Molecular profiling of circulating tumor cells predicts clinical outcome in head and neck squamous cell carcinoma

Keywords: circulating tumor cells (CTCs); head and neck squamous cell carcinoma (HNSCC); microfilter; *MET*; *CD274*

Running title: Molecular profiling of circulating tumor cells

Abstract

Objectives: The relationship between the molecular profiling of circulating tumor cells (CTCs) and clinical factors is a challenge. In this study, we performed molecular detection and characterization of CTCs in patients with head and neck squamous cell carcinoma (HNSCC).

Materials and methods: CTCs captured by microfilter were analyzed for the expression of multiple epithelial markers (*EPCAM, MET, KRT19, and EGFR*) by RT-qPCR. The CTCs-positive samples were further analyzed for the expression of 10 genes (*PIK3CA, CCND1, SNAI1, VIM, CD44, NANOG, ALDH1A1, CD47, CD274*, and *PDCD1LG2*). Finally, we analyzed whether the molecular profiling of CTCs was associated with clinical factors.

Results: Twenty-eight (63.6%) of the 44 HNSCC patients were positive for at least one epithelial-related gene. CTC-positivity was significantly correlated with treatment resistance (*p=0.0363*), locoregional recurrence (*p=0.0151*), and a shorter progression-free survival (PFS) (*p=0.0107*). Moreover, the expression of *MET* in CTCs was associated with a shorter PFS (*p=0.0426*). Notably, patients with *CD274*-positive CTC showed prolonged PFS (*p=0.0346*) and overall survival (*p=0.0378*) compared to those with *CD274*-negative CTC. *Conclusion:* Our results suggest that molecular profiling characterized by the gene expression of CTCs influences clinical factors in patients with HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for around 5% of all cancers and is the sixth most frequent cancer in the world.[1, 2] Despite advances in diagnosis techniques and treatment modalities, over 50% of HNSCC patients develop locoregional recurrence and/or distant metastasis. Even after complete, margin-negative resection, the recurrence rate remains around 20%.[3, 4] For the prevention, as well as early detection, of tumor recurrence and distant metastasis, the development of new diagnostic, predictive and/or prognostic tools is urgently needed. To this end, liquid biopsy using circulating tumor cells (CTCs), cell-free DNA (cfDNA), and exosome has been recently attracting attention.

For the clinical application of CTCs in HNSCC, evidence has shown that the existence or enumeration of CTCs is associated with various clinical factors, such as clinical stage, treatment response, and prognosis.[5-9] In a previous study, we demonstrated the feasibility of the detection and quantification of CTCs using microfilter and a low-pressure filtration system;[10] however, the characterization of CTCs is a problem that remains to be solved. Similar to tumor cells within tumor tissue, isolated CTCs also consist of heterogeneous cell populations with different functional and phenotypic characteristics. Their comprehensive characterization is indispensable for evaluating the clinical significance of CTCs. Despite the development of various methods,[11] it is generally agreed that the molecular characterization of CTCs is difficult, especially due to the fact that samples are contaminated

with normal leukocytes. A number of studies regarding the molecular analysis of CTCs have estimated gene expression in CTCs as the relative gene expression level quantified by the control samples obtained from healthy donors.[12-14] However, leukocytes, as well as CTCs, from cancer patients have also been exposed to various factors caused by tumor burden; therefore, the molecular characteristics of leukocytes are likely to change with the disease condition compared to that of healthy donors. For a more accurate evaluation of the molecular profiling of CTCs, we first captured CTCs using a low-pressure filtration system equipped with precision microfilters, as described previously.[10] The remaining sample was then passed through a second microfilter to capture the control leukocytes. Using this microfiltration-based RT-qPCR method, we first performed the molecular detection of CTCs in HNSCC patients using four epithelial-related genes (*KRT19, EPCAM, EGFR*, and *MET*). Then, we analyzed the molecular characteristics of CTCs. For the molecular analysis of CTCs in each patient, four phenotypic analysis (cell growth, epithelial-mesenchymal transition (EMT), cancer stemness, and immune regulatory) were performed. Finally, we analyzed whether the molecular profiling of CTCs was associated with clinical factors.

Materials and Methods

Cell line

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

Patients and blood sample collection

A total of 44 patients with pathologically and clinically confirmed HNSCC were enrolled in this study. Staging was undertaken according to the *Union Internationale Contre le Cancer*/American Joint Committee on Cancer TNM classification, 8th edition.[15] We evaluated several clinical factors, including age, sex, primary tumor, T status, N status, stage, response to initial treatment, locoregional recurrence, and distant metastasis. This study was approved by the Institutional Review Board of Gunma University (No.12-12). Written informed consent was obtained from each patient. Patients did not receive any anticancer drugs, radiotherapy, or surgery before blood collection. Blood samples were collected with K2EDTA Vacutainer® (BD Bioscience) from the middle of the vein in order to avoid contamination of the blood sample with epithelial cells from the skin. Samples were analyzed within 4 hours of collection.

Isolation of CTCs and control leukocytes

Peripheral blood samples (7.5 mL) were diluted 2× PBS, placed into a 30-mL syringe, and passed through the CellSieve™ microfilter with 7-μm diameter

pores. The filter was then washed 3× PBS and transferred into a new tube, labelled as CTCs. The blood sample passed through the first filter was passed through the second filter to capture the control leukocytes. The second filter was washed 3× PBS and transferred into another tube, labelled as control leukocytes.

Immunocytochemistry and spike-in experiments

To confirm the presence of CTCs on the microfilter and for the establishment of the experimental systems, the immunocytochemistry of the CTCs captured by microfilters and the spiked-in experiment using SAS were performed as previously described.[10, 16] Briefly, the CTCs captured by the microfilter were stained with an antibody cocktail (FITC-conjugated anti-CK 8, 18, and 19, PE-conjugated anti-EpCAM, and cyanine 5-conjugated anti-CD45 antibodies; Creatv MicroTech, Inc.). The filter was then washed with PBS+0.1% Tween-20 and PBS, placed onto a microscope slide, and mounted using Fluoromount-G with DAPI to obtain an image of the filter by using Zeiss Axioskop2 plus instrument (Carl Zeiss Microscopy GmbH, Jena, Germany). Likewise, SAS was spiked into 7.5 ml of whole blood from a healthy donor with 10, 100, and 1000 cells. Each sample was passed through the microfilter. After washing the microfilter, gene expression analysis was performed.

Gene expression analysis

Gene expression analysis was conducted as previously described [16]. The total RNA from the CTCs and control leukocytes was extracted using the

RNeasy micro kit (QIAGEN) according to the manufacturer's instructions. Each RNA sample was treated with DNase to remove contaminating genomic DNA. cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (QIAGEN). A further pre-amplification step was performed using the TaqMan™ PreAmp Master Mix kit (Applied Biosystems) for 14 cycles. The pre-amplified products were then analyzed by RT-qPCR (Applied Biosystems) for target gene expression. Firstly, the expression of four epithelial-related genes (*EPCAM, MET, KRT19,* and *EGFR*) in the CTC filters was determined. When at least one gene out of the four epithelial-related genes was detected, the sample was defined as positive for CTCs. The CTCs-positive samples were further analyzed for the expression of the following 10 genes, grouped into 4 gene signatures; cell growth (*PIK3CA* and *CCND1*), EMT (*SNAI1* and *VIM*), cancer stemness (*CD44*, *NANOG*, and *ALDH1A1*), and immune regulatory (*CD47*, *CD274*, and *PDCD1LG2*). A total of fifteen primers for the fourteen targets and the *ACTB* (β-actin) control were purchased from Applied Biosystems (TaqMan™ Gene Expression Assays). The information regarding the PCR primers for the fifteen genes tested is shown in Supplementary Table 1. The Ct value of the control leukocytes captured by the second microfilter was used as the control in each sample. The Ct values of the target genes were normalized to a reference gene (*ACTB*), and the expression levels of each target molecule in the CTCs was estimated as the fold change compared to the control leukocytes by the relative quantification $2^{-\Delta\Delta Ct}$ method.[17]

Statistical analysis

GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses. Chi-squared test for independence and Fisher's exact test were used to examine differences in categorical variables. Kaplan-Meier curves were plotted and compared using the Log-rank test. Two-sided *P*-values < 0.05 were considered to be statistically significant. To divide the patients into two groups (high-expressing CTCs and low-expressing CTCs), CTC-positive patients were clustered into 2 groups according to k-means clustering based on the fold change of the 8 gene expression levels using the cluster package in R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Preliminary experiments

To confirm whether the CTCs were captured by the microfilter, immunocytochemistry was performed using the blood sample obtained from the patient with hypopharyngeal cancer. As shown in Figure 1A, DAPI+, CK+, EpCAM+, and CD45- cells were successfully detected as CTCs. Thus, we confirmed that CTCs exist in the peripheral blood of patients with HNSCC, and are capturable by microfilter.

In another preliminary experiment, we spiked a low number of SAS cells (10, 100, and 1000 cells) into 7.5 ml of whole blood from a healthy donor. Each sample was passed through the microfilter in the same way as the patient samples. We then extracted mRNA from the microfilter, synthesized the cDNA, pre-amplified the 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 using peripheral blood samples from healthy donors has been previously shown.[16]

Detection of CTCs and correlation with clinical factors in patients with HNSCC

The detection of CTCs and their epithelial-related markers expression are summarized in Fig. 2A. Twenty-eight (63.6%) of the 44 patients with HNSCC were positive for at least one epithelial-related gene. Among the 44 patients tested, *EPCAM* was detected in 6 (13.6 %), *MET* in 12 (27.3 %), *KRT19* in 21 (47.7 %), and *EGFR* in 7 (15.9 %) patients. In 28 patients with CTCs, three

(10.7 %) patients were positive for all four markers, 1 (3.6 %) for three markers, 7 (25.0 %) for two markers, and 17 (60.7 %) for one marker.

Next, we analyzed whether the presence of CTCs in HNSCC is correlated with clinical factors (Table 1). Among the clinical factors assessed, CTC-positive patients significantly showed a non-complete response after the initial treatment (*p=0.0363*). Moreover, during the follow-up period, CTC-positive patients had a significantly higher rate of locoregional recurrence (*p=0.0151*) and a trend toward distant metastasis (*p=0.0891*). Most importantly, CTC-positive patients had a poorer prognosis in terms of progression-free survival (PFS) (*p=0.0107*), but not in overall survival (OS) (*p=0.1653*) (Fig. 2B and 2C); therefore, next, we analyzed which epithelial marker contributed to the clinical outcome of HNSCC. Of note, patients with *MET*-positive CTCs had a shorter PFS than those with *MET*-negative CTCs (*p=0.0426*) (Table 2).

Gene expression of CTCs and correlation with clinical outcome

The CTCs obtained from 28 HNSCC patients were further investigated for the expression of 10 genes grouped into 4 gene signatures: cell growth (*PIK3CA* and *CCND1*), EMT (*SNAI1* and *VIM*), cancer stemness (*CD44, NANOG*, and *ALDH1A1*), and immune regulatory (*CD47*, *CD274*, and *PDCD1LG2*). If the $2^{-\Delta\Delta Ct}$ value is more than 1, the sample was assessed as positive for the expression of the molecule. As shown in Fig. 3A, the CTCs obtained from each HNSCC patient showed variable expression levels and the positive rate of each gene expression ranged from 28.6% to 57.1%, suggesting the phenotypic heterogeneity of CTCs among patients. We

investigated whether the expression of each gene in the CTCs was associated with the clinical outcome. Interestingly, patients with *CD274*-positive CTC showed prolonged PFS (*p=0.0346*) and OS (*p=0.0378*) compared to those with *CD274*-negative CTC (Table 3). The corresponding Kaplan-Meier survival curves are shown in Fig. 3B and 3C. The expression of the three genes *PIK3CA*, *CCND1*, and *CD44* in the CTCs was not significantly but weakly correlated with a worse clinical status. CTC-positive patients were further divided into two groups (high expressing CTCs and low expressing CTCs) according to k-means clustering based on the expression of 8 genes, not including *CD274* or *PDCD1LG2* (Supplementary Fig. 1). The resulting association between these groups and the clinical factors was analyzed. Although there was no significant difference in prognosis (Supplementary Fig. 2), patients with high expression levels of CTCs progressed to significantly advanced disease stages (*p=0.0228*) (Supplementary Table 2).

Discussion

The detection and/or enumeration of CTCs in cancer patients has been reported to be associated with clinical factors, including prognosis.[18-20] The CellSearch system, based on the positive immunomagnetic enrichment of EpCAM positive cells, is currently the only CTC detection method available for clinical use.[21-23] In this study, four representative epithelial-related markers were used for the detection of CTCs in HNSCC. As previously reported, the detection rate of CTCs increased by multiple gene markers, with variations in its expression pattern. Importantly, although the presence of CTCs was not statistically significance with distant metastasis, a significant relevance was found with two important clinical factors: the response to initial treatment and locoregional recurrence. Thus, our results suggest that, in HNSCC, the presence of CTCs at initial diagnosis have the potential to be used as predictive markers for therapeutic effects. Four epithelial-related markers are known to play various important roles in cell regulation, cell biology, and signal transduction in tumor cells. Therefore, next, we analyzed whether the expression of epithelial-related markers was associated with the clinical outcome. From the 4 epithelial-related markers, patients with *MET*-positive CTCs showed a poorer clinical outcome. c-Met is the receptor of hepatocyte growth factor, and has been reported to regulate cell proliferation, migration, and invasion through its tyrosine kinase function.[24, 25] The meta-analysis of c-Met expression in head and neck cancer showed that patients with higher c-Met expression levels had a poor OS and PFS.[26] Furthermore, in several malignancies, c-Met has been used as a maker for

the detection of CTCs, and has been associated with having a clinical impact.[27-29] These findings suggest that c-Met is an attractive marker for the detection, as well as assessment, of malignant potential in the clinical application of CTCs. Compared to the presence of CTCs or epithelial marker expression in CTC, determining the relationship between molecular profiling of CTCs and clinical significance remains a challenge. So far, numerous methods for the isolation and detection of CTCs have been developed; however, leukocyte contamination cannot be avoided due to the overlapping of cell size and characteristics between leukocytes and CTCs. Thus, leukocyte contamination has become a hurdle to the evaluation of more precise molecular characteristics of CTCs. To minimize the effect of gene expression in leukocytes, we used the gene expression in leukocytes on a second microfilter in serial filtration as a background.

In 28 patients with CTCs, the expression levels of 10 genes based on 4 gene signatures were investigated, and their associations with clinical outcome were analyzed. Interestingly, *CD274* expression in CTCs was significantly correlated with a better prognosis in both PFS and OS. In HNSCC, a number of studies concerning the prognostic role of CD274 (PD-L1) expression on tumor cells within the tumor microenvironment have been reported;[30-34] however, the results obtained so far are controversial. Yang et al. demonstrated that PD-L1 expression detected by immunohistochemistry (IHC) was not recommended to predict survival in HNSCC patients through a systemic review and meta-analysis.[35] In general, the mechanisms of PD-L1 expression in tumor cells consist of two different pathways: the oncogenic

pathway, and a reflection of the immune responses mediated by JAK/STAT signaling.[36] In particular, the latter could cause the heterogeneity and the longitudinal fluctuation of PD-L1 expression associated with disease activity and the treatment modalities in cancer patients. Compared to IHC in tumor tissue, the assessment of PD-L1 expression in CTCs may provide a more comprehensive and precise evaluation. However, little is currently known about the relationship between PD-L1 expression in CTCs and clinical outcomes. With regards to HNSCC, Strati et al. recently demonstrated that PD-L1 mRNA expression in EpCAM-positive CTCs predicts the clinical outcomes when collected from patients with HNSCC after curative treatment.[37] The results also indicated that patients who had PD-L1 overexpression in the EpCAM-positive CTCs prior to initial treatment did not have significantly different PFS and OS compared with those without PD-L1 overexpression, which does not correlate with our findings. This is most likely due to the different CTC detection methods used. In the tumor microenvironment, IFN-γ secreted from cytotoxic T lymphocytes and NK cells as antitumor immune responses induces PD-L1 on tumor cells. Moreover, it is generally well-recognized that patients with a high number of tumor-infiltrating lymphocytes have a favorable prognosis for many types of tumors.[38-40] Thus, PD-L1 expression on CTCs may partially reflect the tumor immune microenvironment, that is, increased immune cell infiltration and PD-L1 overexpression in tumor cells. Besides HNSCC, findings regarding the clinical significance of PD-L1 expression in CTCs in non-small cell lung cancer have also been reported.[41-45] Several studies have demonstrated

that longer PFS and OS were observed in patients with PD-L1 expression in CTCs,[41, 43, 45] while other studies have found that patients with PD-L1-positive CTCs had a shorter PFS compared to those with PD-L1 negative CTCs.[42, 44] Interestingly, recent reports have suggested that PD-L1 expression on CTCs or alteration of PD-L1 expression on CTCs along with treatment may be a predictive marker for patient response to immune checkpoint inhibitors in several solid tumors.[44-47] For future clinical application of PD-L1 in CTCs, not only a sufficiently large sample size, but also validation at the both protein and mRNA levels will be needed.

The expression of three genes, *PIK3CA, CCND1*, and *CD44*, in CTCs was found to be weakly correlated with a worse clinical status. As such, CTC-positive patients were clustered into two groups (high- and low-expressing groups) based on the expression of 8 genes, not including *CD274* or *PDCD1LG2*. These two genes were excluded since they are regulated by interferon receptor signaling pathways, in particular, interferon gamma signaling,[48] and their biological functions in the tumor cells remain unclear. As expected, the CTCs highly expressing these targets were significantly observed in patients with advanced disease, but were not considered a predictive marker of clinical outcome. This result suggests that the molecular characterization of CTCs by combining the expression of several genes may be useful for predicting the clinical outcome of tumors, as well as improving our understanding of circulating tumor cell biology; however, the selection of target genes in various types of gene signatures is crucial.

Despite using a small sample size, this study suggested that molecular profiles characterized by the gene expression of CTCs indicated their effects on clinical factors, including clinical outcome in patients with HNSCC. Although further studies with a larger number of patient samples are necessary to determine whether molecular profiling of CTCs contribute to the development of personalized cancer treatment, CTC-relating technologies are a promising tool for predicting treatment response and prognosis in patients with HNSCC. In addition to the detection and enumeration of CTCs, the molecular profiling of CTCs at the single-cell and bulk-cell level may provide a new avenue to fully realize the potential of precision medicine in cancer patients.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

References

[1] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65:87-108.

[2] Vigneswaran N, Williams MD. Epidemiologic trends in head and neck cancer and aids in diagnosis. Oral Maxillofac Surg Clin North Am. 2014;26:123-41.

[3] Sutton DN, Brown JS, Rogers SN, Vaughan ED, Woolgar JA. The prognostic implications of the surgical margin in oral squamous cell carcinoma. Int J Oral Maxillofac Surg. 2003;32:30-4.

[4] Leemans CR, Tiwari R, Nauta JJ, van der Waal I, Snow GB. Recurrence at the primary site in head and neck cancer and the significance of neck lymph node metastases as a prognostic factor. Cancer. 1994;73:187-90.

[5] Jatana KR, Balasubramanian P, Lang JC, Yang L, Jatana CA, White E, et al. Significance of circulating tumor cells in patients with squamous cell carcinoma of the head and neck: initial results. Arch Otolaryngol Head Neck Surg. 2010;136:1274-9.

[6] 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.

[7] Buglione M, Grisanti S, Almici C, Mangoni M, Polli C, Consoli F, et al. Circulating tumour 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.

[8] Grisanti S, Almici C, Consoli F, Buglione M, Verardi R, Bolzoni-Villaret A, et al. Circulating tumor cells in patients with recurrent or metastatic head and neck carcinoma: prognostic and predictive significance. PLoS One. 2014;9:e103918.

[9] Tinhofer I, Konschak R, Stromberger C, Raguse JD, Dreyer JH, Johrens K, et al. Detection of circulating tumor cells for prediction of recurrence after adjuvant chemoradiation in locally advanced squamous cell carcinoma of the head and neck. Ann Oncol. 2014;25:2042-7.

[10] 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.

[11] Banko P, Lee SY, Nagygyorgy V, Zrinyi M, Chae CH, Cho DH, et al. Technologies for circulating tumor cell separation from whole blood. J Hematol Oncol. 2019;12:48.

[12] Bredemeier M, Edimiris P, Mach P, Kubista M, Sjoback R, Rohlova E, et al. Gene Expression Signatures in Circulating Tumor Cells Correlate with Response to Therapy in Metastatic Breast Cancer. Clin Chem. 2017;63:1585-93.

[13] Markou A, Lazaridou M, Paraskevopoulos P, Chen S, Swierczewska M, Budna J, et al. Multiplex Gene Expression Profiling of In Vivo Isolated Circulating Tumor Cells in High-Risk Prostate Cancer Patients. Clin Chem. 2018;64:297-306.

[14] Obermayr E, Agreiter C, Schuster E, Fabikan H, Weinlinger C, Baluchova K, et al. Molecular Characterization of Circulating Tumor Cells Enriched by A Microfluidic Platform in Patients with Small-Cell Lung Cancer. Cells. 2019;8.

[15] Amin MB ES, Greene F, Byrd DR, Brookland RK, Washington MK, Gershenwald JE, Compton CC, Hess KR, Sullivan DC, Jessup JM, Brierley JD, Gaspar LE, Schilsky RL, Balch CM, Winchester DP, Asare EA, Madera M, Gress DM, Meyer LR. (Eds.). AJCC Cancer Staging Manual (8th edition). New York: Springer International Publishing: American Joint Commission on Cancer; 2017.

[16] Chikamatsu K, Tada H, Takahashi H, Kuwabara-Yokobori Y, Ishii H, Ida S, et al. Expression of immune-regulatory molecules in circulating tumor cells derived from patients with head and neck squamous cell carcinoma. Oral Oncol. 2019;89:34-9.

[17] 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.

[18] Alix-Panabieres C, Pantel K. Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy. Cancer Discov. 2016;6:479-91.

[19] Gingras I, Salgado R, Ignatiadis M. Liquid biopsy: will it be the 'magic tool' for monitoring response of solid tumors to anticancer therapies? Curr Opin Oncol. 2015;27:560-7.

[20] Tinhofer I, Staudte S. Circulating tumor cells as biomarkers in head and neck cancer: recent advances and future outlook. Expert Rev Mol Diagn. 2018;18:897-906.

[21] Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781-91.

[22] de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res. 2008;14:6302-9.

[23] Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. Ann Oncol. 2009;20:1223-9.

[24] Rothenberger NJ, Stabile LP. Hepatocyte Growth Factor/c-Met Signaling in Head and Neck Cancer and Implications for Treatment. Cancers (Basel). 2017;9.

[25] Szturz P, Raymond E, Abitbol C, Albert S, de Gramont A, Faivre S. Understanding c-MET signalling in squamous cell carcinoma of the head & neck. Crit Rev Oncol Hematol. 2017;111:39-51.

[26] Li L, Sun Z, Huang X, Li X, Sun L, Zhang L, et al. Role of c-Met expression on prognosis of head and neck cancer: A literature review and meta-analysis. Head Neck. 2019;41:1999-2006.

[27] Uen YH, Lin SR, Wu CH, Hsieh JS, Lu CY, Yu FJ, et al. Clinical significance of MUC1 and c-Met RT-PCR detection of circulating tumor cells in patients with gastric carcinoma. Clin Chim Acta. 2006;367:55-61.

[28] Zhou J, Hu L, Yu Z, Zheng J, Yang D, Bouvet M, et al. Marker expression in circulating cancer cells of pancreatic cancer patients. J Surg Res. 2011;171:631-6.

[29] Zhang T, Boominathan R, Foulk B, Rao C, Kemeny G, Strickler JH, et al. Development of a Novel c-MET-Based CTC Detection Platform. Mol Cancer Res. 2016;14:539-47.

[30] Ukpo OC, Thorstad WL, Lewis JS, Jr. B7-H1 expression model for immune evasion in human papillomavirus-related oropharyngeal squamous cell carcinoma. Head Neck Pathol. 2013;7:113-21.

[31] Lin YM, Sung WW, Hsieh MJ, Tsai SC, Lai HW, Yang SM, et al. High PD-L1 Expression Correlates with Metastasis and Poor Prognosis in Oral Squamous Cell Carcinoma. PLoS One. 2015;10:e0142656.

[32] Maruse Y, Kawano S, Jinno T, Matsubara R, Goto Y, Kaneko N, et al. Significant association of increased PD-L1 and PD-1 expression with nodal metastasis and a poor prognosis in oral squamous cell carcinoma. Int J Oral Maxillofac Surg. 2018;47:836-45.

[33] Takahashi H, Sakakura K, Arisaka Y, Tokue A, Kaira K, Tada H, et al. Clinical and Biological Significance of PD-L1 Expression Within the Tumor Microenvironment of Oral Squamous Cell Carcinoma. Anticancer Res. 2019;39:3039-46.

[34] Ahn H, Yang JM, Kim H, Chung JH, Ahn SH, Jeong WJ, et al. Clinicopathologic implications of the miR-197/PD-L1 axis in oral squamous cell carcinoma. Oncotarget. 2017;8:66178-94.

[35] Yang WF, Wong MCM, Thomson PJ, Li KY, Su YX. The prognostic role of PD-L1 expression for survival in head and neck squamous cell carcinoma: A systematic review and meta-analysis. Oral Oncol. 2018;86:81-90.

[36] Shi Y. Regulatory mechanisms of PD-L1 expression in cancer cells. Cancer Immunol Immunother. 2018;67:1481-9.

[37] Strati A KG, Papaxoinis G, Angelidis I, Zavridou M, Economopoulou P, Kotsantis I, Avgeris M, Mazel M, Perisanidis C, Sasaki C, Alix-Panabieres C, Lianidou E, Psyrri A. 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.

[38] Huang Z, Xie N, Liu H, Wan Y, Zhu Y, Zhang M, et al. The prognostic role of tumour-infiltrating lymphocytes in oral squamous cell carcinoma: A meta-analysis. J Oral Pathol Med. 2019.

[39] Mao Y, Qu Q, Chen X, Huang O, Wu J, Shen K. The Prognostic Value of Tumor-Infiltrating Lymphocytes in Breast Cancer: A Systematic Review and Meta-Analysis. PLoS One. 2016;11:e0152500.

[40] Yao W, He JC, Yang Y, Wang JM, Qian YW, Yang T, et al. The Prognostic Value of Tumor-infiltrating Lymphocytes in Hepatocellular Carcinoma: a Systematic Review and Meta-analysis. Sci Rep. 2017;7:7525.

[41] Adams DL, Adams DK, He J, Kalhor N, Zhang M, Xu T, et al. Sequential Tracking of PD-L1 Expression and RAD50 Induction in

Circulating Tumor and Stromal Cells of Lung Cancer Patients Undergoing Radiotherapy. Clin Cancer Res. 2017;23:5948-58.

[42] Wang Y, Kim TH, Fouladdel S, Zhang Z, Soni P, Qin A, et al. PD-L1 Expression in Circulating Tumor Cells Increases during Radio(chemo)therapy and Indicates Poor Prognosis in Non-small Cell Lung Cancer. Sci Rep. 2019;9:566.

[43] Ilie M, Szafer-Glusman E, Hofman V, Chamorey E, Lalvee S, Selva E, et al. Detection of PD-L1 in circulating tumor cells and white blood cells from patients with advanced non-small-cell lung cancer. Ann Oncol. 2018;29:193-9.

[44] Nicolazzo C, Raimondi C, Mancini M, Caponnetto S, Gradilone A, Gandini O, et al. Monitoring PD-L1 positive circulating tumor cells in non-small cell lung cancer patients treated with the PD-1 inhibitor Nivolumab. Sci Rep. 2016;6:31726.

[45] Dhar M, Wong J, Che J, Matsumoto M, Grogan T, Elashoff D, et al. Evaluation of PD-L1 expression on vortex-isolated circulating tumor cells in metastatic lung cancer. Sci Rep. 2018;8:2592.

[46] Guibert N, Delaunay M, Lusque A, Boubekeur N, Rouquette I, Clermont E, et al. PD-L1 expression in circulating tumor cells of advanced non-small cell lung cancer patients treated with nivolumab. Lung Cancer. 2018;120:108-12.

[47] Kloten V, Lampignano R, Krahn T, Schlange T. Circulating Tumor Cell PD-L1 Expression as Biomarker for Therapeutic Efficacy of Immune Checkpoint Inhibition in NSCLC. Cells. 2019;8.

[48] Garcia-Diaz A SD, Moreno BH, Saco J, Escuin-Ordinas H, Rodriguez GA, Zaretsky JM, Sun L, Hugo W, Wang X, Parisi G, Saus CP, Torrejon DY, Graeber TG, Comin-Anduix B, Hu-Lieskovan S, Damoiseaux R, Lo RS, Ribas A. Interferon receptor signaling pathways regulating PD-L1 and PD-L2 expression. Cell Rep. 2017;19:1189-201.

Figure legends

Figure 1. Immunocytochemistry of CTC in a patient with HNSCC and detection of tumor cells spiked into peripheral blood sample from a healthy donor. (A) CTCs were detected in the form of DAPI+, CK+, EpCAM+, and CD45- cells in a blood sample obtained from a patient with HNSCC (magnification ×40). (B) Standard curve of the cycle threshold (Ct) values calculated from serial dilutions of tumor cells using RT-qPCR. The Ct values shown represent the mean values of triplicate reactions.

Figure 2. Detection of CTCs and correlation with clinical factors in patients with HNSCC. (A) Heat map depicting the expression of 4 epithelial-related genes in patients with HNSCC. The orange-red and light-blue squares denote positive and negative for gene expression, respectively. Kaplan-Meier survival analysis in HNSCC patients according to the CTCs status. (B) Progression-free survival based on CTCs positive and negative. (C) Overall survival based on CTCs positive and negative.

Figure 3. Gene expression of CTCs and correlation with clinical outcome. (A) Heat map depicting fold change of 10 genes compared to control leukocytes. The red square indicates positive for each gene expression. Genes were grouped into 4 gene signatures: cell growth (*PIK3CA* and *CCND1*), EMT (*SNAI1* and *VIM*), cancer stemness (*CD44*, *NANOG*, and *ALDH1A1*), and immune regulatory (*CD47*, *CD274*, and *PDCD1LG2*). Kaplan-Meier survival analysis in HNSCC patients with CTCs according to *CD274*

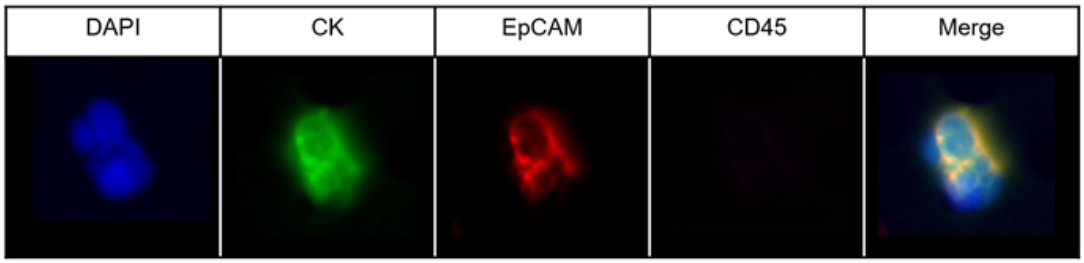
expression status. (B) Progression-free survival based on *CD274* expression on CTCs. (C) Overall survival based on *CD274* expression on CTCs.

Supplementary Figure 1. Heat map depicting fold change of 8 genes compared to control leukocytes. CTC-positive patients were divided into two groups (high- and low-expressing CTCs) by k-means clustering based on the expression of 8 genes, not including *CD274* or *PDCD1LG2*.

Supplementary Figure 2. Kaplan-Meier survival analysis patients with HNSCC. A: Progression-free survival based on high and low expressing CTCs. B: Overall survival based on high- and low-expressing CTCs. CTCs were divided into two groups (high- and low-expressing CTCs) by clustering based on 8 gene expression levels.

Figure 1

A


DAPI
CK
EpCAM
CD45
Merge

B

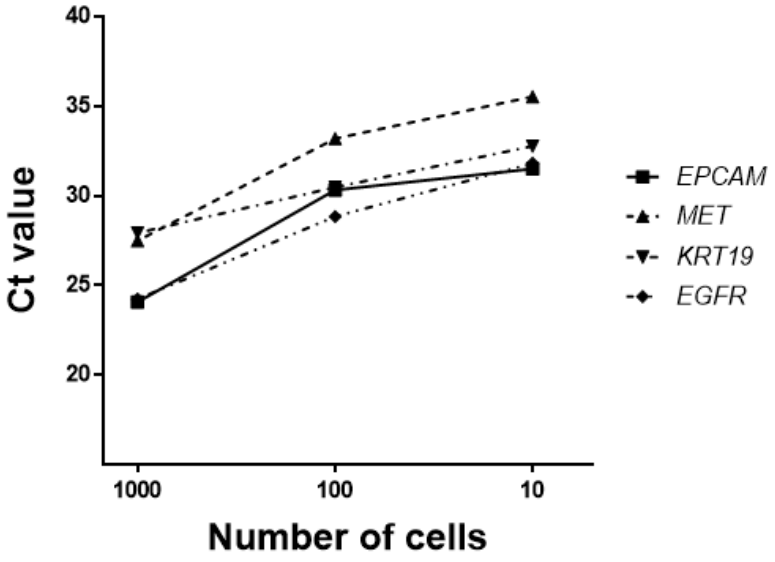

40
35
30
25
20
Ct value
1000
100
10
Number of cells
EPCAM
MET
KRT19
EGFR

Figure 2

A

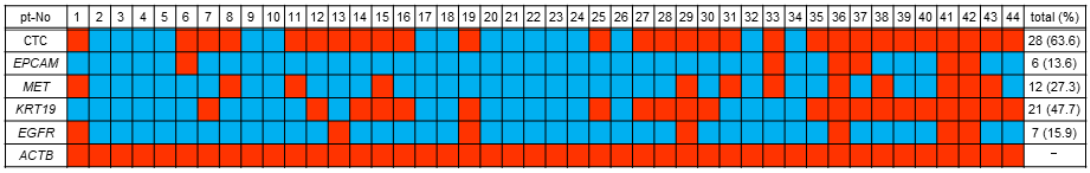
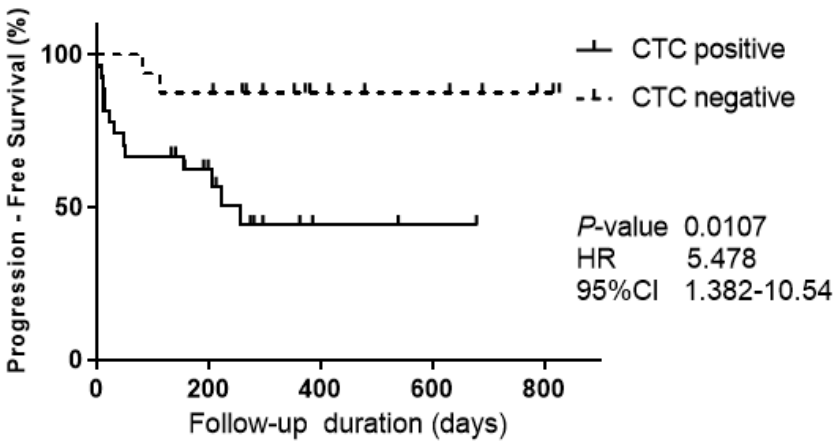

pt-No 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 total (%)
CTC 28 (63.6)
EPCAM 6 (13.6)
MET 12 (27.3)
KRT19 21 (47.7)
EGFR 7 (15.9)
ACTB –

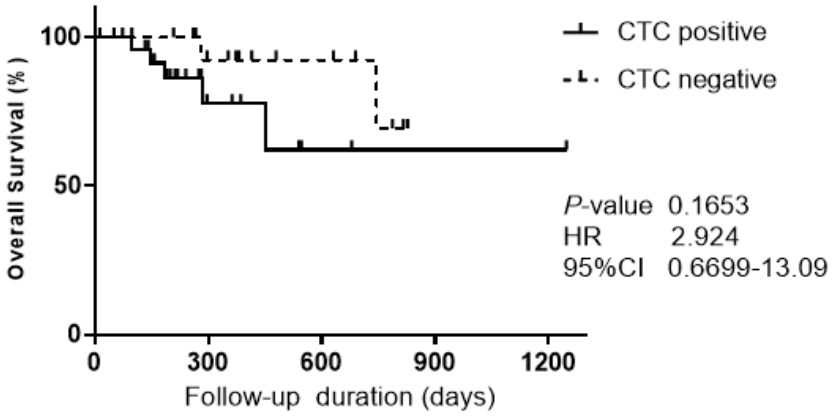
Figure 2

B



Number at risk					
CTC positive	28	12	3	2	1
CTC negative	16	15	8	6	3

C



Number at risk					
CTC positive	28	8	3	2	2
CTC negative	16	12	7	1	1

Figure 3

A

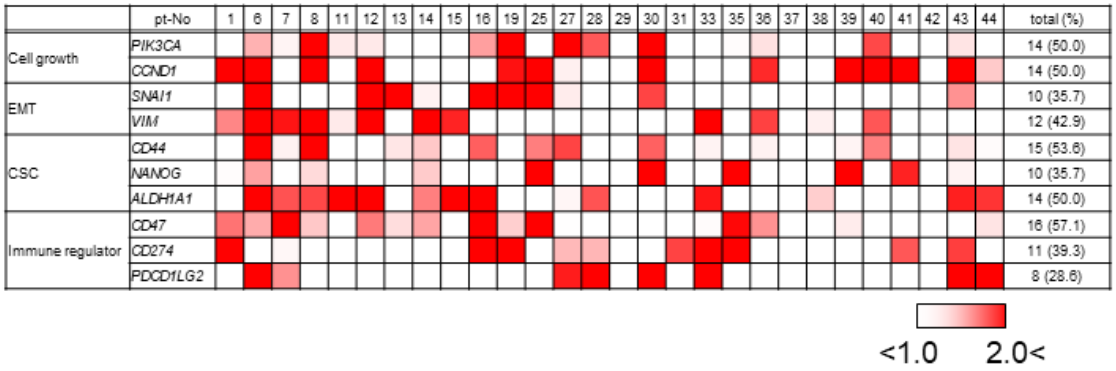
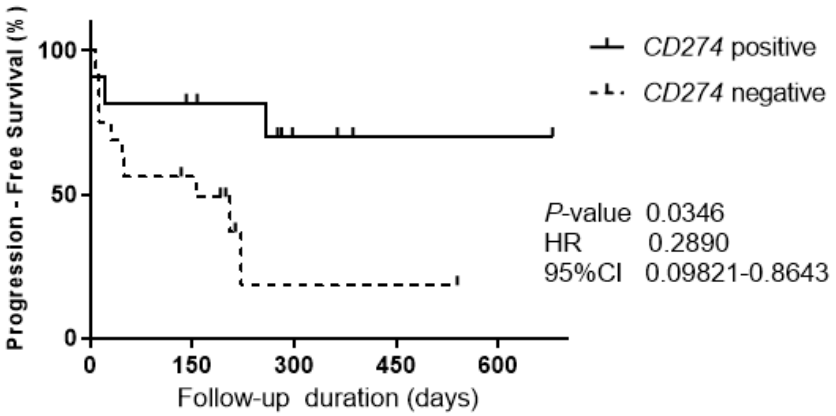

pt-No
1
6
7
8
11
12
13
14
15
16
19
25
27
28
29
30
31
33
35
36
37
38
39
40
41
42
43
44
total (%)
Cell growth
PIK3CA
14 (50.0)
CCND1
14 (50.0)
EMT
SNAI1
10 (35.7)
VIM
12 (42.9)
CSC
CD44
15 (53.6)
NANOG
10 (35.7)
ALDH1A1
14 (50.0)
Immune regulator
CD47
16 (57.1)
CD274
11 (39.3)
PDCD1LG2
8 (28.6)
<1.0
2.0<

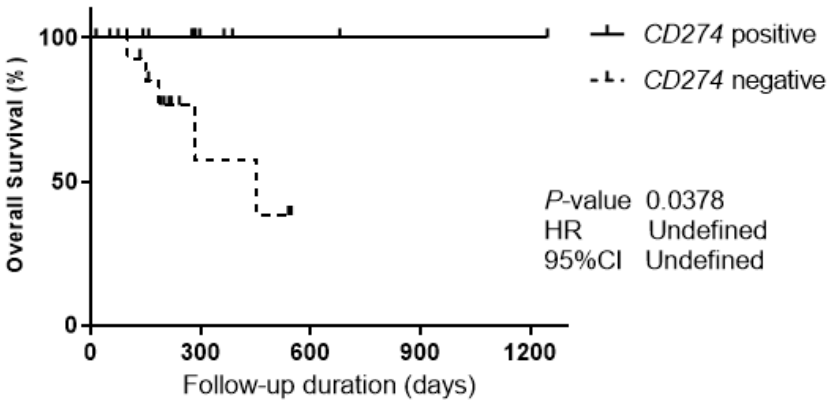
Figure 3

B



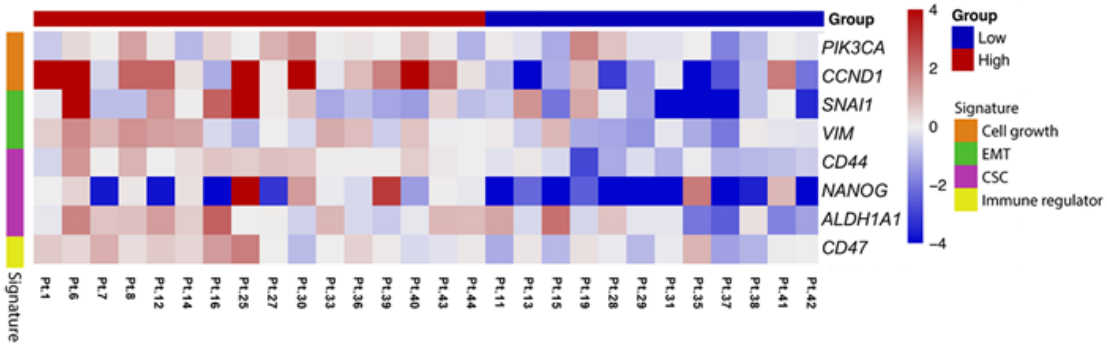
Number at risk					
CD274 positive	11	9	4	2	2
CD274 negative	7	9	2	2	1

C



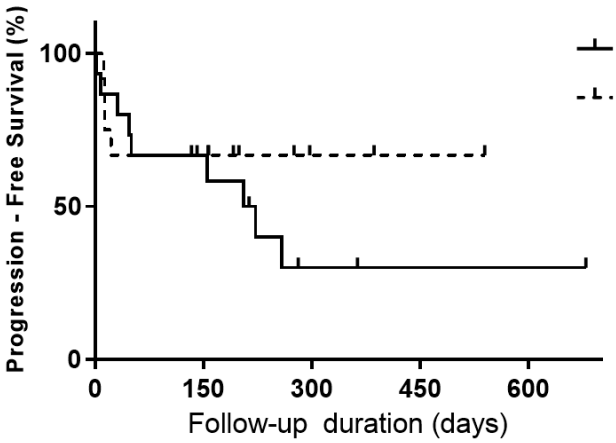
Number at risk					
CD274 positive	11	5	3	2	2
CD274 negative	17	4	1	1	1

Supplementary Figure 1



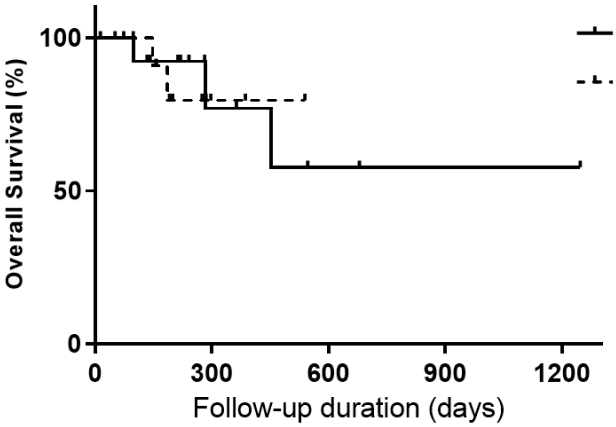
Supplementary Figure 2

A



Number at risk					
High	16	9	3	2	1
Low	12	9	3	2	1

B



Number at risk					
High	16	6	3	2	1
Low	12	3	1	1	1

Table1. Patient demographics and clinical characteristics.				
Clinical variable		CTC (+) n=28	CTC (-) n=16	*P*-value
Age (median 66 y.o.)	< 66	14	7	0.7606
	≧ 66	14	9	
Sex	Male	26	15	1
	Female	2	1	
Primary site	nasal cavity	2	1	0.2878
	oral cavity	3	1	
	nasopharynx	0	2	
	oropharynx	10	7	
	hypopharynx	9	5	
	larynx	4	0	
T factor	T1-2	9	4	0.7385
	T3-4	19	12	
N factor	N0	6	4	1
	N1-3	22	12	
M factor	M0	25	16	0.2896
	M1	3	0	
Stage	I - II	7	3	0.7237
	III - IV	21	13	
Locoregional recurrence	(+)	12	1	0.0151
	(-)	16	15	
Distant metastasis	(+)	11	2	0.0891
	(-)	17	14	
Initial treatment	CR	18	15	0.0363
	PR/SD/PD	10	1	

CTC: circulating tumor cell, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease

Table 2. Prognostic value of the epithelial-related markers in CTCs.					
Progression-free survival					
Gene symbol			*P* -value	HR	95% CI
EPCAM	(+)	6	0.7886	0.7613	0.1277-4.742
	(-)	38			
MET	(+)	12	0.0426	2.746	1.065-13.68
	(-)	32			
KRT19	(+)	21	0.8873	0.93	0.3374-2.557
	(-)	23			
EGFR	(+)	7	0.4712	1.582	0.3947-7.530
	(-)	37			
Overall survival					
Gene symbol			*P* -value	HR	95% CI
EPCAM	(+)	6	0.0972	4.787	0.6006-2225
	(-)	38			
MET	(+)	12	0.6003	1.543	0.2616-10.21
	(-)	32			
KRT19	(+)	21	0.8129	0.8296	0.1681-3.971
	(-)	23			
EGFR	(+)	7	0.1989	2.771	0.4705-40.80
	(-)	37			

Table 3. Prognostic value of the gene expression in CTCs.

Progression-free survival

Gene symbol			*P*-value	HR	95% CI
PIK3CA	(+)	14	0.0776	0.3677	0.1234 - 1.097
	(-)	14			
CCND1	(+)	14	0.6755	1.258	0.4262 - 3.755
	(-)	14			
SNAI1	(+)	10	0.8211	1.137	0.3663 - 3.554
	(-)	18			
VIM	(+)	12	0.6402	1.294	0.4299 - 3.961
	(-)	16			
CD44	(+)	15	0.3007	1.781	0.6011 - 5.304
	(-)	13			
NANOG	(+)	10	0.325	1.713	0.5651 - 5.670
	(-)	18			
ALDH1A1	(+)	14	0.7388	0.8327	0.2795 - 2.460
	(-)	14			
CD47	(+)	16	0.446	1.56	0.5135 - 4.686
	(-)	12			
CD274	(+)	11	0.0346	0.289	0.0982 - 0.8643
	(-)	17			
PDCD1LG2	(+)	8	0.7434	0.8079	0.2395 - 2.766
	(-)	20			

Overall survival					
Gene symbol			*P* -value	HR	95% CI
PIK3CA	(+)	14	0.6999	1.414	0.2452 - 8.184
	(-)	14			
CCND1	(+)	14	0.4544	1.951	0.3357 - 11.53
	(-)	14			
SNAI1	(+)	10	0.191	3.069	0.5447 - 22.77
	(-)	18			
VIM	(+)	12	0.8723	1.139	0.2007 - 6.912
	(-)	16			
CD44	(+)	15	0.5706	1.661	0.2888 - 9.665
	(-)	13			
NANOG	(+)	10	0.9691	0.966	0.1621 - 5.739
	(-)	18			
ALDH1A1	(+)	14	0.842	1.191	0.2063 - 7.037
	(-)	14			
CD47	(+)	16	0.4503	2.209	0.3331 - 12.97
	(-)	12			
CD274	(+)	11	0.0378	Undefined	Undefined
	(-)	17			
PDCD1LG2	(+)	8	0.966	1.048	0.1137 - 9.697
	(-)	20			
CTCs: circulating tumor cells					

Supplementary Table 1. PCR primers used in this study

Gene symbol	Assay ID
ACTB	Hs01060665_g1
EPCAM	Hs00158980_m1
MET	Hs01565576_m1
KRT19	Hs00761767_s1
EGFR	Hs01076090_m1
PIK3CA	Hs00907957_m1
CCND1	Hs00765553_m1
SNAI1	Hs00195591_m1
VIM	Hs00958111_m1
CD44	Hs01075861_m1
NANOG	Hs04399610_g1
ALDH1A1	Hs00946916_m1
CD47	Hs00179953_m1
CD274	Hs01125301_m1
PDCD1LG2	Hs01057777_m1

Supplementary Table 2. The relation between high/low expressing CTCs and clinical characteristics.

Clinical variable		Gene expression		*P*-value
		High	Low	
T factor	T1-2	4	5	0.4319
	T3-4	12	7	
N factor	N0	5	1	0.1965
	N1-3	11	11	
M factor	M0	14	11	1
	M1	2	1	
Stage	I-II	1	6	0.0228
	III-IV	15	6	
Initial treatment	CR	10	8	1
	PR/SD/PD	6	4	
Locoregional recurrence	(+)	8	4	0.4589
	(-)	8	8	
Distant metastasis	(+)	8	3	0.253
	(-)	8	9	
Recurrence/ Metastasis	(+)	10	4	0.2519
	(-)	6	8	

CTCs: circulating tumor cells, CR: complete response, PR: partial response, SD: stable disease, PD: progressive disease