Expression of immune-regulatory molecules in circulating tumor cells derived from patients with head and neck squamous cell carcinoma

Kazuaki Chikamatsu[a,*], Hiroe Tada[a], Hideyuki Takahashi[a], Yuki Kuwabara-Yokobori[a], Hiroki Ishii[b], Shota Ida[a], Masato Shino[a]

[a] Department of Otolaryngology-Head and Neck Surgery, Gunma University Graduate School of Medicine, Gunma, Japan
[b] Laboratory of Cancer Biology and Genetics, National Cancer Institute, NIH, Bethesda, MD, USA

ABSTRACT

Objectives: Circulating tumor cells (CTCs) are cells that have shed from tumor tissue into the bloodstream, and the detection and characterization of CTCs in head and neck squamous cell carcinoma (HNSCC) still remain a challenge.
Materials and methods: CTCs were isolated from 30 patients with HNSCC with recurrent and/or distant metastasis, via the depletion of CD45-positive cells with magnetic beads and the expression of multiple epithelial markers (CK19, EpCAM, EGFR, and c-Met) was analyzed by RT-qPCR with a low concentration of RNA from the CTC population. We next investigated the expression of the immune-regulatory molecules, PD-L1, PD-L2, and CD47, in CTC-positive patients and the PD-L1 expression in CTCs was compared with that in tumor tissues.
Results: Twenty-four (80.0%) of the 30 patients were positive for at least one epithelial-related gene. Among the 24 CTC-positive patients, 19 (79.2%), 20 (83.3%), and 17 (70.8%) patients were positive for CD47, PD-L1, and PD-L2, respectively. Interestingly, the expression of these three immune-regulatory molecules was positively correlated to each other. As expected, PD-L1 expression in the tumor tissue did not correspond completely with that in the CTCs.
Conclusion: Although clinical application and/or characterization of CTCs are still developing, our findings suggest that the CTCs are rapidly becoming a powerful tool in cancer treatments that involve the use of immune checkpoint inhibitors.

Introduction

Circulating tumor cells (CTCs) are cells that have shed from the tumor tissue into the bloodstream, and technological advances in detection and enumeration of such cells have resulted in a new type of blood test referred to as 'liquid biopsy', for cancer detection, diagnosis, treatment efficacy, and monitoring of disease [1–3]. Several studies including our previous study have demonstrated the existence of CTCs in head and neck squamous cell carcinoma (HNSCC) [4–7]. Liquid biopsy has several advantages over tissue biopsy. Liquid biopsy is recognized as an easily accessible, less invasive, and repeatable examination. More importantly, it allows comprehensive analysis of tumor tissue profile.

The emergence of immune checkpoint inhibitors has been drastically changing the cancer treatment paradigm for certain cancers such as melanoma, non-small cell lung carcinoma, renal cell carcinoma, and head and neck carcinoma [8,9]. PD-L1 expression is recognized as a useful biomarker for the efficacy of immune checkpoint inhibitors, and therefore, the precise evaluation of PD-L1 expression on tumor cells is an urgent requirement. To date, although the expression of PD-L1 in tumor cells has been assessed using immunohistochemistry, the type of antibody, evaluation method, and cutoff value are still controversial [10,11]. Furthermore, the heterogeneity of PD-L1 expression in the tumor tissue samples and the longitudinal fluctuation of PD-L1 expression associated with disease activity and the treatment modalities in cancer patients render the assessment even more complicated.

HNSCC is well-known as one of the malignancies with a high level of PD-L1 expression [12]. However, whether the high level of PD-L1 in tumor cells is associated with better response to immune checkpoint inhibitors still remains unclear. Recently, another ligand of PD-1, PD-L2, has been shown to not only be highly expressed in HNSCC, but also to significantly correlate with PD-L1 expression [13]. Importantly, PD-

L2 status was a significant predictor of the progression-free survival after treatment with an anti-PD-1 antibody, pembrolizumab, suggesting that the clinical response to pembrolizumab might be related to the blockade of PD-1/PD-L2 interaction. Another complicated issue to note is that PD-L1 is expressed not only in tumor cells but also in a variety of stroma cells including T cells, macrophages, fibroblasts, and endothelial cells; and moreover, PD-L1 expression in tumor-infiltrating immune cells, but not tumor cell has been reported to be a favorable prognostic factor in resected HNSCC [14].

In this study, firstly, we investigated whether CTCs could be detected in the bloodstream of patients with recurrent and/or metastatic HNSCC, using the CD45-depletion method. Secondly, the expression of the immune-regulatory molecules, PD-L1, PD-L2, and CD47, in the CTCs and the correlation between those gene expressions were analyzed. Finally, in regard to PD-L1, its expression in primary tumors was compared with that in CTCs.

Materials and methods

Patients

A total of 30 patients with HNSCC with recurrent and/or distant metastasis (R/M HNSCC) were enrolled in this study. This study was approved by the Ethical Committee of the Gunma University hospital (No. 12-12) and a written informed consent was obtained from all the patients. Patients' characteristics are shown in Table 1. The origin of the tumors was as follows: oral cavity (n = 3), nasopharynx (n = 1), oropharynx (n = 3), hypopharynx (n = 12), larynx (n = 4), and paranasal sinuses (n = 6), and parotid gland (n = 1). The median age of the cancer patients was 70.5 years (range 53–86). The numbers of recurrence at the local, regional, and distant sites, including the overlapping cases, were 12 (50.0%), 17 (70.8%), and 20 (83.3%), respectively.

Cell line

The human tongue cancer cell line, SAS was used for preliminary experiments. SAS was cultured in DMEM supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 100 u/ml penicillin, and 100 µg/ml streptomycin (all from Gibco-BRL).

CTC isolation and gene expression analysis

Blood samples were collected with K2EDTA Vacutainer® (BD Bioscience) at the middle of vein puncture in order to avoid contamination of the blood sample with epithelial cells from the skin, and were analyzed within 4 h. The peripheral blood (7.5 ml) obtained from patients was layered over the Ficoll-Paque PLUS (GE Healthcare). Following the centrifugation, the layer containing peripheral blood nuclear cells (PBMCs) was harvested, washed, and the contaminating erythrocytes were further lysed with the red blood cell lysis buffer (Roche). The cell suspension was incubated with human CD45 depletion cocktail for 15 min, and then 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 were transferred into a new tube labelled as CTCs.

Total RNA from CTCs was extracted using the RNeasy micro kit (QIAGEN) according to the manufacturer's instruction. Each RNA sample was treated with DNase to remove contaminating genomic DNA. cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (QIAGEN), and further pre-amplification step was performed using the TaqMan™ PreAmp Master Mix kit (Applied Biosystems) with 14 cycles. The preamplified products were then analyzed by RT-qPCR (Applied Biosystems) for the eight target genes. Eight primers for the seven targets (CK19, EpCAM, EGFR, c-Met, PD-L1, PD-L2, and CD47) and the beta actin as control, were purchased from

Table 1
Patient characteristics tested in this study and PD-L1 expression in tumor tissue and CTCs.

Pt. #	Age/sex	Primary site	Recurrence/metastasis			Initial treatment			PD-L1 expression	
			Local	Regional	Distant	Surgery	Radiotherapy	Chemotherapy	Tumor tissue	CTCs
1	73M	maxillary	−	+	+	−	+	+	+	+
2	69M	oropharynx	−	+	+	+	+	+	+	−
3	67M	hypopharynx	−	−	+	+	+	+	−	+
4	67M	oropharynx	−	−	+	−	−	+	+	+
5	57M	hypopharynx	+	+	+	−	+	+	+	+
6	86M	maxillary	+	−	−	−	+	+	+	+
7	75M	hypopharynx	−	−	−	−	−	−	−	+
8	68M	larynx	−	+	+	+	+	+	+	−
9	72F	larynx	−	+	+	+	+	+	−	+
10	77M	maxillary	+	−	−	−	+	+	+	+
11	69M	parotid gland	−	−	+	+	+	+	−	+
12	66M	hypopharynx	−	−	+	+	+	+	+	−
13	54F	hypopharynx	−	+	+	−	+	+	−	+
14	72M	hypopharynx	−	−	+	+	+	+	−	+
15	55M	hypopharynx	−	+	+	−	−	+	+	+
16	71M	larynx	+	−	+	−	+	+	+	+
17	59M	larynx	+	−	−	+	+	+	−	+
18	71M	hypopharynx	+	+	+	−	+	+	−	+
19	70M	oral cavity	−	+	+	+	+	+	+	+
20	69M	nasopharynx	−	+	+	−	+	+	+	+
21	53M	maxillary	+	−	−	−	+	+	−	+
22	60M	hypopharynx	−	−	+	+	+	+	−	+
23	68M	oropharynx	−	+	−	−	+	+	+	+
24	72M	oral cavity	+	+	−	−	−	+	NA	−
25	85M	maxillary	+	−	−	+	−	+	ND	NA
26	55M	hypopharynx	+	+	+	−	−	+	ND	NA
27	61M	maxillary	+	+	−	+	+	+	ND	NA
28	75M	hypopharynx	−	+	−	+	+	−	ND	NA
29	69F	oral cavity	+	+	+	+	+	+	ND	NA
30	68M	hypopharynx	−	+	+	+	+	+	ND	NA

NA. not available; ND, not done; CTCs, circulating tumor cells.

Applied Biosystems (TaqMan™ Gene Expression Assays) for this study. All samples were analyzed in triplicate. Only samples that were positive for beta actin were enrolled in this study. For every PCR run, positive control (SAS cells) and no-template control (a water control) were included. The information regarding the PCR primers for the eight genes tested is shown in Supplementary Table 1. Target gene expression in the CTCs was determined using a relative quantification method. When at least one gene out of the four epithelial related genes was detected, the sample was defined as positive for CTCs. The CTC-positive samples were further analyzed for the expression of the immune-regulatory molecules, PD-L1, PD-L2, and CD47. Since samples obtained by negative selection are invariably contaminated with leukocytes, the average Ct value of 20 healthy donor samples which were processed and analyzed in exactly the same way as patient samples was used as a baseline of the control group. Ct values of PD-L1, PD-L2, and CD47 were normalized to a reference gene (beta actin), and the expression levels of the immune-regulatory molecules in CTCs was estimated as the fold change compared to that in healthy donor samples, by the relative quantification 2-deltadelta Ct method [15].

Immunohistochemistry for PD-L1

PD-L1 expression in tumor tissues was evaluated by immunohistochemistry with anti-PD-L1 monoclonal antibody (PD-L1 IHC 28-8 pharmDx, Dako). Briefly, sections were deparaffinized, and the antigen retrieval was performed by immersion in citrate buffer at 97 °C for 20 min. The slides were then stained using Dako Autostainer Link 48 (Agilent). An experienced pathologist assessed quantitatively the PD-L1 expression in tumor cells as the percentage of cells with moderate to strong membranous staining. PD-L1-positive was defined as membranous PD-L1 expression in 1% or more.

Statistical analyses

Data were analyzed using the Statistical Package for Social Science version 22.0 (SPSS, IBM, Armonk, NY, USA). Correlation between the expression of immune-regulatory molecules in CTCs was determined by the Spearman's rank test and correlation between PD-L1 tumor tissue expression and the presence of PD-L1-positive CTCs was determined by the Fisher's exact test. Significance was defined at p < 0.05.

Results

Preliminary experiments using a HNSCC cell line and healthy donor samples

First, for the establishment of the experimental systems, we extracted mRNA, synthesized cDNA, preamplified cDNA, and performed RT-qPCR using the HNSCC cell line, SAS. Four epithelial markers, EpCAM, CK19, EGFR, and c-Met, were selected for the detection of CTCs. These four target genes and the internal control gene, beta actin, were investigated to confirm whether the serial dilution of cell number, from 1 to 1000, could be detected by RT-qPCR. As shown in Fig. 1A, Ct values were inversely correlated with the number of tumor cells.

Next, SAS was spiked into 7.5 ml of whole blood from a healthy donor with 10, 100, and 1000 cells. Each sample was processed with Ficoll density gradient, red blood cell lysis, and depletion of CD45+ cells. We then extracted mRNA from the enriched fraction, synthesized cDNA, preamplified cDNA, and performed RT-qPCR. Four epithelial markers were successfully detected in all spiked-in blood samples (Fig. 1B).

The diagnostic specificity of this assay was evaluated by peripheral blood samples from 20 healthy donors. Only 1 of 20 (5%) samples was positive for CK-19, whereas any other epithelial-related genes were not detected in these healthy donors (data not shown).

Detection of CTCs in patients with R/M HNSCC

Twenty-four (80.0%) of the 30 patients with R/M HNSCC were positive for at least one target gene. Among the 24 CTC-positive patients, EpCAM was detected in 10 (41.7%), CK-19 in 19 (79.2%), EGFR in 10 (41.7%), and c-Met in 16 (66.7%) patients (Fig. 2). Only one (4.2%) patient was positive for all the four markers, 12 (50.0%) for three markers, 4 (16.7%) for two markers, and 7 (29.2%) for one marker.

Immune-regulatory molecule expression in CTCs

Next, we investigated the expression of the immune-regulatory molecules, PD-L1, PD-L2, and CD47, by molecular analysis of the CTCs. It is previously known that these molecules are also expressed in various hematopoietic cells beside the tumor cells [16,17], and the CTC samples obtained from PBMCs by the CD45-depletion method are invariably contaminated with leukocytes. To eliminate the background arising from the contaminating leukocytes, the average of delta Ct values ($Ct_{target} - Ct_{beta\ actin}$) of CD45-depleted samples from 20 healthy donors was used as the control, and the expression levels of the immune-regulatory molecules were calculated using the 2-deltadelta Ct method. If the 2-deltadelta Ct value is more than 1, the sample was assessed as positive for the expression of the molecule.

Among the 24 CTC-positive patients, 19 (79.2%), 20 (83.3%), and 17 (70.8%) patients were positive for CD47, PD-L1, and PD-L2, respectively (Fig. 3). Interestingly, the expression of these three immune-regulatory molecules was positively correlated with each other (Fig. 4).

Comparison of PD-L1 expression between tumor tissue and CTCs

The PD-L1 expression was assessed by immunohistochemistry (IHC) in 23 patients with HNSCC who were positive for CTCs in their peripheral blood (Fig. 5). Clinical utility of PD-L1 IHC 28-8 pharmDx has been approved as a companion diagnostic assay for nivolumab in HNSCC. Therefore, the PD-L1 expression in the primary tumors was assessed by IHC and compared with that in CTCs. CTCs expressed PD-L1 in 20 of the 23 patients, while PD-L1 expression in tumor tissue was detected in 13 of the 23 patients (Table 1). Concordance of the PD-L1 expression between the tumor tissue and the CTCs was observed in 10 (43.5%) of the 23 patients; whereas, in the remaining 13 patients, the PD-L1 expression in the tumor tissue did not correspond with that in the CTCs (Table 2). There was no significant correlation between tumor tissue and CTCs PD-L1 expression (*p = 0.23*).

Discussion

The objective of this study was the detection and immune-characterization of CTCs in patients with R/M HNSCC. We succeeded in this objective, and the following findings are particularly important: (1) increased detection rate of CTCs using multiple epithelial markers, (2) the assessment of immune-regulatory molecules in CTCs, and (3) the comparison of PD-L1 expression between tumor tissue and CTCs.

To date, various techniques for CTC detection have been developed and their effectiveness has been validated as a new tool for cancer detection, diagnosis, and treatment [1–3]. In the present study, for molecular analysis of CTCs, we chose the negative selection method involving the depletion of CD45-positive cells with magnetic beads. We previously reported the feasibility of detection and quantification of CTCs in patients with HNSCC, using a low-pressure filtration system equipped with precision microfilters [7]. CTCs could be detected in around 90% of the patients tested; however, there were some areas of concern, which are the presence of false positive cells and the presence of cells with no epithelial cell markers. Similarly, the positive selection system such as the CELLSEARCH® system could not enrich the cells that lost the epithelial cell-specific molecules and/or acquire mesenchymal
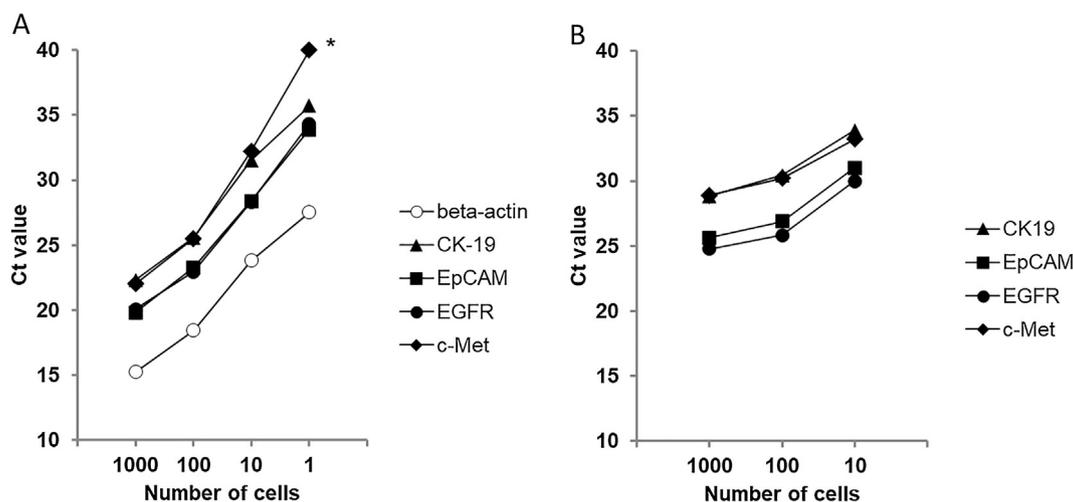
Fig. 1. Standard curve of the cycle threshold (Ct) values calculated from serial dilutions of tumor cells using real time RT-qPCR. Ct values shown are mean values for triplicate reactions. Plots represent cycle threshold versus the number of tumor cells. (A) SAS cells alone and (B) SAS cells spiked into blood sample from a healthy donor. *, as the Ct value of c-Met gene from one SAS culture was not determined, Ct value is indicated as 40.

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22	#23	#24	total (%)
β-actin																									-
EpCAM																										10 (41.7)
CK-19																										19 (79.2)
EGFR																										10 (41.7)
c-MET																										16 (66.7)

Fig. 2. Heat map depicting the expression of four epithelial-related genes in CTC-positive patients. The red square indicates positive for each gene expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phenotypes, as compared to the negative selection system. To increase the real-CTCs detection rate, we performed mRNA expression analysis of multiple epithelial-related markers by RT-qPCR. Four genes used in this study have been previously reported to be predominantly epithelial-specific genes [18] and used for the detection of CTCs in various cancers including HNSCC [19–22]. As shown in Fig. 1A, Ct values obtained with RT-qPCR exhibited excellent correlation with the serially diluted tumor cells. Moreover, 10, 100, and 1000 tumor cells spiked in whole blood were also detected (Fig. 1B). Finally, CTCs were detected in 24 (80.0%) of the 30 patients with R/M HNSCC by our system. Although CTC detection rate by single gene expression in 30 patients tested ranged from 33.3% to 63.3% (EGFR, 33.3%; EpCAM, 33.3%; c-Met, 53.3%; CK-19, 63.3%), the combination of four genes increased the detection rate up to 80.0%. So far, a number of reports have shown that the RT-PCR method using multi-marker gene analysis is superior to that using single gene analysis in various cancers [21,23,24]. Similarly, our results demonstrated the usefulness of multigene test in the detection of CTCs.

CTCs have different malignant potential even in the same patient; especially, the presence of CTCs harboring EMT phenotype would play an important role in the determination of the clinical outcome of cancer

patients. Therefore, the combination of multiple epithelial-specific genes not only increases the sensitivity, but also may enables the assessment of the malignant phenotype.

Next, we were able to detect three immune-regulatory molecules, PD-L1, PD-L2, and CD47, in the CTCs. Strati et al. have earlier investigated PD-L1 expression in EpCAM-positive CTCs in patients with locally advanced HNSCC and PD-L1 expression was found in 24 (25.5%) out of the 94 patients [25]. Moreover, PD-L1 overexpression at the end of the treatment was an independent prognostic factor. The difference in positive rate of PD-L1 expression in CTCs may be due to difference in the objectives and methodology. PD-L2 is also known to bind to PD-1 on T cell and inhibit T cell functions such as T cell proliferation and cytokines production. Yearly et al. have demonstrated certain important findings concerning PD-L2 expression in HNSCC [13]; firstly, more than half of the HNSCC samples expressed PD-L2 and it showed the highest percentage among the tumor types assessed. Although it was evaluated by a scoring system using combined expression in both tumor and non-tumor cells, the overall expression of PD-L1 and PD-L2 were significantly correlated. Similar to that in tumor tissue, our findings showed that there was a significant correlation between PD-L1 and PD-L2 expression in CTCs, suggesting that the characteristics of

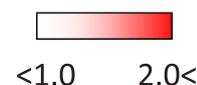|	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22	#23	#24	total (%)
CD47																									19 (79.2)
PD-L1																									20 (83.3)
PD-L2																									17 (70.8)

<1.0   2.0<

Fig. 3. Heat map depicting the mRNA expression level of the immune-regulatory molecules, CD47, PD-L1, and PD-L2 in CTCs. The red square indicates positive for each gene expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
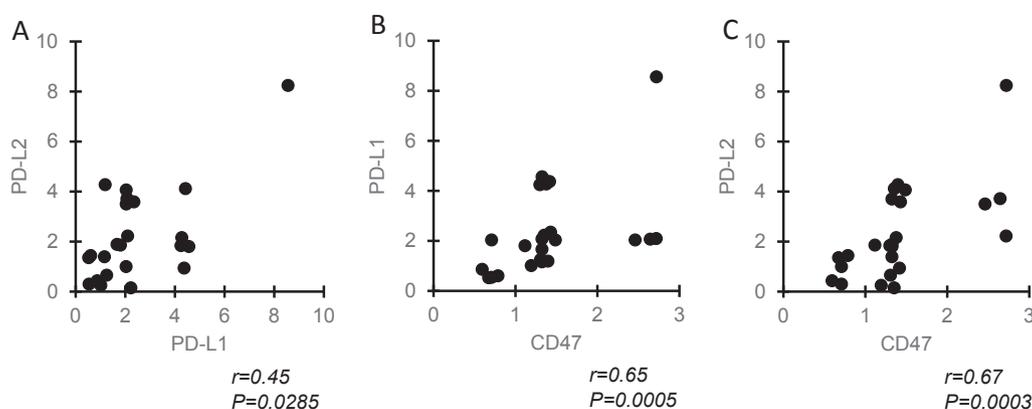
Fig. 4. The mRNA expression level of each immune-regulatory molecule correlated positively with each other. (A) PD-L1 vs PD-L2, *p = 0.0285*; (B) PD-L1 vs CD47, *p = 0.0005*; (C) PD-L2 vs CD47, *p = 0.0003*.

CTCs may closely resemble those of tumor cells from the tumor tissue. As another immune-regulatory molecule, CD47 expression in CTCs was also assessed. CD47 is an integrin associated protein and is expressed in hematopoietic cells as well as in various types of cancers including HNSCC, as previously described [26]. CD47 on tumor cells binds signal-regulator protein alpha (SIRPα) on the phagocytic cells such as macrophages, and inhibits phagocytosis, and functions as an innate immune checkpoint [27]. Therefore, blocking the CD47 checkpoint enhances the engulfment of tumor cells by the antigen-presenting cells, and results in enabling the efficient processing and presentation of the tumor antigens to CD8^{+} effector T cells [28]. CD47 expression in CTCs was found in 19 (79.2%) out of the 24 patients who were detected with CTCs, and this frequency is higher than that in a previous report using immunohistochemistry [26]. Notably, the expression of each immune-regulatory molecule in CTCs was correlated with each other. Regarding the expression of CD47 and PD-L1 in tumor cells, Casey et al. have demonstrated that MYC, which plays critical roles in stem cell maintenance and tumorigenesis, directly binds to the promoters of the *Cd47* and *Pd-l1* genes [29]. Meanwhile, as the expression of PD-L1 and PD-L2 was regulated by the interferon receptor signaling pathways, particularly the interferon gamma signaling [30], it might suggest that the expression of PD-L1 and PD-L2 in tumor cells is correlated. Thus, CTCs in some patients may evade, by the expression of multiple immune-regulatory molecules, both innate and acquired immune responses and lead to an immune-resistant phenotype.

Finally, we compared the expression of PD-L1 in CTCs with that in tumor tissue. As expected, PD-L1 expression in tumor tissue did not correspond to that in CTCs. It is known that PD-L1 expression on tumor cells not only shows localization within the tumor tissue, but also

Table 2
Comparison of PD-L1 expression between tumor tissue and CTCs.

		CTCs		Total	
		+	−		
Tumor tissue	+	10 (43.5)	3 (13.0)	13 (56.5)	p = 0.23
	−	10 (43.5)	0 (0.0)	10 (43.5)	
	Total	20 (87.0)	3 (13.0)	23 (100.0)	

Parentheses indicate percentage. CTCs, circulating tumor cells.

changes dramatically through the treatment process. Ock et al. have shown that PD-L1 expression in HNSCC cell lines is significantly up-regulated after cisplatin treatment [31]. Leduc et al. have also shown that TPF induction chemotherapy increases the PD-L1 expression on both tumor cells and immune cells in HNSCC [32]. Alternatively, PD-L1 expression in several cancer cell lines has been reported to be up-regulated in response to ionizing radiation in a time- and dose-dependent manner [33]. Thus, the expression of PD-L1 in tumor cells varies with different conditions including disease progression, treatment modality, and the immune status and temporal changes. For the development of effective approaches to immune checkpoint inhibitors, precise evaluation of PD-L1 expression would be mandatory and the evaluation of PD-L1 expression in CTCs may enable real-time assessment as well as the continuous monitoring of the PD-L1 status in tumor cells.

In conclusion, our study shows that it is possible to detect CTCs and assess the immunological characteristics of CTCs in patients with R/M HNSCC by real time RT-qPCR. Although clinical application and/or
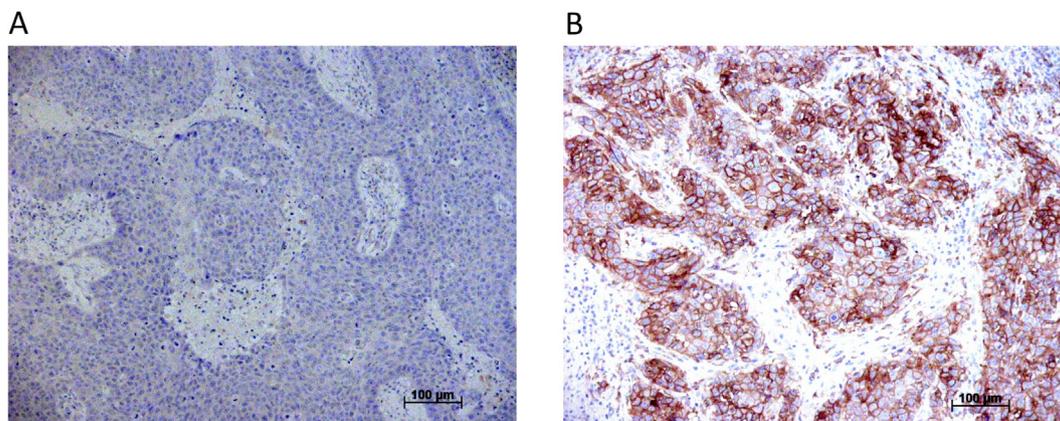

Fig. 5. Representative microphotograph of immunohistochemical staining of PD-L1 using PD-L1 IHC 28-8 pharmDx. (A) a PD-1-negative case (pt-14; magnification ×100) and (B) a PD-1-positive case (pt-19; magnification ×100).

characterization of CTCs are still developing, our findings suggest that CTCs are rapidly becoming a powerful tool in cancer treatment.

Acknowledgements

Conflict of interest statement

The authors declare that they have no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2018.12.002.

References

[1] Friedlander TW, Premasekharan G, Paris PL. Looking back, to the future of circulating tumor cells. Pharmacol Ther 2014;142:271–80.

[2] Economopoulou P, Kotsantis I, Kyrodimos E, Lianidou ES, Psyrri A. Liquid biopsy: an emerging prognostic and predictive tool in head and neck squamous cell carcinoma (HNSCC). Focus on circulating tumor cells (CTCs). Oral Oncol 2017;74:83–9.

[3] Kulasinghe A, Perry C, Jovanovic L, Nelson C, Punyadeera C. Circulating tumor cells in metastatic head and neck cancers. Int J Cancer 2015;136:2515–23.

[4] Hristozova T, Konschak R, Stromberger C, Fusi A, Liu Z, Weichert W, et al. The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN). Ann Oncol 2011;22:1878–85.

[5] Nichols AC, Lowes LE, Szeto CC, Basmaji J, Dhaliwal S, Chapeskie C, et al. Detection of circulating tumor cells in advanced head and neck cancer using the Cell Search system. Head Neck 2012;34:1440–4.

[6] Buglione M, Grisanti S, Almici C, Mangoni M, Polli C, Consoli F, et al. Circulating tumor cells in locally advanced head and neck cancer: preliminary report about their possible role in predicting response to non-surgical treatment and survival. Eur J Cancer 2012;48:3019–26.

[7] Kawada T, Takahashi H, Sakakura K, Ida S, Mito I, Toyoda M, et al. Circulating tumor cells in patients with head and neck squamous cell carcinoma: feasibility of detection and quantitation. Head Neck 2017;39:2180–6.

[8] Shrma P, Allison JP. The future of immune checkpoint therapy. Science 2015;348:56–61.

[9] Pennock GK, Chow LQ. The evolving role of immune checkpoint inhibitors in cancer treatment. Oncologist 2015;20:812–22.

[10] McLaughlin J, Han G, Schalper KA, Carvajal-Hausdorf D, Pelekanou V, Rehman J, et al. Quantitative assessment of the heterogeneity of PD-L1 expression in non-small-cell lung cancer. JAMA Oncol 2016;2:46–54.

[11] De Meulenaere A, Vermassen T, Aspeslagh S, Huvenne W, Van Dorpe J, Ferdinande L, et al. Turning the tide: clinical utility of PD-L1 expression in squamous cell carcinoma of the head and neck. Oral Oncol 2017;70:34–42.

[12] Heineman TE, Widman A, Kuan EC, St John M. The genetic landscape of programmed death ligand-1 (PD-L1) alterations in head and neck cancer. Laryngoscope Investig Otolaryngol 2017;2:99–103.

[13] Yearley JH, Gibson C, Yu N, Moon C, Murphy E, Juco J, et al. PD-L2 expression in human tumors: relevance to anti-PD-1 therapy in cancer. Clin Cancer Res 2017;23:3158–67.

[14] Kim HR, Ha SJ, Hong MH, Heo SJ, Koh YW, Choi EC, et al. PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. Sci Rep 2016;6:36956.

[15] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 2001;25:402–8.

[16] Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat Immunol 2007;8:239–45.

[17] Oldenborg PA. CD47: A cell surface glycoprotein which regulates multiple functions of hematopoietic cells in health and disease. ISRN Hematol 2013;2013:614619.

[18] Sieuwerts AM, Kraan J, Bolt-de Vries JB, van der Spoel P, Mostert B, Martens JWM, et al. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. Breast Cancer Res Treat 2009;118:455–68.

[19] Shi J, Li Y, Liang S, Zeng J, Liu G, Mu F, et al. Analysis of circulating tumor cells in colorectal cancer liver metastasis patients before and after cryosurgery. Cancer Biol Ther 2016;17:935–42.

[20] Ilie M, Szafer-Glusman E, Hofman V, Long-Mira E, Suttmann R, Darbonne W, et al. Expression of MET in circulating tumor cells correlates with expression in tumor tissue from advanced-stage lung cancer patients. Oncotarget 2017;8:26112–21.

[21] Xi L, Nicastri DG, El-Hefnawy T, Hughes SJ, Luketich JD, Godfrey TE. Optimal markers for real-time quantitative reverse transcription PCR detection of circulating tumor cells from melanoma, breast, colon, esophageal, head and neck, and lung cancers. Clin Chem 2007;53:1206–15.

[22] Braig F, Voigtlaender M, Schieferdecker A, Busch C-J, Laban S, Grob T, et al. Liquid biopsy monitoring uncovers acquired RAS-mediated resistance to cetuximab in a substantial proportion of patients with head and neck squamous cell carcinoma. Oncotarget 2016;7:42988–95.

[23] de Albuquerque A, Kaul S, Breier G, Krabisch P, Fersis N. Multimarker analysis of circulating tumor cells in peripheral blood of metastatic breast cancer patients: a step forward in personalized medicine. Breast Care (Basel) 2012;7:7–12.

[24] Obermayr E, Sanchez-Cabo F, Tea MK, Singer CF, Krainer M, Fischer MB, et al. Assessment of a six gene panel for the molecular detection of circulating tumor cells in the blood of female cancer patients. BMC Cancer 2010;10:666.

[25] Strati A, Koutsodontis G, Papaxoinis G, Angelidis I, Zavridou M, Economopoulou P, et al. Prognostic significance of PD-L1 expression on circulating tumor cells in patients with head and neck squamous cell carcinoma. Ann Oncol 2017;28:1923–33.

[26] Sakakura K, Takahashi H, Kaira K, Toyoda M, Murata T, Ohnishi H, et al. Relationship between tumor-associated macrophage subsets and CD47 expression in squamous cell carcinoma of the head and neck in the tumor microenvironment. Lab Invest 2016;96:994–1003.

[27] Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, et al. The CD47-signal regulatory protein alpha (SIRPα) interaction is a therapeutic target for human solid tumors. Proc Natl Acad Sci USA 2012;109:6662–7.

[28] Liu X, Pu Y, Cron K, Deng L, Kline J, Frazier WA, et al. CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. Nat Med 2015;21:1209–15.

[29] Casey SC, Tong L, Li Y, Do R, Walz S, Fitzgerald KN, et al. MYC regulates the antitumor immune response through CD47 and PD-L1. Science 2016;352:227–31.

[30] Garcia-Diaz A, Shin DS, Moreno BH, Saco J, EWscuin-Ordinas H, Rodriguez GA, et al. Interferon receptor signaling pathways regulating PD-L1 and PD-L2 expression. Cell Rep 2017;19:1189–201.

[31] Ock C-Y, Kim S, Keam B, Kim S, Ahn Y-O, Chung E-J, et al. Changes in programmed death-ligand 1 expression during cisplatin treatment in patients with head and neck squamous cell carcinoma. Oncotarget 2017;8:97920–7.

[32] Leduc C, Adam J, Louvet E, Sourisseau T, Dorvault N, Bernard M, et al. TPF induction chemotherapy increases PD-L1 expression in tumor cells and immune cells in head and neck squamous cell carcinoma. ESMO Open 2018;3:e000257.

[33] Sato H, Niimi A, Yasuhara T, Permata TBM, Hagiwara Y, Isono M, et al. DNA double-strand break repair pathway regulates PD-L1 expression in cancer cells. Nat Commun 2017;8:1751.