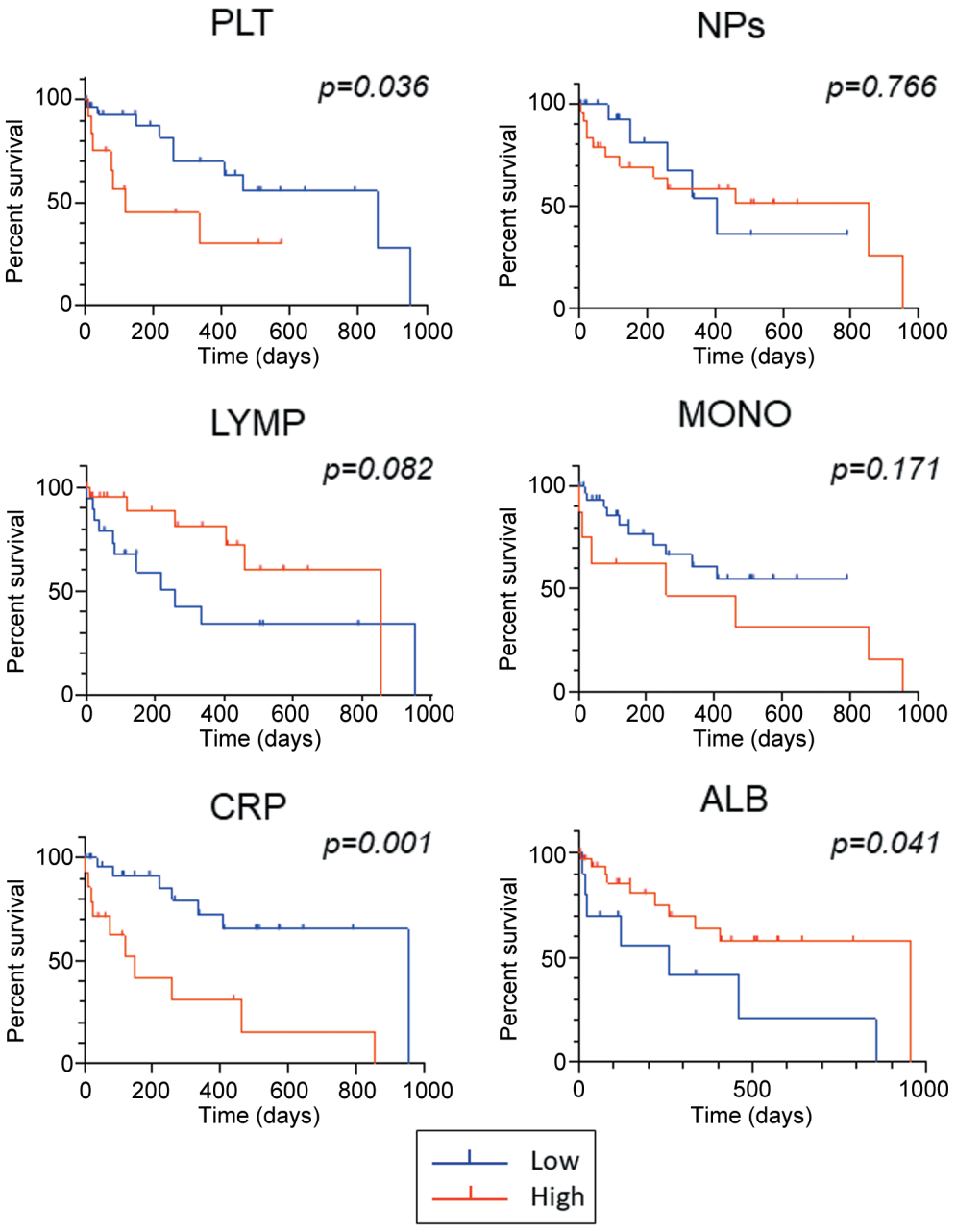

Figure 4. *Continued*

enriched subpopulation of CTCs with both EMT and stemness phenotypes. During tumor blood dissemination, CTCs in the blood not only encounter various host cells, including leukocytes, platelets, and other CTCs but also interact with the blood environment. Thus, CTCs may acquire survival and metastatic potential through phenotypic alterations due to environmental exposure.

The blood environment comprises several factors. Systemic inflammatory markers are associated with disease progression, treatment responses, and prognosis in various cancers, including HNSCC (11, 12, 24-26). Our results indicated that platelet count, CRP level, albumin level, NLR, PNI, and CAR were prognostic markers in patients with R/M HNSCC, suggesting that these markers might be useful for evaluating life expectancy in R/M HNSCC.

In this study, lymphocyte- and albumin-related markers were associated with *PIK3CA*, *CD44*, *ALDH1A1*, and *PDCD1LG2* expression in CTCs. Both peripheral blood

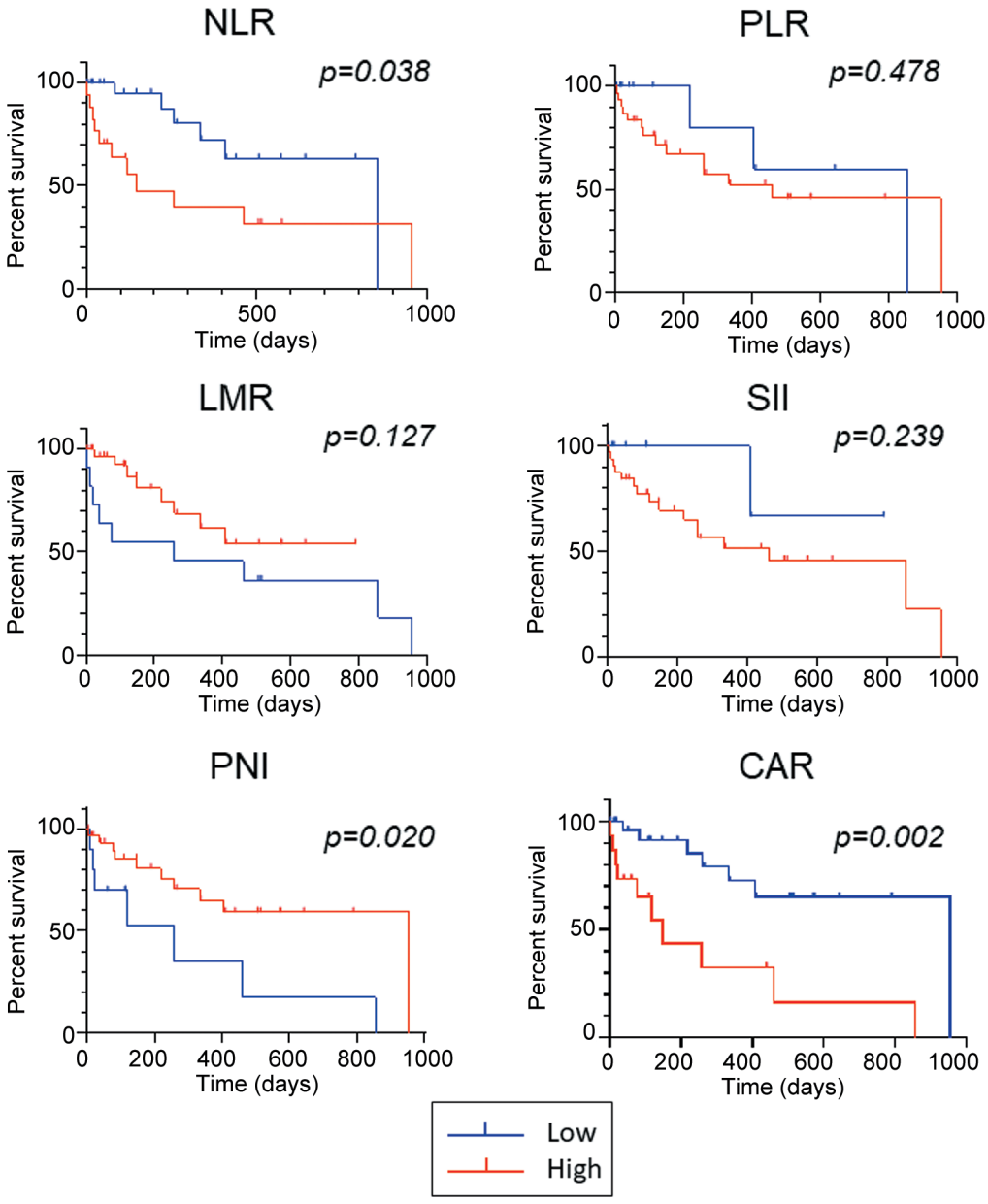

Figure 4. *Kaplan–Meier survival analysis in recurrent/metastatic head and neck squamous cell carcinoma patients according to systemic inflammatory makers. The optimal cutoff values of systemic inflammatory markers for overall survival were determined based on receiver operating characteristics curve analysis.*

lymphocytes and serum levels of albumin, a parameter of nutritional status, influence immune cell function (27, 28); therefore, high lymphocyte counts and high serum levels of albumin may contribute to antitumor immune responses. As they have left the immune-suppressive tumor micro-environment, CTCs in the bloodstream are more vulnerable to antitumor immune responses in the peripheral blood compared to primary tumors and metastatic lesions. Thus, the circulatory immune system might select for CTCs with immune-resistant phenotypes. The immune escape mechanisms employed by CTCs in the peripheral blood include human leukocyte antigen down-regulation and Fas ligand, CD47, and programmed death-ligand 1 (PD-L1) expression (29-31). Additionally, our findings suggest that molecular alterations in CTCs allow them to escape immune surveillance. Growing evidence supports the association between immunosuppressive phenotypes and the gene signatures tested in this study. Using a model of pancreatic

A

	PLT	NPs	LYMP	MONO	CRP	ALB	NLR	PLR	LMR	SII	PNI	CAR
PIK3CA	0.1873	-0.05154	0.3935	0.002661	-0.04538	0.02364	-0.4162	-0.223	0.3487	-0.2367	0.1366	-0.0493
	0.281	0.769	0.019	0.988	0.796	0.893	0.013	0.198	0.04	0.171	0.434	0.779
CCND1	-0.3425	0.07143	0.2879	0.1661	-0.1002	0.04911	-0.1908	-0.4868	0.05798	-0.3325	0.187	-0.09692
	0.044	0.683	0.094	0.34	0.567	0.779	0.272	0.003	0.741	0.051	0.282	0.58
SNAI1	0.1504	0.07003	0.1957	0.2712	-0.1049	0.3055	-0.09664	-0.08095	-0.0944	-0.02605	0.3244	-0.1305
	0.388	0.689	0.26	0.115	0.549	0.074	0.581	0.644	0.59	0.882	0.057	0.455
VIM	0.06177	0.121	0.348	0.268	-0.04524	0.2807	-0.209	-0.2826	-0.005602	-0.1263	0.3451	-0.05938
	0.724	0.489	0.041	0.12	0.796	0.102	0.228	0.1	0.975	0.47	0.042	0.735
ZEB2	0.02395	-0.04762	0.3732	0.1174	-0.1794	0.2365	-0.3132	-0.284	0.1429	-0.2101	0.3208	-0.1983
	0.891	0.786	0.027	0.502	0.302	0.171	0.067	0.098	0.413	0.226	0.06	0.253
CD44	0.05939	0.008964	0.3504	0.1048	-0.0818	0.3619	-0.3154	-0.3056	0.1843	-0.2104	0.4347	-0.1078
	0.735	0.959	0.039	0.549	0.64	0.033	0.065	0.074	0.289	0.225	0.009	0.537
NANOG	-0.02535	-0.07255	0.07817	0.01415	-0.1888	0.3804	-0.1258	-0.1333	0.01429	-0.1218	0.4268	-0.2042
	0.885	0.679	0.655	0.936	0.277	0.024	0.472	0.445	0.935	0.486	0.011	0.239
ALDH1A1	-0.09048	-0.05546	0.4063	0.1343	-0.223	0.3153	-0.384	-0.3863	0.1695	-0.3294	0.411	-0.2317
	0.605	0.752	0.015	0.442	0.198	0.065	0.023	0.022	0.33	0.053	0.014	0.181
CD47	-0.0755	-0.3398	0.1307	-0.1142	-0.2661	0.1919	-0.4465	-0.1731	0.2924	-0.3852	0.2164	-0.2751
	0.666	0.046	0.454	0.514	0.122	0.269	0.007	0.32	0.088	0.022	0.212	0.11
CD274	-0.09216	-0.1955	0.09022	-0.08026	-0.1621	0.1635	-0.207	-0.1196	0.08964	-0.2109	0.1376	-0.1773
	0.598	0.26	0.606	0.647	0.352	0.348	0.233	0.494	0.609	0.224	0.431	0.308
PDCD1LG2	-0.1544	-0.3602	0.3879	-0.1221	-0.3205	0.3951	-0.5818	-0.3417	0.4501	-0.5123	0.4754	-0.3367
	0.376	0.034	0.021	0.485	0.061	0.019	<.001	0.045	0.007	0.002	0.004	0.048

r
p-value

B

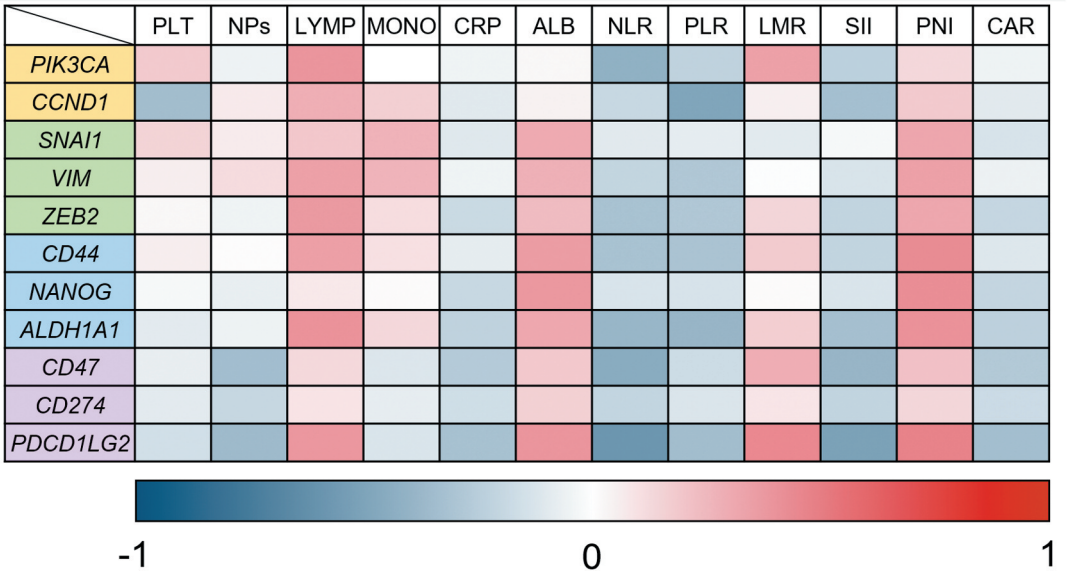

Figure 5. *Correlations between gene expression levels in circulating tumor cells and systemic inflammatory markers in patients with recurrent/metastatic head and neck squamous cell carcinoma. (A) Correlation coefficient (upper row) and significance levels (lower row). (B) Correlation heatmap with correlation coefficients.*

cancer, Sivaram *et al*. showed that PIK3CA-AKT signaling in tumors reduced major histocompatibility complex class I and CD80 expression on the cell surface to promote immune evasion (32). In contrast, CD44+ tumor-initiating cells in HNSCC not only have an EMT phenotype but are also less immunogenic, and selectively express PD-L1 compared to CD44 cells (33). Similarly, EMT is strongly associated with an inflammatory tumor microenvironment in lung cancer, with increased levels of multiple immune checkpoint molecules (34). Although these findings were based on tumor tissues or xenografts and these phenomena might be mirrored within the tumor microenvironments, CTCs in the blood likely exhibit similar biological behaviors to acquire immune-suppressive properties.

The main limitations of the present study are its small sample size and heterogeneous population comprising different primary sites and recurrence patterns. We are currently conducting a large-scale study in pretreated HNSCC patients to assess the molecular phenotypic alterations of CTCs under blood environmental stress

including antitumor immunity. Taken together, the present study investigated CTCs from patients who had received various treatments and had recurrent and/or metastatic lesions; therefore, it was difficult to determine the molecular mechanism of CTCs within the cascade of metastasis. However, our results suggest the potent survival advantage of CTCs through various phenotypic alterations in the hostile blood environment, specifically against immune functions defined by lymphocyte counts and serum albumin levels.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

Kazuaki Chikamatsu conceived and designed the study. Hiroe Tada, Shota Ida, Ikko Mito, and Toshiyuki Matsuyama performed the experiments, and collected data. Hideyuki Takahashi and Kazuaki Chikamatsu analyzed and interpreted the data. All Authors read and approved the final manuscript.

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