ANTICANCER RESEARCH *40*: 3559-3564 (2020)
doi:10.21873/anticanres.14345

Epithelial–Mesenchymal Transition Status of Circulating Tumor Cells Is Associated With Tumor Relapse in Head and Neck Squamous Cell Carcinoma

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Abstract. *Background/Aim: We aimed to elucidate the clinical implication of the epithelial–mesenchymal plasticity of circulating tumor cells (CTCs) in patients with head and neck squamous cell carcinoma (HNSCC). Patients and Methods: CTCs isolated from 44 patients with non-recurrent/metastatic HNSCC and 42 with recurrent/metastatic (R/M) HNSCC were classified into four epithelial–mesenchymal transition (EMT) statuses based on the expression of epithelial (keratin 19) and mesenchymal (vimentin) markers and the relationships between EMT status in CTCs and clinical factors were investigated. Results: E^+M^- CTC phenotype was more frequent in patients without recurrence/metastasis (p=0.0468) and was also more frequent in those with a complete response (p=0.0346). The E^+M^+ phenotype constituted the major proportion of the CTCs detected in patients with R/M HNSCC (p=0.0374). Conclusion: CTCs may play unique roles at various stages of metastasis through transitioning from epithelial to mesenchymal phenotypes.*

Despite continued improvements in surgical techniques and the development of new chemotherapeutic agents for the treatment of head and neck squamous cell carcinoma (HNSCC), patients with locoregional recurrence or distant metastasis still have less satisfactory outcomes (1, 2). Accumulating evidence has indicated that circulating tumor cells (CTCs) that are shed into the bloodstream from a primary tumor play pivotal roles as metastasis-initiating cells in metastatic dissemination (3-5); however, the mechanisms involved in metastasis are not fully understood.

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Key Words: Epithelial–mesenchymal transition, circulating tumor cell, head and neck squamous cell carcinoma, recurrence, metastasis.

Epithelial–mesenchymal transition (EMT) is a complex transitional process causing the transformation from epithelial to mesenchymal states and enabling tumor cells to acquire migration and invasive potential (6, 7). In general, a subpopulation of cells in the primary tumor undergo EMT, facilitate intravasation into the bloodstream, and circulate as CTCs. Mesenchymal–epithelial transition, which is the reverse process to that of EMT, may be needed by such cells in order to regain the ability to proliferate and colonize at secondary sites (8, 9). Moreover, recent studies indicated that tumor cells with partial EMT or hybrid epithelial/mesenchymal phenotype have greater survival advantage and metastatic potential compared to other types of tumor cells (10, 11). In HNSCC, Puram *et al.* demonstrated, by single-cell transcriptomic analysis, high partial EMT scores were an independent predictor of nodal metastasis, grade, and adverse pathological features and were associated with metastasis (12).

Thus far, we have reported that molecular characteristics of CTCs isolated from two cohorts, namely patients with previously untreated HNSCC and those with recurrent/metastatic (R/M) HNSCC, correlated with clinical factors including disease progression and prognosis (13, 14). In order to elucidate the relationships between epithelial–mesenchymal plasticity of CTCs and tumor relapse, CTCs isolated from these cohorts were divided into four different phenotypic groups based on the expression of keratin 19 (*KRT19*) as epithelial marker and vimentin (*VIM*) as mesenchymal marker, and the correlation of this status with clinical factors was investigated. Our findings may provide new insights into the role of epithelial–mesenchymal plasticity of CTCs in HNSCC.

Patients and Methods

Selection of CTC-positive patients. Molecular analysis data from CTCs isolated in previous studies were used (13, 14). Blood samples were collected from 44 previously patients with untreated HNSCC and 42 with R/M HNSCC. CTCs were isolated using

CellSieve™ microfilter (Creatv MicroTech, Inc., Potomac, MD, USA) for the untreated group and CD45-negative selection for the R/M group. Total RNA was extracted from CTCs using RNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen). A further preamplification step was performed using the TaqMan™ PreAmp Master Mix kit (Applied Biosystems, Waltham, MA, USA) for 14 cycles. The preamplified products were then analyzed by real-time quantitative polymerase chain reaction (RT-qPCR) (Applied Biosystems) for the expression of four epithelial-related genes: epithelial cell adhesion molecule (*EPCAM*) (Hs00158980_m1), MET proto-oncogene, receptor tyrosine kinase (*MET*) (Hs01565576_m1), *KRT19* (Hs00761767_s1), and epidermal growth factor receptor (*EGFR*) (Hs01076090_m1). When at least one of the four epithelial-related genes were detected, the sample was defined as positive for CTCs. Finally, 25 patients with untreated HNSCC and no distant metastases and 35 with R/M HNSCC were enrolled in this study. Their characteristics are shown in Table I. This study was approved by the Ethical Committee of the Gunma University hospital (No. 12-12) and written informed consent was obtained from each patient.

Gene-expression analysis and EMT phenotypic classification. The CTC-positive samples were further analyzed for the expression of various genes including *VIM* (Hs00958111_m1) (14). The threshold cycle (Ct) value for control leukocytes was used as the control in each sample. Control leukocytes were obtained from use of a second filter that the blood sample passed through for the untreated group and from healthy donors using CD45-negative selection for the R/M group (13, 14).

The Ct values of the target genes were normalized to the reference gene actin beta (*ACTB;* Hs01060665_g1), and the expression level of *VIM* in the CTCs was estimated as the fold change compared to the control leukocytes by the relative quantification $2^{-\Delta\Delta Ct}$ method (15). Based on the expression of epithelial marker (E) *KRT19* and mesenchymal marker (M) *VIM*, CTC-positive patients were divided into four groups: E^-M^- (*KRT19*$^-$ and *VIM*$^-$), E^+M^- (*KRT19*$^+$ and *VIM*$^-$), E^-M^+ (*KRT19*$^-$ and *VIM*$^+$), E^+M^+ (*KRT19*$^+$ and *VIM*$^+$) phenotypes.

Statistical analysis. Data were analyzed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). The chi-squared test of independence was used to examine differences in categorical variables. Two-sided *p*-values of less than 0.05 were considered statistically significant. Survival curves were analyzed by the Kaplan–Meier method and compared using the log-rank test.

Results

Expression of KRT19 and VIM in CTCs and EMT status in the non-R/M HNSCC cohort. Twenty-five patients previously untreated and without distant metastasis of HNSCC were CTC-positive. Among them, 20 (80.0%) and 11 (44.0%) patients were positive for *KRT19* and *VIM*, respectively (Figure 1). The CTC phenotype for each patient was classified as E^-M^- in one patient (4.0%), E^+M^- in 13 patients (52.0%), E^-M^+ in four patients (16.0%), and E^+M^+ in seven patients (28.0%).

Table I. *Patient characteristics*.

Clinical variable	Non-R/M HNSCC cohort (n=25)	R/M HNSCC cohort (n=35)
Age, years		
Median (range)	65 (47-86)	69 (53-86)
Gender, n		
Male	23	32
Female	2	3
Tumor site, n		
Sinonasal cavity	1	4
Oral cavity	3	3
Nasopharynx	0	1
Oropharynx	8	5
Hypopharynx	9	16
Larynx	4	5
Parotid	0	1

R/M HNSCC: Recurrent/metastatic head and neck squamous cell carcinoma.

Correlations between EMT status and clinical factors. Next, we investigated whether the EMT status of CTCs was associated with clinical factors. There was no relationship with T classification, N classification, stage, local recurrence, and distant metastasis. Interestingly, the E^+M^- CTC phenotype was more frequent in patients without recurrence/metastasis (p=0.0468, Table II). Regarding treatment response, the E^+M^- CTC phenotype was also more frequent in those with a complete response (p=0.0346). To evaluate the prognostic significance of EMT status of CTCs, Kaplan–Meier survival analyses were performed among three groups E^+M^-, E^+M^+ and E^-M^+ (Figure 2). However, there was no significant difference among the three groups.

EMT status of CTCs in the R/M HNSCC cohort and comparison with that in the HNSCC cohort without R/M. Finally, we investigated the EMT status of CTCs from patients with locoregional or distant metastasis of HNSCC and compared it to that in previously patients with untreated HNSCC and without metastasis. In patients with R/M, 29 out of 35 (82.9%) had *KRT19*-positive CTCs. The percentage of *VIM*-positive CTCs in the R/M cohort was significantly higher than that in the non-R/M cohort (74.3% *vs*. 44.0%, p=0.0303; Table III). E^-M^- CTCs were found in two patients (5.7%), E^+M^- in seven (20.0%), E^-M^+ in four (11.4%), and E^+M^+ in 22 (62.9%). The distribution of CTC EMT status in patients with R/M HNSCC was found to be significantly different from that in patients without R/M of HNSCC (p=0.0374; Table IV); the E^+M^+ phenotype constituted the major proportion of the CTCs detected in patients with R/M HNSCC.

Figure 1. *The expression pattern of keratin 19 (KRT19) and vimentin (VIM) in circulating tumor cells (CTCs) detected in patients without (A) and with (B) recurrent/metastasis (R/M) of head and neck squamous cell carcinoma (HNSCC). The red and blue squares denote positive and negative gene expression, respectively. CTCs were isolated by microfilter-based or CD45-depletion methods and analyzed by real-time quantitative polymerase chain reaction for the expression of four epithelial-related genes (epithelial cell adhesion molecule, MET proto-oncogene receptor tyrosine kinase, KRT19, and epidermal growth factor receptor). When at least one out of the four epithelial-related genes were detected, the sample was defined as positive for CTCs. All patients were classified as having CTCs.*

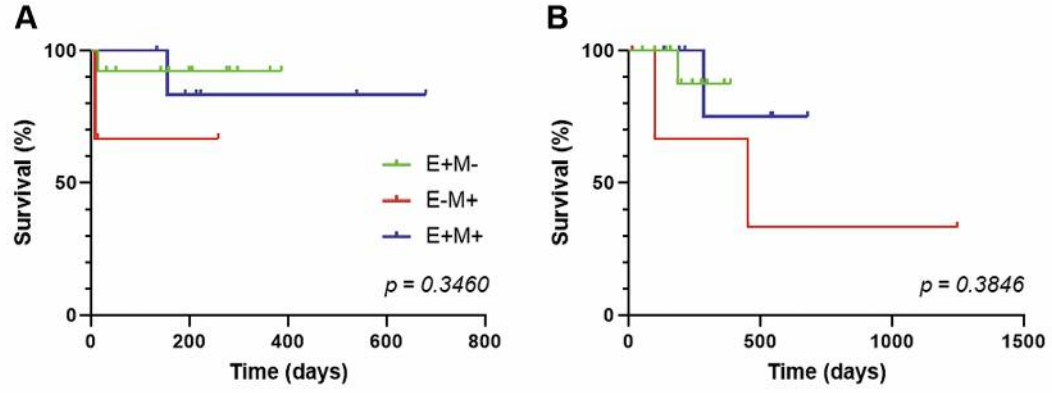

Figure 2. *Kaplan–Meier survival analysis in patients with non-recurrent/metastatic head and neck squamous cell carcinoma. Progression-free (A) and overall (B) survival based on the epithelial–mesenchymal transition phenotype of circulating tumor cells (CTCs). Phenotype was defined by the expression pattern of epithelial marker keratin 19 (KRT19) and mesenchymal marker vimentin (VIM): E^+M^-: $KRT19^+$ and VIM^-, E^-M^+: $KRT19^-$ and VIM^+, E^+M^+: $KRT19^+$ and VIM^+.*

Discussion

Although EMT is known to contribute to many of the steps in the invasion–metastasis cascade, it is difficult to evaluate the EMT status accurately because of the existence of various epithelial and mesenchymal markers and constant fluctuations in the tumor environment. To date, as epithelial markers for CTC identification in HNSCC, epithelial cell adhesion molecule (EpCAM) and cytokeratin have been well-studied (16-19). However, these epithelial markers may be down-regulated or lost during EMT. As previously reported, we firstly detected CTCs by expression of multiple epithelial-related gene (*KRT19, EPCAM, EGFR*, and *MET*), and then analyzed the molecular characteristics of CTCs. Our study revealed that of the four epithelial-related genes tested, expression of *KRT19* was most frequent (14). Additionally, McMullen *et al*. described cytokeratin as being more sensitive in the detection of CTCs in head and neck cancer (20). Vimentin found in mesenchymal cells is recognized as a classical EMT marker and its expression in tumor cells is associated with cancer invasion and poor prognosis in numerous types of cancer, including HNSCC (21-25). Based on these findings, EMT status was defined as the combination of expression of both *KRT19* and *VIM* in the present study.

In the non-R/M HNSCC cohort, CTC EMT status did not affect the prognosis; however, the number of E^+M^- patients was significantly higher in the subgroups without R/M and complete response. These results suggest that E^+M^- CTCs (*i.e.* those with epithelial phenotype) may have a low tumorigenic potential and be more sensitive to therapy. In general, EMT links various malignant traits, such as cancer stemness, immune suppression, and treatment resistance (26-28); therefore, CTC EMT status prior to treatment might be an important indicator for treatment outcomes in patients with HNSCC.

Our study demonstrated that in the R/M HNSCC cohort, the population of *VIM*-positive CTCs was significantly higher than in the non-R/M HNSCC cohort. E^+M^+ was the predominant

Table II. *Associations between epithelial–mesenchymal transition (EMT) phenotype of circulating tumor cells (CTCs) and clinical factors in the cohort without recurrence/metastasis of head and neck squamous cell carcinoma. EMT phenotype was defined by the expression pattern of epithelial marker keratin 19 (KRT19) and mesenchymal marker vimentin (VIM).*

Clinical variable		n	EMT phenotype, n (%)				*p*-Value
			E^-M^-	E^+M^-	E^-M^+	E^+M^+	
T Classification	T1-2	8	0 (0.0%)	5 (62.5%)	1 (12.5%)	2 (25.0%)	0.838
	T3-4	17	1 (5.9%)	8 (47.1%)	3 (17.6%)	5 (29.4%)	
N Classification	N0	5	0 (0.0%)	2 (40.0%)	1 (20.0%)	2 (40.0%)	0.8478
	N+	20	1 (5.0%)	11 (55%)	3 (15.0%)	5 (25.0%)	
Stage	I-II	7	0 (0.0%)	5 (71.4%)	0 (0.0%)	2 (28.6%)	0.4486
	III-IV	18	1 (5.6%)	8 (44.4%)	4 (22.2%)	5 (27.8%)	
Local recurrence	Yes	9	1 (11.1%)	3 (33.3%)	3 (33.3%)	2 (22.2%)	0.137
	No	16	0 (0.0%)	10 (62.5%)	1 (6.3%)	5 (31.3%)	
Distant metastasis	Yes	8	1 (12.5%)	3 (37.5%)	2 (25.0%)	2 (25.0%)	0.3569
	No	17	0 (0.0%)	10 (58.8%)	2 (11.8%)	5 (29.4%)	
Recurrence/metastasis	Yes	11	1 (9.1%)	4 (36.4%)	4 (36.4%)	2 (18.2%)	**0.0468**
	No	14	0 (0.0%)	9 (64.3%)	0 (0.0%)	5 (35.7%)	
Treatment response	CR	18	0 (0.0%)	11 (61.1%)	1 (5.6%)	6 (33.3%)	**0.0346**
	Non-CR	7	1 (14.3%)	2 (28.6%)	3 (42.9%)	1 (14.3%)	

CR: Complete response; E^-M^-: *KRT19*$^-$ and *VIM*$^-$, E^+M^-: *KRT19*$^+$ and *VIM*$^-$, E^-M^+: *KRT19*$^-$ and *VIM*$^+$, E^+M^+: *KRT19*$^+$ and *VIM*$^+$. Bold values indicate statistical significance.

Table III. *Epithelial–mesenchymal transition phenotype as defined by the expression pattern of keratin 19 (KRT19) and vimentin (VIM) in circulating tumor cells in cohorts with and without recurrence/metastasis (R/M) of head and neck squamous cell carcinoma.*

	n	*KRT19*, n (%)			*VIM*, n (%)		
HNSCC cohort		Positive	Negative	*p*-Value	Positive	Negative	*p*-Value
Non-R/M	25	20 (80.0%)	5 (20.0%)	>0.9999	11 (44.0%)	14 (56.0%)	**0.0303**
R/M	35	29 (82.9%)	6 (17.1%)		26 (74.3%)	9 (25.7%)	

Bold value indicates statistical significance.

Table IV. *Epithelial–mesenchymal transition (EMT) phenotype of circulating tumor cells in cohorts with and without recurrence/metastasis (R/M) of head and neck squamous cell carcinoma. EMT phenotype was defined by the expression pattern of epithelial marker keratin 19 (KRT19) and mesenchymal marker vimentin (VIM).*

HNSCC cohort	n	EMT phenotype (%)				*p*-Value
		E^-M^-	E^+M^-	E^-M^+	E^+M^+	
Non-R/M	25	1 (4.0%)	13 (52.0%)	4 (16.0%)	7 (28.0%)	**0.0374**
R/M	35	2 (5.7%)	7 (20.0%)	4 (11.4%)	22 (62.9%)	

E^-M^-: *KRT19*$^-$ and *VIM*$^-$, E^+M^-: *KRT19*$^+$ and *VIM*$^-$, E^-M^+: *KRT19*$^-$ and *VIM*$^+$, E^+M^+: *KRT19*$^+$ and *VIM*$^+$. Bold value indicates statistical significance.

CTC phenotype in the R/M HNSCC cohort. The following two possibilities should therefore be considered. Once metastasis has been established, CTCs are shed from metastatic sites, circulate in the bloodstream, and the heterogeneity among CTCs at the EMT level would be increased. EMT is essential to dissemination into the bloodstream, whereas re-acquisition of epithelial characteristics following EMT is required for metastatic establishment at the secondary site. In fact, several studies have indicated that epithelial markers are expressed at metastatic sites as well as at primary sites. Chao *et al.,* using

paired primary and metastatic tumors from breast and prostate cancer patients, demonstrated that metastases exhibited increased expression of the epithelial marker E-cadherin compared to primary tumors, whereas the expression of the mesenchymal marker vimentin was mostly unchanged (29). Similarly, samples of brain metastases from various primary tumor sites have been reported to coexpress E-cadherin and vimentin as markers for MET and EMT (30). Thus, some populations of E^+M^+ CTCs may be derived from metastatic lesions. Another possibility is that E^+M^+ CTCs may be a subpopulation which has acquired resistance over the course of various treatments. Recent data on CTC EMT status have shown that CTCs exhibiting both epithelial and mesenchymal phenotypes are able to form CTC clusters, responsible for treatment resistance, and have a higher metastatic potential (31). In metastatic breast cancer, Papadaki *et al*. showed that CTCs with stemness and partial EMT represent a chemoresistant subpopulation and their detection is an independent factor predictive of an increased risk of relapse, and as expected, the incidence of such CTCs was increased after first-line chemotherapy (32). Since the patients with R/M HNSCC tested in this study had already received various treatments, CTCs have been exposed to various extrinsic pressures such as nutrient deprivation, irradiation/chemothera-peutic agents and, therefore, most of the CTCs might be expected to have the E^+M^+ phenotype while other CTC types would disappear.

Our results suggest that CTCs may play unique roles at various stages of metastasis through transitioning from epithelial to mesenchymal phenotype. In particular, CTCs with E^+M^+ status may be the cause of treatment resistance or relapse of disease. Therapeutic strategies targeting EMT processes in CTCs are urgently needed to control the metastatic dissemination of tumor cells.

Conflicts of Interest

None of the Authors have any financial or personal relationship with other people or organizations that could have inappropriately influenced this study.

Authors' Contributions

Kazuaki Chikamatsu conceived and designed the study. Hiroe Tada, Shota Ida, and Yurino Nagata performed the experiments, and collected data. Hideyuki Takahashi and Kazuaki Chikamatsu analyzed and interpreted the data. All Authors read and approved the final article.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (grants no. 19K18794 to Shota Ida and no. 17K11374 to Kazuaki Chikamatsu) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1 Vermorken JB and Specenier P: Optimal treatment for recurrent/metastatic head and neck cancer. Ann Oncol Suppl *7*: vii252-261, 2010. PMID: 20943624. DOI: 10.1093/annonc/mdq453

2 Allen CT, Law JH, Dunn GP and Uppaluri R: Emerging insights into head and neck cancer metastasis. Head Neck *35(11)*: 1669-1678, 2013. PMID: 23280716. DOI: 10.1002/hed.23202

3 Massague J and Obenauf AC: Metastatic colonization by circulating tumour cells. Nature *529(7586)*: 298-306, 2016. PMID: 26791720. DOI: 10.1038/nature17038

4 Micalizzi DS, Maheswaran S and Haber DA: A conduit to metastasis: Circulating tumor cell biology. Genes Dev *31(18)*: 1827-1840, 2017. PMID: 29051388. DOI: 10.1101/gad.305805.117

5 Follain G, Herrmann D, Harlepp S, Hyenne V, Osmani N, Warren SC, Timpson P and Goetz JG: Fluids and their mechanics in tumour transit: Shaping metastasis. Nat Rev Cancer *20(2)*: 107-124, 2020. PMID: 31780785. DOI: 10.1038/s41568-019-0221-x

6 Dongre A and Weinberg RA: New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol *20(2)*: 69-84, 2019. PMID: 30459476. DOI: 10.1038/s41580-018-0080-4

7 Pearson GW: Control of invasion by epithelial-to-mesenchymal transition programs during metastasis. J Clin Med *8(5)*: pii: E646, 2019. PMID: 31083398. DOI: 10.3390/jcm8050646

8 Yao D, Dai C and Peng S: Mechanism of the mesenchymal–epithelial transition and its relationship with metastatic tumor formation. Mol Cancer Res *9(12)*: 1608-1620, 2011. PMID: 21840933. DOI: 10.1158/1541-7786.MCR-10-0568

9 Jolly MK, Ware KE, Gilja S, Somarelli JA and Levine H: EMT and MET: Necessary or permissive for metastasis? Mol Oncol *11(7)*: 755-769, 2017. PMID: 28548345. DOI: 10.1002/1878-0261.12083

10 Jolly MK, Boareto M, Huang B, Jia D, Lu M, Ben-Jacob E, Onuchic JN and Levine H: Implications of the hybrid epithelial/mesenchymal phenotype in metastasis. Front Oncol *5*: 155, 2015. PMID: 26258068. DOI: 10.3389/fonc.2015.00155

11 Jolly MK, Somarelli JA, Sheth M, Biddle A, Tripathi SC, Armstrong AJ, Hanash SM, Bapat SA, Rangarajan A and Levine H: Hybrid epithelial/mesenchymal phenotypes promote metastasis and therapy resistance across carcinomas. Pharmacol Ther *194*: 161-184, 2019. PMID: 30268772. DOI: 10.1016/j.pharmthera.2018.09.007

12 Puram SV, Tirosh I, Parikh AS, Patel AP, Yizhak K, Gillespie S, Rodman C, Luo CL, Mroz EA, Emerick KS, Deschler DG, Varvares MA, Mylvaganam R, Rozenblatt-Rosen O, Rocco JW, Faquin WC, Lin DT, Regev A and Bernstein BE: Single-cell transcriptomic analysis of primary and metastatic tumor ecosystems in head and neck cancer. Cell *171(7)*: 1611-1624, 2017. PMID: 29198524. DOI: 10.1016/j.cell.2017.10.044

13 Chikamatsu K, Tada H, Takahashi H, Kuwabara-Yokobori Y, Ishii H, Ida S and Shino M: Expression of immune-regulatory molecules in circulating tumor cells derived from patients with head and neck squamous cell carcinoma. Oral Oncol *89*: 34-39, 2019. PMID: 30732956. DOI: 10.1016/j.oraloncology.2018.12.002

14 Tada H, Takahashi H, Kuwabara-Yokobori Y, Shino M and Chikamatsu K: Molecular profiling of circulating tumor cells predicts clinical outcome in head and neck squamous cell carcinoma. Oral Oncol *102*: 104558, 2020. PMID: 32044652. DOI: 10.1016/j.oraloncology.2019.104558

15 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2[−delta delta C(t)] method. Methods *25(4)*: 402-408, 2001. PMID: 11846609. DOI: 10.1006/meth.2001.1262

16 Jatana KR, Balasubramanian P, Lang JC, Yang L, Jatana CA, White E, Agrawal A, Ozer E, Schuller DE, Teknos TN and Chalmers JJ: Significance of circulating tumor cells in patients with squamous cell carcinoma of the head and neck: Initial results. Arch Otolaryngol Head Neck Surg *136(12)*: 1274-1279, 2010. PMID: 21173379. DOI: 10.1001/archoto.2010.223

17 Nichols AC, Lowes LE, Szeto CC, Basmaji J, Dhaliwal S, Chapeskie C, Todorovic B, Read N, Venkatesan V, Hammond A, Palma DA, Winquist E, Ernst S, Fung K, Franklin JH, Yoo J, Koropatnick J, Mymryk JS, Barrett JW and Allan AL: Detection of circulating tumor cells in advanced head and neck cancer using the CellSearch system. Head Neck *34(10)*: 1440-1444, 2012. PMID: 22076949. DOI: 10.1002/hed.21941

18 Buglione M, Grisanti S, Almici C, Mangoni M, Polli C, Consoli F, Verardi R, Costa L, Paiar F, Pasinetti N, Bolzoni A, Marini M, Simoncini E, Nicolai P, Biti G and Magrini SM: 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 *48(16)*: 3019-3026, 2012. PMID: 22682019. DOI: 10.1016/j.ejca.2012.05.007

19 Grisanti S, Almici C, Consoli F, Buglione M, Verardi R, Bolzoni-Villaret A, Bianchetti A, Ciccarese C, Mangoni M, Ferrari L, Biti G, Marini M, Ferrari VD, Nicolai P, Magrini SM and Berruti A: Circulating tumor cells in patients with recurrent or metastatic head and neck carcinoma: Prognostic and predictive significance. PLoS One *9(8)*: e103918, 2014. PMID: 25105871. DOI: 10.1371/journal.pone.0103918

20 McMullen KP, Chalmers JJ, Lang JC, Kumar P and Jatana KR: Circulating tumor cells in head and neck cancer: A review. World J Otorhinolaryngol Head Neck Surg *2(2)*: 109-116, 2016. PMID: 29204555. DOI: 10.1016/j.wjorl.2016.05.003

21 Thiery JP and Sleeman JP: Complex networks orchestrate epithelial–mesenchymal transitions. Nat Rev Mol Cell Biol *7(2)*: 131-142, 2006. PMID: 16493418. DOI: 10.1038/nrm1835

22 Smith A, Teknos TN and Pan Q: Epithelial to mesenchymal transition in head and neck squamous cell carcinoma. Oral Oncol *49(4)*: 287-292, 2013. PMID: 23182398. DOI: 10.1016/j.oraloncology.2012.10.009

23 Liu CY, Lin HH, Tang MJ and Wang YK: Vimentin contributes to epithelial–mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. Oncotarget *6(18)*: 15966-15983, 2015. PMID: 25965826. DOI: 10.18632/oncotarget.3862

24 Wangmo C, Charoen N, Jantharapattana K, Dechaphunkul A and Thongsuksai P: Epithelial–mesenchymal transition predicts survival in oral squamous cell carcinoma. Pathol Oncol Res, 2019. PMID: 31471883. DOI: 10.1007/s12253-019-00731-z

25 Jung AR, Jung CH, Noh JK, Lee YC and Eun YG: Epithelial-mesenchymal transition gene signature is associated with prognosis and tumor microenvironment in head and neck squamous cell carcinoma. Sci Rep *10(1)*: 3652, 2020. PMID: 32107458. DOI: 10.1038/s41598-020-60707-x

26 Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, Brisken C, Yang J and Weinberg RA: The epithelial–mesenchymal transition generates cells with properties of stem cells. Cell *133(4)*: 704-715, 2008. PMID: 18485877. DOI: 10.1016/j.cell.2008.03.027

27 Gloushankova NA, Zhitnyak IY and Rubtsova SN: Role of epithelial–mesenchymal transition in tumor progression. Biochemistry *83(12)*: 1469-1476, 2018. PMID: 30878022. DOI: 10.1134/S0006297918120052

28 Nieto MA, Huang RY, Jackson RA and Thiery JP: EMT: 2016. Cell *166(1)*: 21-45, 2016. PMID: 27368099. DOI: 10.1016/j.cell.2016.06.028

29 Chao Y, Wu Q, Acquafondata M, Dhir R and Wells A: Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases. Cancer Microenviron *5(1)*: 19-28, 2012. PMID: 21892699. DOI: 10.1007/s12307-011-0085-4

30 Jeevan DS, Cooper JB, Braun A, Murali R and Jhanwar-Uniyal M: Molecular pathways mediating metastases to the brain *via* epithelial-to-mesenchymal transition: Genes, proteins, and functional analysis. Anticancer Res *36(2)*: 523-532, 2016. PMID: 26851006.

31 Jia D, Li X, Bocci F, Tripathi S, Deng Y, Jolly MK, Onuchic JN and Levine H: Quantifying cancer epithelial–mesenchymal plasticity and its association with stemness and immune response. J Clin Med *8(5)*: pii: E725, 2019. PMID: 31121840. DOI: 10.3390/jcm8050725

32 Papadaki MA, Stoupis G, Theodoropoulos PA, Mavroudis D, Georgoulias V and Agelaki S: Circulating tumor cells with stemness and epithelial-to-mesenchymal transition features are chemoresistant and predictive of poor outcome in metastatic breast cancer. Mol Cancer Ther *18(2)*: 437-447, 2019. PMID: 30401696. DOI: 10.1158/1535-7163.MCT-18-0584

Received April 11, 2020
Revised April 19, 2020
Accepted April 21, 2020