Systemic immune responses are associated with molecular characteristics of circulating tumor cells in head and neck squamous cell carcinoma

HIROE TADA*, YURINO NAGATA*, HIDEYUKI TAKAHASHI, TOSHIYUKI MATSUYAMA, SHOTA IDA, IKKO MITO and KAZUAKI CHIKAMATSU

Department of Otolaryngology-Head and Neck Surgery, Gunma University Graduate School of Medicine, Maebashi, Gunma 3718511, Japan

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Abstract. Systemic immunity mediated by circulating immune cells may affect clinical features, as well as the characteristics of circulating tumor cells (CTCs) in patients with head and neck squamous cell carcinoma (HNSCC). The present study aimed to analyze the influence of circulating immune cells, using their markers, on clinical features to investigate the association between systemic immunity and the molecular characteristics of CTCs. Circulating immune-cell markers were associated with disease progression and clinical outcomes in patients with HNSCC. Meanwhile, there was no significant association between the presence of CTCs and systemic immune-related markers. Moreover, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit a expression in CTCs was significantly associated with higher lymphocyte counts (P=0.035) and an increased prognostic nutrition index (P=0.0157). Patients with CTCs expressing *CD47* exhibited significantly higher neutrophil (P=0.0031) and monocyte (P=0.0016) counts. Patients with CTCs expressing programmed cell death 1 ligand 2 exhibited lower C-reactive protein (CRP) levels (P=0.0271) and a decreased CRP/albumin ratio (P=0.0207). The current results suggested that the interaction between CTCs and circulating immune cells may provide survival advantages via molecular alterations to CTCs.

Correspondence to: Professor Kazuaki Chikamatsu, Department of Otolaryngology-Head and Neck Surgery, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 3718511, Japan
E-mail: tikamatu@gunma-u.ac.jp

*Contributed equally

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Introduction

Recently developed immune checkpoint-blockade therapies have been demonstrated to have remarkable benefits for the treatment of patients with recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC) (1,2). Many studies have focused on achieving a deeper understanding of the interaction between tumor cells and associated immune cells within the tumor microenvironment, to not only predict but also improve immunotherapeutic responsiveness (3,4). Similarly, in the bloodstream complex interactions between tumor cells and immune cells play an important role in the formation of distant metastases and in clinical outcomes (5-7).

Most tumor cells that enter the bloodstream through intravasation from primary sites die by anoikis induced by detachment from the extracellular matrix, the shearing force of blood pressure, or immune surveillance. However, some are able to survive in the bloodstream through phenotypic and functional alterations that confer resistance to environmental stress and are called circulating tumor cells (CTCs). Among the three forms of cell death mentioned, immune surveillance is the most complex and fluctuates depending on the patient's disease progression, nutritional status, and treatment pressure. In contrast, protumoral skewing of the immune system supports evasion of immune surveillance and promotes tumor cell dissemination. To date, the role of systemic immunity in cancer patients has been exclusively investigated and reported with regard to its clinical significance (8,9); however, the relationship between systemic immunity and CTCs remains unclear. Interestingly, in patients with breast cancer, the presence of CTCs has been correlated with a reduction in $CD3^+$, $CD4^+$, and $CD8^+$ T cells (10). Another study in lung-cancer patients showed that numbers of CTCs were negatively correlated with those of $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ T cells (11). However, further studies are needed to more precisely evaluate the impact of systemic immunity on CTCs in the bloodstream.

To address this issue, we first investigated whether the proportions of circulating immune cells were correlated with clinical features. We then analyzed the potential correlations with the molecular characteristics of CTCs.

Materials and methods

Patients. This study enrolled 44 patients with untreated HNSCC from a previous study (12). Their median age was 66 years (range: 47-86). The tumor origins included the oral cavity (n=4), nasopharynx (n=2), oropharynx (n=17), hypopharynx (n=14), larynx (n=4), and nasal cavity (n=3). We evaluated several clinical variables, including age, T factor, N factor, stage, locoregional recurrence, distant metastasis, initial treatment response, and presence of CTCs. This study was approved by the Ethical Committee of Gunma University Hospital (no. 12-12), and written informed consent was obtained from each patient.

Data acquisition and systemic immune-related markers. Laboratory data, including neutrophil, lymphocyte, monocyte, and platelet counts, as well as serum C-reactive protein (CRP) and albumin levels, were collected from patients' clinical records within 2 weeks of blood collection for CTC isolation. The neutrophil-lymphocyte ratio (NLR), platelet-lymphocyte ratio (PLR), and lymphocyte-monocyte ratio (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: SII = (platelet count x neutrophil count)/lymphocyte count (13), PNI = (10 x serum albumin) + (0.005 x lymphocyte count) (14), and CAR = CRP/albumin (15).

CTC detection and gene expression. CTC detection and molecular data from a previous study were used (12). Blood samples were collected from 44 patients with untreated HNSCC; CTCs were isolated using a CellSieve™ microfilter (Creatv MicroTech, Inc.). In brief, peripheral blood samples (7.5 ml) were passed through a CellSieve™ microfilter. The filter was then washed with phosphate-buffered saline three times and transferred into a new tube, labeled as CTCs. The first filtrate was passed through a second filter to capture control leukocytes. The second filter was washed, transferred into another tube, and used as a control.

Total RNA from the CTCs was extracted using an RNeasy Micro Kit (Qiagen, Inc.) according to the manufacturer's instructions. Complementary DNA synthesis was performed using the QuantiTect Reverse Transcription kit (Qiagen, Inc.) with 14 cycles of preamplification using the TaqMan™ PreAmp Master Mix Kit (Applied Biosystems). The products were analyzed using the real-time quantitative polymerase chain reaction (Applied Biosystems). Primers for the 14 target genes epithelial cell adhesion molecule [*EPCAM*: Hs00158980_m1], *MET*: Hs01565576_m1, keratin 19 [*KRT19*: Hs00761767_s1], epidermal growth factor receptor [*EGFR*: Hs01076090_m1], phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha [*PIK3CA*: Hs00907957_m1], cyclin D1 [*CCND1*: Hs00765553_m1], snail family transcriptional repressor 1 [*SNAI1*: Hs00195591_m1], vimentin [*VIM*: Hs00958111_m1], *CD44*: Hs01075861_m1, nanog homeobox [*NANOG*: Hs04399610_g1], aldehyde dehydrogenase 1 family member A1 [*ALDH1A1*: Hs00946916_m1], *CD47*: Hs00179953_m1, *CD274*: Hs01125301_m1, and programmed cell death 1 ligand 2 [*PDCD1LG2*: Hs01057777_m1] and *ACTB* (Hs01060665_g1, normalization control) were purchased from Applied Biosystems (TaqMan™ Gene Expression Assays). CTC gene expression levels were 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. The cycle threshold (Ct) values of the target genes were normalized to those of the reference gene *ACTB*. Expression levels were estimated as fold changes and compared to those in the control leukocyte group using the $2^{-\Delta\Delta Ct}$ relative quantification method (16). When the $2^{-\Delta\Delta Ct}$ value was >1, the sample was assessed as positive for the expression of the gene.

Statistical analysis. GraphPad Prism version 8.0 for Windows (GraphPad Software, Inc.) was used for all analyses. Mann-Whitney U tests were used to assess differences in continuous variables. Kaplan-Meier curves were plotted and compared using log-rank tests to compare survival curves between subgroups. The optimal cutoff values of circulating immune cells as well as their related markers for progression-free survival (PFS) and overall survival (OS) were determined based on receiver operating characteristic curve analysis. Two-sided P-values <0.05 were considered to be statistically significant.

Results

Clinical significance of systemic immune-related markers. A total of 44 treatment-naïve patients with HNSCC were enrolled in this study. Their characteristics are listed in Table I. Twenty-eight (63.6%) of the 44 were positive for CTCs as described previously (12). First, we determined whether systemic immune-related markers were associated with clinical factors and the presence of CTCs. Monocyte counts in elderly patients were significantly higher than those in younger patients (P=0.0339). Patients with advanced-stage disease exhibited significantly higher monocyte counts and LMR values than those with early-stage disease. Interestingly, patients with distant metastases during the follow-up period had significantly lower lymphocyte counts, NLR, and LMR. Meanwhile, there was no significant correlation between the presence of CTCs and any systemic immune-related markers. Next, we analyzed the prognostic significance of systemic immune-related markers in these 44 patients (Table II). As expected, various systemic immune-related markers, including lymphocyte counts, NLR, PLR, LMR, SII, and PNI, were associated with PFS (lymphocyte counts, P=0.0021; NLR, P=0.005; PLR, P=0.04; LMR, P=0.013; SII, P=0.046; and PNI, P=0.015). Monocyte counts and PLR were significantly associated with OS (monocyte counts, P=0.049; PLR, P=0.0441). The corresponding Kaplan-Meier survival curves are shown in Fig. 1.

Association between systemic immune-related markers and the molecular characteristics of CTCs. Finally, we analyzed whether systemic immune-related markers were associated with specific molecular characteristics of CTCs. Gene expression tests revealed that the expression levels of five genes, *PIK3CA*, *CD44*, *NANOG*, *CD47*, and *PDCD1LG2*,

Table I. Clinicopathological characteristics of patients with head and neck squamous cell carcinoma (n=44).

Clinical variable	N (%)	Neutrophils, x10^9/l	P-value	Lymphocytes, x10^9/l	P-value	Monocytes, x10^9/l	P-value	CRP, mg/dl	P-value	NLR	P-value	PLR	P-value	LMR	P-value	SII	P-value	PNI	P-value	CAR	P-value
Age, years																					
<66	21 (47.7)	4.190 (3.760-5.685)	0.2855	1.700 (1.355-2.030)	0.7054	0.320 (0.250-0.390)	0.0339[a]	0.18 (0.065-0.730)	0.4878	2.661 (1.888-3.947)	0.4419	138.8 (103.5-173.2)	0.9629	5.813 (3.710-6.842)	0.0605	586.1 (453.5-1090.0)	0.6086	52.20 (48.93-55.43)	0.0639	0.0311 (0.0143-0.1156)	0.2736
≥66	23 (52.3)	4.910 (4.220-7.090)		1.610 (0.940-2.330)		0.410 (0.290-0.560)		0.23 (0.080-0.480)		3.273 (1.923-5.517)		143.5 (102.8-166.7)		3.927 (2.789-5.935)		736.5 (443.6-1090.0)		48.65 (45.80-51.50)		0.0548 (0.0205-0.1346)	
T status																					
T1-2	13 (29.5)	4.150 (3.570-5.645)	0.1317	1.670 (0.980-2.390)	0.7460	0.280 (0.230-0.520)	0.1421	0.08 (0.040-0.580)	0.1039	2.742 (1.764-4.625)	0.7032	122.2 (99.6-213.3)	0.8589	5.015 (2.947-6.921)	0.6633	523.1 (465.6-1168.0)	0.6474	53.15 (43.40-55.43)	0.4484	0.0205 (0.0091-0.1784)	0.1860
T3-4	31 (70.5)	5.370 (3.960-7.090)		1.700 (1.430-2.300)		0.370 (0.300-0.500)		0.23 (0.100-0.560)		2.974 (1.947-5.517)		143.5 (103.2-160.9)		5.152 (3.115-6.122)		745.6 (442.4-1165.0)		49.35 (46.65-52.20)		0.0536 (0.0214-0.1204)	
N status																					
N0	10 (22.7)	4.655 (4.053-7.963)	0.8402	1.740 (1.308-2.255)	0.9503	0.405 (0.338-0.568)	0.0335[a]	0.12 (0.068-0.705)	0.4937	2.473 (1.686-5.020)	0.7097	142.8 (111.3-170.6)	0.8148	4.354 (1.948-5.457)	0.2061	527.5 (436.9-1358.0)	0.8571	48.93 (46.46-52.29)	0.6062	0.0262 (0.0165-0.1923)	0.6164
N1-3	34 (77.3)	4.850 (3.815-6.540)		1.645 (1.105-2.308)		0.315 (0.260-0.448)		0.25 (0.080-0.500)		2.972 (1.941-5.193)		139.5 (101.7-171.8)		5.227 (3.255-6.496)		711.9 (443.3-1173.0)		50.25 (46.18-55.31)		0.0548 (0.0187-0.1209)	
Stage																					
I-II	10 (22.7)	4.345 (3.428-5.440)	0.1135	1.750 (1.435-2.158)	0.6537	0.275 (0.220-0.345)	0.0202[a]	0.23 (0.063-0.870)	0.9945	2.551 (1.675-3.357)	0.1682	128.3 (99.0-183.3)	0.8148	6.435 (4.615-8.466)	0.0389[a]	505.7 (466.2-804.7)	0.3538	52.80 (49.91-55.39)	0.1226	0.0494 (0.0144-0.2158)	>0.9999
III-IV	34 (77.3)	5.140 (3.940-7.230)		1.615 (1.043-2.340)		0.375 (0.298-0.528)		0.22 (0.078-0.500)		3.124 (2.047-5.563)		145.2 (102.0-167.6)		4.467 (2.933-6.032)		757.8 (421.7-1205.0)		49.28 (45.56-52.29)		0.050 (0.0184-0.1209)	

Table I. Continued.

Clinical variable	N (%)	Neutrophils, x10^9/l	P-value	Lymphocytes, x10^9/l	P-value	Monocytes, x10^9/l	P-value	CRP, mg/dl	P-value	NLR	P-value	PLR	P-value	LMR	P-value	SII	P-value	PNI	P-value	CAR	P-value
Locoregional recurrence																					
(+)	13 (29.5)	4.300 (3.850-6.515)	0.8149	1.560 (0.950-2.205)	0.3900	0.390 (0.275-0.510)	0.6889	0.22 (0.070-0.565)	0.8838	3.729 (1.818-5.758)	0.5760	111.7 (88.3-187.1)	0.4300	3.600 (2.828-6.181)	0.3300	470.6 (322.7-1238.0)	0.5417	47.75 (44.95-52.38)	0.1816	0.050 (0.0184-0.1359)	0.9434
(-)	31 (70.5)	4.910 (3.840-7.090)		1.700 (1.270-2.300)		0.330 (0.270-0.470)		0.22 (0.080-0.480)		2.724 (1.947-3.969)		146.9 (115.6-170.4)		5.152 (3.560-6.684)		687.4 (473.8-1141.0)		51.20 (47.50-53.35)		0.0495 (0.0178-0.1241)	
Distant metastasis																					
(+)	13 (29.5)	5.370 (4.000-7.040)	0.5213	1.060 (0.865-1.850)	0.0289[a]	0.380 (0.295-0.510)	0.7847	0.23 (0.100-0.475)	0.7076	4.349 (3.087-6.035)	0.0239[a]	160.9 (111.2-237.9)	0.1921	3.115 (2.325-5.874)	0.0491[a]	1141.0 (443.0-1252.0)	0.2104	48.65 (43.70-51.70)	0.1162	0.0548 (0.0222-0.1291)	0.5085
(-)	31 (70.5)	4.780 (3.660-6.390)		1.780 (1.490-2.330)		0.330 (0.270-0.470)		0.20 (0.070-0.650)		2.494 (1.829-3.608)		135.6 (98.5-151.3)		5.227 (3.808-6.684)		531.2 (464.5-982.0)		51.40 (47.50-53.35)		0.0433 (0.0175-0.1328)	
Initial treatment																					
CR	33 (75.0)	4.910 (3.860-7.115)	0.4343	1.780 (1.350-2.235)	0.2083	0.330 (0.275-0.510)	0.9202	0.27 (0.080-0.755)	0.2852	2.724 (2.014-4.527)	0.7284	143.5 (106.8-165.6)	0.8100	5.152 (3.521-6.435)	0.3593	687.4 (472.2-1153.0)	0.4535	51.00 (47.63-53.25)	0.2569	0.0555 (0.0182-0.1624)	0.3983
PR/SD/PD	11 (25.0)	4.190 (3.740-6.040)		1.440 (0.930-2.460)		0.390 (0.250-0.500)		0.13 (0.070-0.350)		3.729 (1.713-5.698)		118.6 (78.1-207.5)		3.600 (2.789-6.240)		443.6 (309.6-1279.0)		47.00 (44.10-52.55)		0.0302 (0.0184-0.0972)	
CTC																					
Positive	28 (63.6)	5.390 (3.715-7.128)	0.6949	1.740 (1.198-2.493)	0.6508	0.330 (0.288-0.550)	0.5661	0.28 (0.080-0.890)	0.2177	2.579 (1.761-5.237)	0.7084	147.5 (99.3-222.8)	0.5065	5.129 (3.435-5.984)	0.7673	636.8 (478.3-1448.0)	0.6037	51.30 (45.40-55.14)	0.5997	0.0587 (0.0180-0.2205)	0.4231
Negative	16 (36.4)	4.655 (3.870-6.168)		1.645 (1.055-2.268)		0.365 (0.253-0.463)		0.16 (0.070-0.365)		3.108 (1.962-4.900)		128.9 (105.1-164.6)		4.885 (3.029-6.590)		634.7 (442.7-1165.0)		49.33 (46.39-52.53)		0.0394 (0.0184-0.1164)	

[a]P<0.05. The values of circulating immune cells and their associated markers are indicated as the median and interquartile range. CTC, circulating tumor cell; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CRP, C-reactive protein; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LMR, lymphocyte-monocyte ratio; SII, systemic immune-inflammation index; PNI, prognostic nutrition index; CAR, CRP/albumin ratio.

Table II. Prognostic value of systemic immune-related markers in patients with head and neck squamous cell carcinoma (n=44).

A, Progression-free survival

Variables	N	P-value	HR	95% CI
Neutrophils, x10^9/l				
<3.670	8	0.1919	3.5330	1.0010-12.4800
≥3.670	36			
Lymphocytes, x10^9/l				
<1.090	10	0.0021[a]	0.2339	0.0608-0.9001
≥1.090	34			
Monocytes, x10^9/l				
<0.375	25	0.2740	1.7460	0.6204-4.9160
≥0.375	19			
CRP, mg/dl				
<0.065	36	0.1440	0.2489	0.0738-0.8397
≥0.065	8			
NLR				
<3.710	28	0.0050[a]	4.1040	1.3960-12.0600
≥3.710	16			
PLR				
<157.1	30	0.0400[a]	2.7610	0.9080-8.3980
≥157.1	14			
LMR				
<3.704	15	0.0130[a]	0.2951	0.0985-0.8842
≥3.704	29			
SII				
<1,027	30	0.0460[a]	2.6870	0.8890-8.1210
≥1,027	14			
PNI				
<49.43	21	0.0150[a]	0.2697	0.0969-0.7504
≥49.43	23			
CAR				
<0.01745	9	0.1010	4.6300	1.4350-14.9300
≥0.01745	35			

B, Overall survival

Variables	N	P-value	HR	95% CI
Neutrophils, x10^9/l				
<4.000	13	0.1053	Undefined	Undefined
≥4.000	31			
Lymphocytes, x10^9/l				
<1.465	15	0.2465	0.4250	0.0910-1.9860
≥1.465	29			
Monocytes, x10^9/l				
<0.375	25	0.0490[a]	4.4270	0.9467-20.7000
≥0.375	19			
CRP, mg/dl				
<0.065	36	0.1320	Undefined	Undefined
≥0.065	8			

Table II. Continued.

B, Overall survival

Variables	N	P-value	HR	95% CI
NLR				
<3.710	28	0.1090	3.4560	0.7702-15.5000
≥3.710	16			
PLR				
<157.1	30	0.0441[a]	4.4910	0.9573-21.0700
≥157.1	14			
LMR				
<6.496	35	0.2147	Undefined	Undefined
≥6.496	9			
SII				
<836	27	0.0975	3.5890	0.7962-16.1800
≥836	17			
PNI				
<43.63	5	0.6657	0.7170	0.1209-4.2520
≥43.63	39			
CAR				
<0.01745	9	0.1060	Undefined	Undefined
≥0.01745	35			

[a]P<0.05. HR, hazard ratio; CI, confidence interval; CRP, C-reactive protein; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LMR, lymphocyte-monocyte ratio; SII, systemic immune-inflammation index; PNI, prognostic nutrition index; CAR, CRP/albumin ratio.

were significantly correlated with systemic immune-related markers (Table III). In particular, *PIK3CA* expression in CTCs was significantly correlated with higher lymphocyte counts (P=0.035) and PNI (P=0.0157). Patients with CTCs expressing *CD47* showed significantly higher neutrophil (P=0.0031) and monocyte counts (P=0.0016). Furthermore, those with CTCs expressing *PDCD1LG2* showed significantly lower CRP (P=0.0271) and CAR (P=0.0207) levels.

Discussion

Circulating immune-related cells extravasate, migrate toward the tumor site, and perform multiple important functions in the immune response against tumor cells. It is well known that the interaction between immune cells and tumor cells in the tumor microenvironment is associated with prognosis and treatment efficacy (3,4). Systemic immunity in cancer patients also plays a crucial role in processes ranging from tumor initiation to metastatic progression. To date, several systemic immune-related markers, including NLR, PLR, and SII, have been reported to reflect disease progression and therapeutic response as well as predict prognosis in patients with HNSCC (17-19). In the present study, despite its small sample size, several immune-related markers were clearly correlated with clinical features and

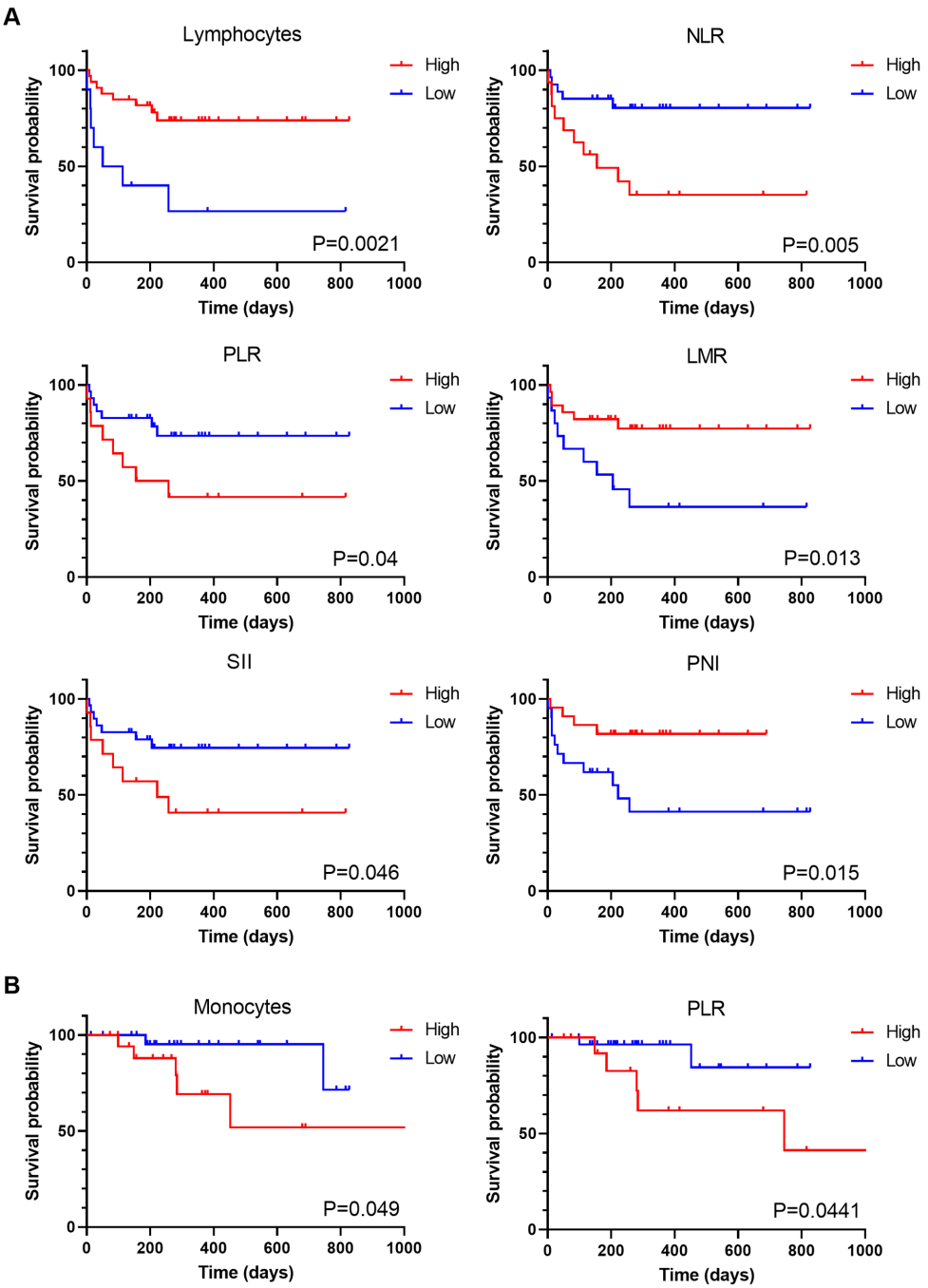

Figure 1. Kaplan-Meier survival analysis in patients with head and neck squamous cell carcinoma according to circulating immune cells and their associated markers. (A) Progression-free survival. (B) Overall survival. Optimal cut-off values were determined by receiver operating characteristic curve analysis. NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LMR, lymphocyte-monocyte ratio; SII, systemic immune-inflammation index; PNI, prognostic nutrition index.

prognosis. Patients with advanced disease exhibited significantly higher monocyte counts and lower LMR than those with early-stage disease. Our previous study on peripheral monocytes in patients with oropharyngeal squamous cell carcinoma (OPSCC) indicated that elevated monocyte counts and lower LMR were independent prognostic factors for PFS and OS, respectively (20). In line with our previous study, in cases of HNSCC including OPSCC, similar findings were observed, suggesting that circulating monocytes in patients with HNSCC are closely related to disease status. Although the reason for this association is not yet fully understood, a high monocyte count generally reflects chronic inflammatory conditions, which can promote angiogenesis, induce cell proliferation, increase reactive oxygen species production, and suppress antitumor immunity (21,22). In our previous study, the presence of CTCs was associated with treatment response, locoregional recurrence, and PFS (12); however, there was no significant correlation between the presence of CTCs and systemic immune-related markers. Therefore, systemic immune-related markers may be insufficient to assess antitumor immunity against CTCs. Notably, lymphocyte-related markers, lymphocyte counts, NLR, and

Table III. Association between systemic immune-related markers and molecular characteristics of CTCs in patients (n=28).

Gene symbol	N (%)	Neutrophils, x10^9/l	P-value	Lymphocytes, x10^9/l	P-value	Monocytes, x10^9/l	P-value	CRP, mg/dl	P-value	NLR	P-value	PLR	P-value	LMR	P-value	SII	P-value	PNI	P-value	CAR	P-value
PIK3CA																					
Positive	14 (50.0)	4.415 (3.800-5.723)	0.8036	1.900 (1.478-2.535)	0.0350[a]	0.380 (0.258-0.410)	0.8299	0.100 (0.070-0.320)	0.1270	2.261 (1.711-3.789)	0.0556	121.6 (96.9-152.5)	0.2273	5.675 (3.817-7.343)	0.0690	506.0 (329.2-886.7)	0.1936	51.85 (49.20-55.35)	0.0157[a]	0.0222 (0.0172-0.0717)	0.0869
Negative	14 (50.0)	4.785 (3.905-7.200)		1.445 (0.868-1.883)		0.310 (0.243-0.563)		0.265 (0.093-0.705)		3.887 (2.609-5.926)		150.0 (107.7-211.9)		3.299 (1.986-6.310)		1020.0 (463.9-1205.0)		47.38 (44.66-49.76)		0.0760 (0.0213-0.1819)	
CCND1																					
Positive	14 (50.0)	4.655 (4.133-5.433)	0.9820	1.875 (1.328-2.535)	0.1139	0.395 (0.283-0.460)	0.3228	0.310 (0.095-0.500)	0.2306	2.556 (1.711-4.248)	0.2273	132.9 (93.4-170.0)	0.8743	4.577 (3.390-7.343)	0.6027	672.7 (353.4-1012.0)	0.4824	50.55 (46.84-53.24)	0.5189	0.0738 (0.0218-0.1254)	0.2405
Negative	14 (50.0)	4.785 (3.670-7.963)		1.525 (0.960-1.920)		0.310 (0.243-0.478)		0.115 (0.063-0.265)		3.650 (2.312-6.035)		128.3 (108.9-167.6)		4.885 (2.502-6.351)		634.4 (459.2-1218.0)		48.95 (46.18-52.68)		0.0262 (0.0145-0.0675)	
SNAI1																					
Positive	10 (35.7)	5.400 (3.890-7.948)	0.4081	1.740 (1.328-2.535)	0.3318	0.395 (0.305-0.455)	0.4567	0.200 (0.078-0.383)	0.8226	3.330 (1.989-5.592)	0.9063	128.9 (102.6-153.1)	0.4642	4.524 (3.479-6.404)	0.9812	783.7 (353.4-1173.0)	0.7595	51.85 (47.51-53.24)	0.3375	0.0450 (0.0198-0.1029)	0.9720
Negative	18 (64.3)	4.375 (3.815-5.678)		1.640 (0.945-2.118)		0.315 (0.243-0.493)		0.135 (0.070-0.418)		2.985 (1.899-4.533)		134.9 (102.0-205.6)		4.885 (2.815-6.919)		523.4 (459.2-1181.0)		49.15 (46.18-51.96)		0.0346 (0.0179-0.1254)	
VIM																					
Positive	12 (42.9)	4.415 (3.643-7.868)	0.9818	1.585 (1.255-2.428)	0.9454	0.395 (0.268-0.455)	0.4850	0.135 (0.070-0.358)	0.8100	3.227 (1.706-5.634)	0.8731	133.4 (84.4-167.4)	0.8017	5.967 (2.895-6.590)	0.8372	687.0 (316.2-1163.0)	>0.9999	49.28 (46.74-52.46)	0.9725	0.0346 (0.0177-0.1177)	0.9549
Negative	16 (57.1)	4.785 (3.870-5.485)		1.685 (0.975-2.153)		0.315 (0.245-0.485)		0.200 (0.073-0.448)		3.108 (2.442-4.303)		128.9 (113.2-162.8)		4.220 (3.029-6.984)		616.9 (448.8-1165.0)		50.18 (45.21-53.13)		0.0450 (0.0189-0.1143)	
CD44																					
Positive	15 (53.6)	5.440 (4.190-8.360)	0.0520	1.620 (0.990-2.460)	0.7168	0.410 (0.320-0.500)	0.0314[a]	0.180 (0.080-0.480)	0.6088	3.691 (1.713-6.253)	0.3627	122.2 (98.5-166.7)	0.4672	3.821 (3.000-6.122)	0.2737	745.6 (359.3-1226.0)	0.6177	49.30 (47.75-52.45)	0.9372	0.0400 (0.0205-0.1200)	0.7077

Table III. Continued.

Gene symbol	N (%)	Neutrophils, x10^9/l	P-value	Lymphocytes, x10^9/l	P-value	Monocytes, x10^9/l	P-value	CRP, mg/dl	P-value	NLR	P-value	PLR	P-value	LMR	P-value	SII	P-value	PNI	P-value	CAR	P-value
Negative	13 (46.4)	4.150 (3.600-5.395)		1.670 (1.120-2.135)		0.270 (0.220-0.380)		0.100 (0.070-0.345)		2.724 (2.002-3.947)		148.8 (110.4-181.7)		6.185 (3.233-7.716)		523.1 (454.0-1020.0)		51.00 (45.33-54.35)		0.0222 (0.0174-0.1210)	
NANOG																					
Positive	10 (35.7)	4.205 (3.800-4.783)	0.1749	1.905 (1.463-2.473)	0.1749	0.355 (0.235-0.530)	0.9343	0.325 (0.078-0.713)	0.2293	2.448 (1.711-3.498)	0.0987	111.2 (75.6-142.9)	0.0800	5.967 (3.390-8.523)	0.3318	453.5 (323.4-1001.0)	0.0400[a]	50.15 (44.06-55.35)	0.8973	0.1095 (0.0198-0.1819)	0.1868
Negative	18 (64.3)	5.425 (3.965-7.200)		1.525 (0.960-2.203)		0.365 (0.265-0.448)		0.135 (0.070-0.322)		3.710 (2.351-5.701)		150.0 (117.1-167.6)		4.273 (2.815-6.351)		836.0 (484.6-1239.0)		49.30 (46.56-52.26)		0.0346 (0.0179-0.0717)	
ALDH1A1																					
Positive	14 (50.0)	4.835 (3.345-8.603)	0.8388	1.525 (1.185-2.308)	0.5714	0.360 (0.258-0.418)	0.8123	0.100 (0.070-0.398)	0.3331	3.330 (1.871-5.926)	0.6347	128.9 (96.9-161.3)	0.6673	5.189 (3.450-6.257)	0.9459	634.4 (329.2-1275.0)	0.9459	50.43 (46.91-52.48)	0.4473	0.0222 (0.0172-0.1093)	0.2502
Negative	14 (50.0)	4.655 (3.930-5.670)		1.735 (0.960-2.280)		0.365 (0.235-0.530)		0.265 (0.093-0.425)		2.953 (2.017-4.211)		134.9 (108.4-176.3)		4.273 (2.657-7.762)		672.7 (459.2-1084.0)		48.85 (44.66-53.84)		0.0622 (0.0213-0.1450)	
CD47																					
Positive	16 (57.1)	5.435 (4.240-8.018)	0.0031[a]	1.615 (1.010-2.250)	0.6642	0.410 (0.368-0.515)	0.0016[a]	0.260 (0.100-0.448)	0.1350	3.849 (2.171-6.144)	0.0593	147.4 (111.0-169.5)	0.2803	3.710 (2.745-6.045)	0.0661	923.2 (466.0-1219.0)	0.0816	49.33 (47.98-52.53)	0.3776	0.0598 (0.0222-0.1213)	0.1182
Negative	12 (42.9)	3.890 (3.115-4.725)		1.685 (1.060-2.290)		0.255 (0.225-0.323)		0.075 (0.048-0.335)		2.693 (1.686-3.518)		118.1 (91.4-150.6)		6.213 (4.011-8.042)		481.0 (322.0-867.4)		48.38 (45.09-52.89)		0.0195 (0.0107-0.0930)	
CD274																					
Positive	11 (39.3)	4.790 (3.840-8.360)	0.5784	1.490 (0.860-2.100)	0.2255	0.360 (0.260-0.470)	0.7902	0.180 (0.080-0.320)	0.9170	3.691 (2.441-5.517)	0.2255	143.5 (121.0-205.0)	0.0659	3.821 (2.660-7.250)	0.4581	821.8 (464.5-1425.0)	0.2441	51.00 (46.30-52.45)	0.9172	0.0400 (0.0205-0.1200)	0.8440
Negative	17 (60.7)	4.530 (3.820-5.915)		1.670 (1.355-2.395)		0.370 (0.250-0.455)		0.140 (0.070-0.465)		2.724 (1.708-4.067)		111.7 (82.8-157.1)		5.813 (3.299-6.496)		523.1 (322.7-1165.0)		49.30 (46.23-52.95)		0.0389 (0.0180-0.1137)	

Table III. Continued.

Gene symbol	N (%)	Neutrophils, x10^9/l	P-value	Lymphocytes, x10^9/l	P-value	Monocytes, x10^9/l	P-value	CRP, mg/dl	P-value	NLR	P-value	PLR	P-value	LMR	P-value	SII	P-value	PNI	P-value	CAR	P-value
PDCD1-LG2																					
Positive	8 (28.6)	4.870 (3.798-9.088)	0.5327	1.475 (0.900-2.458)	0.8227	0.370 (0.268-0.463)	0.6445	0.075 (0.048-0.123)	0.0271[a]	4.244 (1.895-6.579)	0.4385	132.9 (88.0-198.2)	0.8617	4.480 (2.745-5.898)	0.4688	856.9 (341.7-1722.0)	0.5327	50.43 (47.56-52.29)	0.7379	0.0190 (0.0111-0.0282)	0.0207[a]
Negative	20 (71.4)	4.655 (3.870-5.483)		1.645 (1.313-2.153)		0.365 (0.250-0.478)		0.265 (0.100-0.513)		2.985 (1.962-4.116)		128.3 (105.1-157.7)		5.216 (3.207-7.109)		634.7 (448.8-1042.0)		49.18 (45.93-53.13)		0.0622 (0.0222-0.1327)	

[a]P<0.05. The values of circulating immune cells and their associated markers are indicated as the median and interquartile range. CTC, circulating tumor cell; CRP, C-reactive protein; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LMR, lymphocyte-monocyte ratio; SII, systemic immune-inflammation index; PNI, prognostic nutrition index; CAR, CRP/albumin ratio; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit a; CCND1, cyclin D1; SNAI1, snail family transcriptional repressor 1; VIM, vimentin; NANOG, nanog homeobox; ALDH1A1, aldehyde dehydrogenase 1 family member A1; PDCD1LG2, programmed cell death 1 ligand 2.

LMR were significantly associated with distant metastasis. These findings imply that lymphocyte-mediated systemic immune responses may contribute to the prevention of distant metastasis. So far, several studies have investigated the relationship between systemic immune-related markers and primary tumor characteristics using immunohistochemistry or molecular analysis; however, it seems that no meaningful correlations have yet been observed (23-25). In the dynamic multistep process of colonization of distant organs, CTCs that disseminate in the bloodstream and seed new tumors at distant organ sites might have acquired a status of dormancy in the peripheral bloodstream. Therefore, we investigated the relationship between the molecular characteristics of CTCs and systemic immune-related markers.

Among the 10 genes tested, *PIK3CA*, *CD47*, and *PDCD1LG2* expression levels in CTCs were significantly correlated with two systemic immune-related markers. *PIK3CA* is an oncogene that is known to play a role in regulating cell proliferation, invasion, and metabolism (26,27). Chen *et al* demonstrated that *PIK3CA* overexpression promotes the epithelial-mesenchymal transition and enriches cancer stem cells in both murine and human HNSCC cell lines (28). Moreover, *PIK3CA* overexpression was reported to be associated with poor outcomes (29). Patients with *PIK3CA*-positive CTCs showed higher lymphocyte counts and higher PNI, suggesting that CTCs could acquire immune resistance through *PIK3CA* expression. Indeed, in mouse experiments using pancreatic cancer cell lines, PIK3CA-AKT signaling in tumors was found to reduce the cell-surface expression levels of major histocompatibility complex I molecules and CD80, which promote immune evasion (30). Additionally, a tendency toward significance between the other two lymphocyte-related markers (NLR and LMR) and *PIK3CA* expression in CTCs suggests immune evasion by *PIK3CA* expression in CTCs.

CD47-positive CTCs were found in patients with higher neutrophil and monocyte counts. In addition, high NLR, low LMR, and high SII showed a tendency toward *CD47* expression in CTCs. CD47 has been shown to be highly expressed in multiple cancer types, including HNSCC (31-34). We previously reported that CD47 is expressed in approximately half (56.8%) of oral SCC tissues and that CD47 expression correlates with poor OS (34). Most importantly, CD47 acts as a signal to inhibit phagocytic activity by binding to signal regulatory protein α (SIRPα) present on phagocytes (35); thus, it has been suggested that tumor cells evade the host's immune surveillance by expressing CD47 on their surface. As SIRPα is known to be expressed on monocytes, macrophages, neutrophils, and dendritic cells (36,37), in cases of higher circulating monocyte and/or neutrophil counts, CTCs may be more likely to be phagocytosed and killed by these cells. Some CTCs may upregulate CD47 expression to evade the innate immune response mediated by neutrophils and monocytes.

Finally, *PDCD1LG2* expression in CTCs was observed in patients with lower CRP values and lower CARs. Similar to programmed cell death 1 ligand 1 (PD-L1), PD-L2 encoded by the *PDCD1IG2* gene binds to PD-1 on T cells and inhibits T-cell proliferation and effector functions (38). Moreover, the expression of PD-L2 is regulated by interferon receptor

signaling pathways, particularly the interferon-γ (IFN-γ) pathway (39). CRP is an acute inflammatory protein that increases in response to infection and inflammation. Recently, Yoshida *et al* demonstrated that CRP inhibits proliferation, activation-associated phenotypes, and the effector function of activated T cells in patients with melanoma (40). Thus, high CRP levels may impair adaptive immunity and consequently affect PD-L2 expression on CTCs via reduced IFN-γ production.

Although the relatively small sample size partially limits the strength of these findings, our results indicate that circulating immune cells and their related markers are correlated with disease progression and clinical outcomes in patients with HNSCC. Moreover, the interaction between CTCs and circulating immune cells appears to provide survival advantages via molecular alterations to CTCs. Further elucidation of the survival mechanisms of CTCs in the blood microenvironment may provide new insights into novel therapeutic strategies targeting CTCs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HTad and KC conceived and designed the study. HTak, YN, TM, SI and IM acquired the data. HTad and HTak analyzed and interpreted the data. HTad, HTak and KC confirm the authenticity of the raw data. HTad and KC wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of Gunma University Hospital (Maebashi, Japan; approval no. 12-12), and written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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