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# Protein level of epithelial membrane protein (EMP) 1, EMP 2, and EMP 3 in carcinoma of unknown primary

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Carcinoma of unknown primary (CUP) is defined as a metastatic carcinoma whose primary site cannot be determined, and the absence of a known primary tumor in CUP poses a significant challenge in treatment planning. The purpose of this study was to investigate the protein level of epithelial membrane proteins (EMP) 1, EMP 2, and EMP 3 in CUP and explore their clinical implications. Tissue microarrays were constructed using samples from 72 CUP cases. The histologic subtypes were adenocarcinoma (ADC) in 22% of cases, poorly differentiated carcinoma (PDC) in 15%, squamous cell carcinoma (SCC) in 19%, and undifferentiated carcinoma (UDC) in 14%. Clinically, 17 cases (23.6%) were of favorable type, and 55 cases (76.4%) were of unfavorable type. Immunohistochemical staining for EMP 1, EMP 2, and EMP 3 was performed on the tissue microarrays to investigate the correlation between staining results and clinicopathologic parameters. The investigation of EMP 1, EMP 2, and EMP 3 protein levels in CUP revealed that EMP 2 H-score was significantly higher ($p=0.013$) in the favorable type, and there was a higher proportion of stromal EMP 1-positivity ($p=0.034$) and high protein level of tumoral EMP 3 ($p=0.002$). A positive correlation was observed between EMP 1 and EMP 3 ($r=0.425$ and $p<0.001$). In conclusion, CUP exhibits EMP 1, EMP 2, and EMP 3 protein levels, and their protein levels are different according to the clinical subtype.

*Key words: carcinoma; epithelial membrane protein; primary unknown*

Carcinoma of unknown primary (CUP) is defined as a metastatic carcinoma whose primary site cannot be determined through clinical history, physical examination, radiographic findings, laboratory tests, and diagnostic investigations [1]. CUP accounts for approximately 5–15% of malignant tumors [2–4]. However, advances in imaging and molecular techniques have reduced the incidence of CUP to around 1–2% of individuals diagnosed with cancer [5]. Histologically, CUP is comprised of adenocarcinomas (AD) (50–60%), poorly differentiated carcinomas (PD) (30–40%), and other histologic types, including squamous cell carcinomas (SCC) (5–8%) and undifferentiated carcinomas (UD) (2–5%) [4, 6]. The precise nature of CUP remains uncertain, but two main hypotheses exist: 1) the first hypothesis suggests that CUP is a true metastatic tumor with an undetectable primary focus due to its small size; and 2) the second hypothesis suggests that CUP is a distinct entity with independent characteristics, lacking an actual primary lesion due to regression or dormancy, which is therefore referred to as the „true“ or „genuine“ CUP hypothesis [6].

The absence of a known primary tumor in CUP poses a significant challenge in treatment planning, as therapeutic strategies for metastatic carcinoma are usually determined by the type of primary cancer. Traditional diagnostic and treatment algorithms for CUP involve tissue origin-specific therapy for the favorable subgroup, identified through the traditional diagnostic work-up, while the unfavorable subgroup receives either tissue origin-specific therapy or empirical chemotherapy based on the characteristics observed in the specific CUP case [7]. To identify the most suitable tissue origin for a particular CUP case, various tools such as immunohistochemistry (IHC) and molecular techniques such as gene expression profiling, miRNA expression, and DNA methylation analysis are utilized [8]. Additionally, with the advancements in genomic tools, efforts to identify potential treatment targets have continued in order to apply target therapies to CUP [9], and such identification of appropriate treatment targets for CUP is crucial for its effective management.

Epithelial membrane proteins (EMP1, EMP2, and EMP3) belong to the myelin protein 22 kDa (PMP22) gene family

and are known to primarily function in the peripheral nervous system. However, they have been reported to have various roles in different types of tumors [10, 11]. EMP1 acts through the PI3K/AKT pathway [12], affecting tumor cell adhesion. EMP2 affects tumor cell migration through the FAK/SRC pathway [13], while EMP3 is involved in tumor cell survival and metastasis through the ErbB2-PI3K-AKT pathway [14, 15]. However, these EMPs have been reported to exhibit both tumor progressor and tumor suppressor roles in various tumors [16, 17]. Previous studies have investigated the expression of EMP1, EMP2, and EMP3 in different types of human cancer, but research specifically focused on CUP has been limited. Therefore, the purpose of this study is to investigate the protein level of EMP1, EMP2, and EMP3 in CUP and explore their implications.

## Patients and methods

**Patient selection and clinicopathologic evaluation.** This study involved the use of formalin-fixed paraffin-embedded (FFPE) tissue samples from patients diagnosed with CUP at Severance Hospital. The study received ethical approval from the Institutional Review Board of the hospital (IRB number: 4-2023-0670). The patient cohort consisted of individuals diagnosed with metastatic carcinoma between January 1999 and December 2012. Cases with insufficient biopsy material were excluded. A comprehensive review of the archival hematoxylin and eosin (H&E)-stained slides of all cases was done. Clinicopathologic parameters such as patient age, sex, histological type, involved organ, and patient outcome were evaluated for each case. CUP cases were classified into four categories according to following histologic criteria [18]: AD, displaying glandular differentiation; SCC, exhibiting features of squamous differentiation such as intercellular bridges and keratin pearls; PD, lacking differentiation towards any specific lineage; and UD, characterized by syncytial tumor cell nests or scattered tumor cells closely associated with dense lymphoplasmacytic infiltration, resembling nasopharyngeal undifferentiated carcinoma.

Additionally, CUP cases were further classified into favorable and unfavorable subgroups according to international guidelines [19]. The favorable subgroup included poorly differentiated neuroendocrine carcinomas of unknown primary, well-differentiated neuroendocrine tumors of unknown primary, peritoneal adenocarcinomatosis of serous papillary type in females, isolated axillary nodal metastases in females, SCC involving non-supraclavicular cervical lymph nodes, single metastatic deposit from unknown primary, blastic bone metastases or positive immunohistochemical stain result for prostate-specific antigen (PSA) or elevated serum PSA level in males, and SCC involving isolated inguinal adenopathy.

**Tissue microarray.** After reviewing the H&E-stained slides, the most suitable FFPE tumor tissues were collected. The representative tumor area was marked on the FFPE slides, and the selected area was extracted using a punch machine. A 3 mm tissue core was then inserted into a recipient block of dimensions 6×5. Two tissue cores were collected from each case to create the tissue microarray (TMA).

**Immunohistochemistry.** The antibodies used for IHC in this study are shown in Supplementary Table S1. IHC was performed using FFPE tissue sections. Tissue sections with a thickness of 3 μm were cut from the paraffin blocks and then deparaffinized and rehydrated using xylene and alcohol solutions. The staining procedure was conducted using the Ventana Discovery XT automated stainer (Ventana Medical System, Tucson, AZ, USA). Antigen retrieval was performed using CC1 buffer (Cell Conditioning 1; citrate buffer pH 6.0, Ventana Medical System). IHC staining was carried out, with appropriate positive control (adrenal gland, Supplementary Figure S1). The primary antibody incubation step was omitted in the negative control.

**Interpretation of immunohistochemical results.** Immunohistochemical staining results were assessed by light microscopy. The protein levels of EMP 1, EMP 2, and EMP 3 were analyzed according to the semi-quantitative H-score method and scored in tumor cells. H-score yields a total range of 0 to 300, which is obtained by multiplying the dominant staining intensity score (0, no staining; 1, weak or barely detectable staining; 2, distinct brown staining; 3, strong dark brown staining) by the percentage (0–100%) of positive cells [20]. If the H-score was greater than the median value, it was defined as a high expression, otherwise, it was defined as a low expression. The protein level of EMP in tumor stromal tissue was also evaluated, and it was defined as positive when it was observed in 10% or more of stromal cells. For CK7 and CK20, a threshold of 10% was used, where cases with less than 10% staining were considered negative and those with 10% or more staining were classified as positive [21].

**Statistical analysis.** Data were statistically processed using SPSS for Windows, Version 23.0 (SPSS Inc., Chicago, IL, USA). Student's t-test and Fisher's exact test were used for continuous and categorical variables, respectively. For data with multiple comparisons, a corrected p-value with the application of the Bonferroni multiple comparison procedure was used. A p-value $<0.05$ was considered statistically significant. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to survival. Multivariate regression analysis was performed using the Cox proportional hazards model.

## Results

**Basal characteristics of CUP patients according to the histologic subtype and clinical subtype.** Supplementary Table S2 presents the basal characteristics of 72 CUP cases according to histologic subtypes. Adenocarcinoma (ADC) accounted for 22% of cases, poorly differentiated carcinoma (PDC) for 15%, squamous cell carcinoma (SCC) for 19%, and undifferentiated carcinoma (UDC) for 14%. As for clinical

subtypes, 17 cases (23.6%) were classified as favorable type, while 55 cases (76.4%) were classified as unfavorable type (Supplementary Table S3). There was a significant difference in clinical subtypes based on histologic subtypes, with ADC and UDC showing a higher proportion of unfavorable types, while SCC showed a higher proportion of favorable types (p=0.003). Postoperative treatment varied according to histologic subtypes, with chemotherapy being the most common treatment for ADC, chemo-radiation therapy for PDC, and surgery alone for UDC (p=0.007). In terms of CK7/CK20 expression, 37 cases (51.4%) were CK7 (+)/CK20 (–), 3 cases (4.2%) were CK7 (+)/CK20 (+), 3 cases (4.2%) were CK7 (–)/CK20 (+), and 29 cases (40.3%) were CK7 (–)/CK20 (–). However, there was no significant difference in CK7/CK20 expression based on histologic subtypes (p=0.522).

**Table 1. Protein level of EMP 1, EMP 2, and EMP 3 in CUP according to the histologic subtype.**

| EMP status | Total n=72 (%) | Histologic subtype | | | | p-value |
|---|---|---|---|---|---|---|
| | | ADC (n=22) (%) | PDC (n=15) (%) | SCC (n=19) (%) | UDC (n=16) (%) | |
| EMP 1 (T) | | | | | | 0.989 |
| Low | 36 (50.0) | 11 (50.0) | 8 (53.3) | 9 (47.4) | 8 (50.0) | |
| High | 36 (20.0) | 11 (50.0) | 7 (46.7) | 10 (52.6) | 8 (50.0) | |
| EMP 1 (S) | | | | | | 0.150 |
| Negative | 49 (68.1) | 19 (86.4) | 8 (53.3) | 12 (63.2) | 10 (62.5) | |
| Positive | 23 (31.9) | 3 (13.6) | 7 (46.7) | 7 (36.8) | 6 (37.5) | |
| EMP 2 (T) | | | | | | 0.107 |
| Low | 47 (65.3) | 17 (77.3) | 12 (80.0) | 9 (47.4) | 9 (56.3) | |
| High | 25 (34.7) | 5 (22.7) | 3 (20.0) | 10 (25.6) | 7 (43.8) | |
| EMP 2 (S) | | | | | | 0.506 |
| Negative | 63 (87.5) | 19 (86.4) | 14 (93.3) | 15 (78.9) | 15 (93.8) | |
| Positive | 9 (12.5) | 3 (13.6) | 1 (6.7) | 4 (21.1) | 1 (6.3) | |
| EMP 3 (T) | | | | | | 0.868 |
| Low | 36 (50.0) | 12 (54.5) | 8 (53.3) | 8 (42.1) | 8 (50.0) | |
| High | 36 (50.0) | 10 (45.5) | 7 (46.7) | 11 (57.9) | 8 (50.0) | |
| EMP 3 (S) | | | | | | 0.147 |
| Negative | 29 (40.3) | 12 (54.5) | 3 (20.0) | 9 (47.4) | 5 (31.3) | |
| Positive | 43 (59.7) | 10 (45.5) | 12 (80.0) | 10 (52.6) | 11 (68.8) | |

Abbreviations: PD-poorly differentiated carcinoma; AD-adenocarcinoma; SC-squamous cell carcinoma; UD-undifferentiated carcinoma

**Table 2. Protein level of EMP 1, EMP 2, and EMP 3 in CUP according to the CK7 and CK20 pattern.**

| EMP status | Total (n=72) (%) | CK7/CK20 pattern | | | | p-value |
|---|---|---|---|---|---|---|
| | | CK7(+)/CK20(-) (n=37) (%) | CK7(+)/CK20(+) (n=3) (%) | CK7(-)/CK20(+) (n=3) (%) | CK7(-)/CK20(-) (n=29) (%) | |
| EMP 1 (T) | | | | | | 0.531 |
| Low | 36 (50.0) | 16 (43.2) | 2 (66.7) | 1 (33.3) | 17 (58.6) | |
| High | 36 (20.0) | 21 (56.8) | 1 (33.3) | 2 (66.7) | 12 (41.4) | |
| EMP 1 (S) | | | | | | 0.258 |
| Negative | 49 (68.1) | 29 (78.4) | 2 (66.7) | 2 (66.7) | 16 (55.2) | |
| Positive | 23 (31.9) | 8 (21.6) | 1 (33.3) | 1 (33.3) | 13 (44.8) | |
| EMP 2 (T) | | | | | | 0.063 |
| Low | 47 (65.3) | 19 (51.4) | 2 (66.7) | 3 (100.0) | 23 (79.3) | |
| High | 25 (34.7) | 18 (48.6) | 1 (33.3) | 0 (0.0) | 6 (20.7) | |
| EMP 2 (S) | | | | | | 0.104 |
| Negative | 63 (87.5) | 35 (94.6) | 3 (100.0) | 3 (100.0) | 22 (75.9) | |
| Positive | 9 (12.5) | 2 (5.4) | (0.0) | 0 (0.0) | 7 (24.1) | |
| EMP 3 (T) | | | | | | 0.867 |
| Low | 36 (50.0) | 18 (48.6) | 2 (66.7) | 1 (33.3) | 15 (51.7) | |
| High | 36 (50.0) | 19 (51.4) | 1 (33.3) | 2 (66.7) | 14 (48.3) | |
| EMP 3 (S) | | | | | | 0.172 |
| Negative | 29 (40.3) | 19 (51.4) | 0 (0.0) | 1 (33.3) | 9 (31.0) | |
| Positive | 43 (59.7) | 18 (48.6) | 3 (100.0) | 2 (66.7) | 20 (69.0) | |

**Protein level of EMP 1, EMP 2, and EMP 3 in CUP.** The results of EMP 1, EMP2, and EMP3 H-scores in tumor cells of CUP are presented in Supplementary Table S4. The median, mean ± SD, and range of EMP H-scores were as follows: EMP 1: 25, 61.4±70.2, 0–270; EMP 2: 0, 18.6±47.3, 0–240; EMP 3: 65, 79.5±73.0, 0–300. When investigating the EMP 1, EMP 2, and EMP 3 H-scores according to histologic and clinical subtypes of CUP, no statistically significant differences were observed based on histologic subtypes. However, there was a statistically significant difference in EMP 2 H-scores based on clinical subtypes (p=0.013), with the favorable type showing significantly higher EMP 2 H-scores (Supplementary Table S5). When the protein levels of EMP 1, EMP 2, and EMP 3 in tumor cells were assessed as H-scores, and categorized as low and high, there was no statistically significant difference among histologic subtypes (Table 1) and also

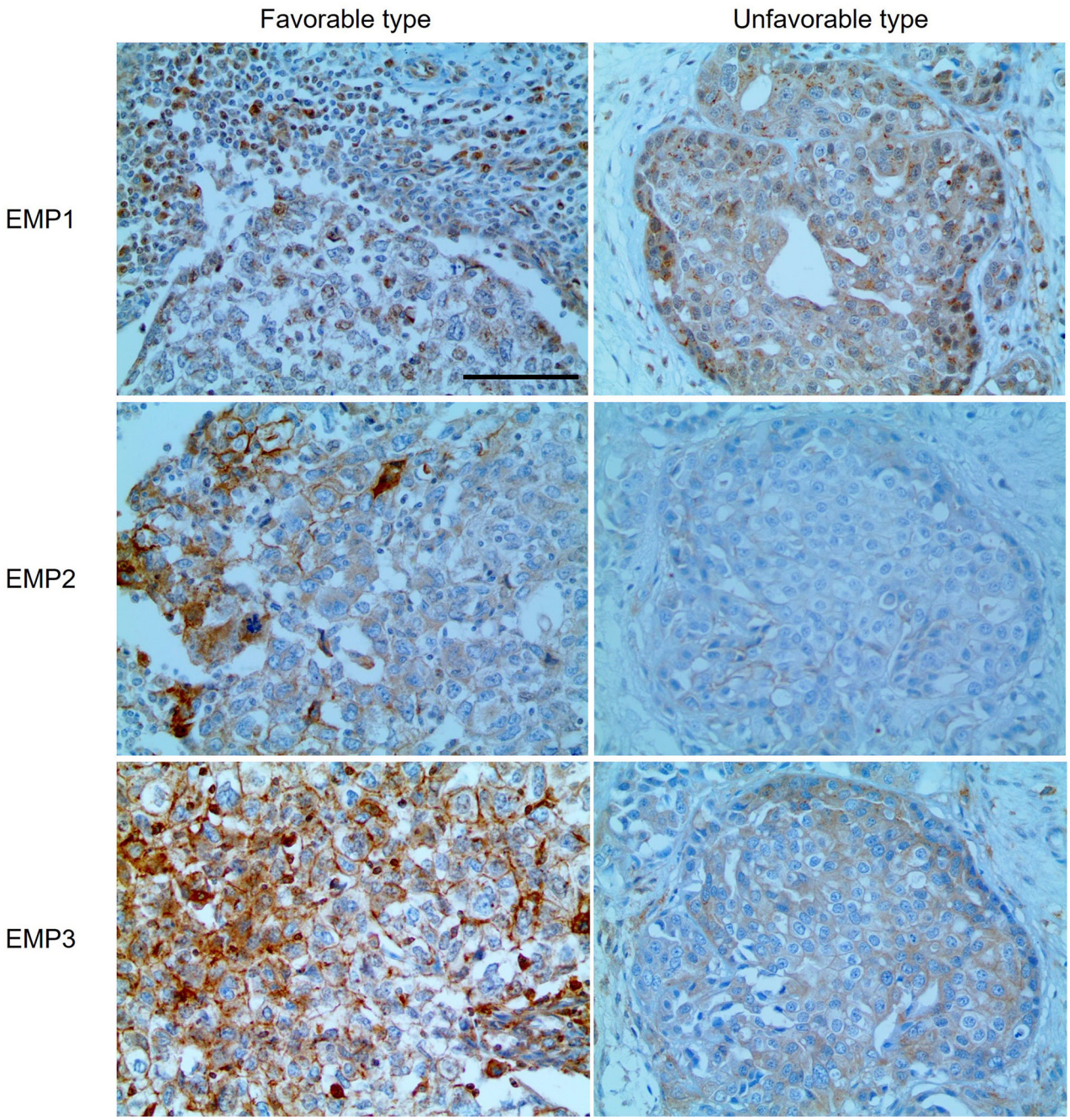


**Figure 1. Protein level of EMP 1, EMP 2, and EMP 3 in CUP clinical subtype. In favorable types, the protein level of EMP 2 and EMP 3 was higher in tumor cells, while the protein level of EMP 1 was higher in stromal cells (Scale bar = 500 μm).**

among different CK7/CK20 staining patterns (Table 2). Also, when their protein level in tumor stroma was categorized as negative and positive, no statistically significant difference was observed as well. However, there was a statistically significant difference between the clinical subtypes. Stromal EMP 1 (p=0.034) and tumoral EMP3 (p=0.002) showed statistically significant differences; a higher proportion of stromal EMP 1 positivity was found in the favorable clinical subtype, and high protein level of tumoral EMP 3 was observed in the favorable subtype (Table 3 and Figure 1). There was a positive correlation between EMP 1 and EMP 3 when the correlation between EMP 1, EMP 2, and EMP 3 H-scores was analyzed in CUP (r=0.425, p<0.001, Table 4).

**Correlation between the clinicopathologic factors and the protein level of EMP 1, EMP 2, and EMP 3 in CUP.** There was a significant association between EMP 1 status in tumor stroma and the involved organ. Specifically, a higher proportion of EMP 1 positivity in tumor stroma was observed in lymph nodes when compared to organs other than lymph nodes (p=0.002, Figure 2).

**Impact of the expression of EMP 1, EMP 2, and EMP 3 on the prognosis of CUP.** The impact of EMP 1, EMP 2, and EMP 3 protein levels on patient prognosis in CUP was analyzed through univariate analysis, but no statistically significant findings were observed (Table 5). However, in subgroup analysis, a significant association was found between EMP 2 H-score and prognosis in the group with lymph node involvement. Specifically, patients with low EMP 2 H-scores showed a poor prognosis (p=0.016, Figure 3).

**Table 3. Protein level of EMP 1, EMP 2, and EMP 3 in CUP according to the clinical subtype.**

| EMP status | Total (n=72) (%) | Clinical subtype | | p-value |
|---|---|---|---|---|
| | | Favorable type (n=17) (%) | Unfavorable type (n=55) (%) | |
| EMP 1 (T) | | | | 0.405 |
| Low | 36 (50.0) | 7 (41.2) | 29 (52.7) | |
| High | 36 (20.0) | 10 (58.8) | 26 (47.3) | |
| EMP 1 (S) | | | | *0.034* |
| Negative | 49 (68.1) | 8 (47.1) | 41 (74.5) | |
| Positive | 23 (31.9) | 9 (52.9) | 14 (25.5) | |
| EMP 2 (T) | | | | 0.071 |
| Low | 47 (65.3) | 8 (47.1) | 39 (70.9) | |
| High | 25 (34.7) | 9 (52.9) | 16 (29.1) | |
| EMP 2 (S) | | | | 0.463 |
| Negative | 63 (87.5) | 14 (82.4) | 49 (89.1) | |
| Positive | 9 (12.5) | 3 (17.6) | 6 (10.9) | |
| EMP 3 (T) | | | | *0.002* |
| Low | 36 (50.0) | 3 (17.6) | 33 (60.0) | |
| High | 36 (50.0) | 14 (82.4) | 22 (40.0) | |
| EMP 3 (S) | | | | 0.931 |
| Negative | 29 (40.3) | 7 (41.2) | 22 (40.0) | |
| Positive | 43 (59.7) | 10 (58.8) | 33 (60.0) | |

**Table 4. Correlation between EMP H-score in CUP.**

| Parameter | Correlation coefficient | p-value |
|---|---|---|
| EMP 1 and EMP 2 | –0.100 | 0.403 |
| EMP 1 and EMP 3 | 0.452 | <0.001 |
| EMP 2 and EMP 3 | 0.060 | 0.617 |

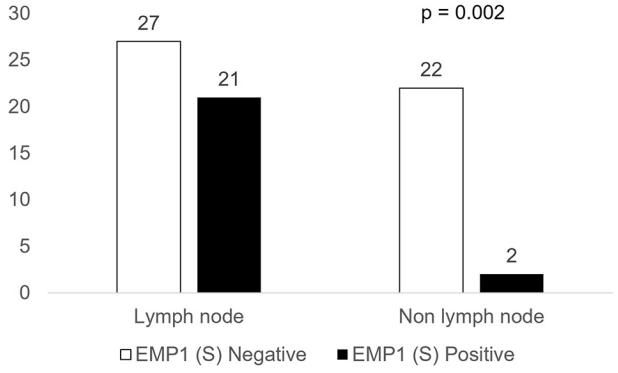


**Figure 2. Correlation between stromal EMP 1 status and lymph node involvement in CUP. A higher proportion of EMP 1 positivity in tumor stroma was observed in CUP with lymph node involvement compared to CUP without lymph node involvement (p=0.002).**

## Discussion

In this study, we investigated the protein level of EMP 1, EMP 2, and EMP 3 in CUP. Firstly, the percentage of CUP cases showing high protein levels of EMP was as follows: EMP 1 (20.0%), EMP 2 (34.7%), and EMP 3 (50.0%). The EMP family is known to exhibit both tumor promoter and tumor suppressor roles depending on tumor types. Consequently, tumors may demonstrate either higher or lower expression of the EMP family compared to normal tissues. Tumors showing high protein levels of each of the EMP family are as follows: 1) head and neck cancer [22, 23], breast cancer [24, 25], and stomach cancer [26, 27] showing high protein levels of EMP 1; 2) nasopharyngeal cancer [28, 29], and uterine endometrial cancer [30–33] showing high protein level of EMP 2; and 3) breast cancer [24, 25] showing high protein level of EMP 3. On the other hand, tumors showing low protein levels of the EMP family are as follows: 1) oral cavity cancer [34, 35] and nasopharyngeal cancer [36] showing low protein level of EMP 1; 2) urothelial cancer [37] showing low protein level of EMP 2; and 3) lung cancer [38] showing low protein level of EMP 3. This suggests that the protein level status of the EMP family may be diverse because CUP groups are heterogeneous and can be associated with different tumor types. In this study, the protein level status of the EMP family was significantly variable according to clinical subtypes. In the favorable type, the EMP 2 H-score was significantly higher compared to the unfavorable type (p=0.013), and there was a higher proportion of stromal EMP 1 positivity (p=0.034) and tumoral EMP 3 high protein level (p=0.002). EMP 2

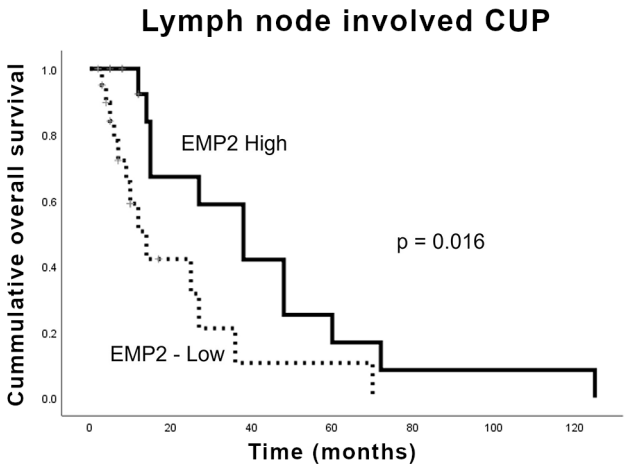


**Figure 3. Impact of the protein level of EMP 2 on the prognosis of CUP with lymph node involvement. In the CUP group with lymph node involvement, cases showing low protein levels of EMP 2 were associated with poor overall survival (p=0.016).**

and EMP 3 are associated with various signaling pathways in tumor biology. Notably, EMP 2 is linked to the FAK/SRC pathway [13], while EMP 3 is associated with the PI3K-AKT pathway [14, 15]. Genomic analysis of approximately 1,800 cases of CUP revealed frequent genetic alterations activating the FAK/SRC pathway and/or PI3K-AKT pathway, including EGFR amplification (17%), PIK3CA amplification (14%), HER-2 amplification (5%), KRAS mutation (18%), and PIK3CA mutation (9%) [39]. Therefore, considering the differences in EMP 2 and EMP 3 protein levels according to the clinical subtypes of CUP, the EMP family-related signaling pathways can be variable depending on the clinical subtype, and further investigation is warranted. The favorable type in CUP is a heterogeneous group. Peritoneal carcinomatosis of a serous papillary type in females may be associated with an ovarian cancer phenotype, in which EMP 2 overexpression has been observed [40]. SCC involving non-supraclavicular cervical lymph nodes may be associated with a nasopharyngeal cancer phenotype, in which EMP 2 overexpression is also observed [28, 29]. Additionally, the favorable type with isolated axillary nodal metastases in females can be associated with a breast cancer phenotype, in which EMP 3 overexpression is observed [24, 25]. Male patients with blastic bone metastases or IHC/serum PSA expression, the favorable type, can be associated with a prostate cancer phenotype, in which EMP 3 overexpression is reported [41]. Hence, it can be suggested that the favorable type of CUP harbors higher expression of EMP 2 and EMP 3.

In this study, the protein level of EMP 1 in tumor stromal cells showed a significant association with the favorable type (p=0.034) and lymph node involvement (p=0.002). The cells comprising the tumor stroma are diverse, but the main cell types are fibroblasts and immune cells. Previous studies have suggested that EMP1, as a specific fibrotic gene expressed in hepatic stem cells and endothelial cells, plays an important role in the fibrotic process following liver injury [42]. EMP 1 has also been reported to exhibit a positive correlation with infiltrating CD8+ T cells, macrophages, neutrophils, and dendritic cells in urothelial carcinoma. It has shown a strong association with immune markers such as CCL-2, CD68,

**Table 5. The impact of clinicopathologic factors and EMP 1, EMP 2, and EMP 3 status on the time to survival by univariate analysis.**

| Parameters | Overall survival | | | p-value |
|---|---|---|---|---|
| | No. of cases | Patient death | Median survival (95% CI) (months) | |
| Sex | | | | 0.267 |
| Male | 32 | 24 | 33 (21–46) | |
| Female | 19 | 14 | 25 (8–41) | |
| Histologic subtype | | | | **0.030** |
| ADC | 17 | 9 | 22 (8–35) | |
| PDC | 14 | 13 | 18 (12–25) | |
| SCC | 16 | 11 | 32 (18–47) | |
| UDC | 9 | 8 | 64 (24–104) | |
| Clinical subtype | | | | 0.239 |
| Favorable type | 15 | 14 | 41 (23–58) | |
| Unfavorable type | 41 | 27 | 28 (14–42) | |
| CK7 | | | | 0.892 |
| Negative | 26 | 19 | 32 (17–47) | |
| Positive | 30 | 22 | 32 (17–48) | |
| CK20 | | | | 0.386 |
| Negative | 52 | 37 | 33 (22–45) | |
| Positive | 4 | 4 | 21 (2–39) | |
| CK7/CK20 pattern | | | | 0.804 |
| CK7 (+)/CK20 (–) | 28 | 20 | 33 (17–50) | |
| CK7 (+)/CK20 (+) | 2 | 2 | 23 (0–52) | |
| CK7(–)/CK20(+) | 2 | 2 | 19 (0–52) | |
| CK7(–)/CK20(–) | 24 | 17 | 33 (17–50) | |
| EMP 1 (T) | | | | 0.403 |
| Low | 29 | 22 | 28 (15–40) | |
| High | 27 | 19 | 39 (21–56) | |
| EMP 1 (S) | | | | 0.154 |
| Negative | 37 | 24 | 26 (13–39) | |
| Positive | 19 | 17 | 41 (23–59) | |
| EMP 2 (T) | | | | 0.175 |
| Low | 37 | 27 | 28 (14–42) | |
| High | 19 | 14 | 40 (23–56) | |
| EMP 2 (S) | | | | 0.829 |
| Negative | 49 | 35 | 33 (21–45) | |
| Positive | 7 | 6 | 28 (8–47) | |
| EMP 3 (T) | | | | 0.219 |
| Low | 28 | 20 | 26 (12–40) | |
| High | 28 | 21 | 38 (22–54) | |
| EMP 3 (S) | | | | 0.851 |
| Negative | 20 | 15 | 32 (13–51) | |
| Positive | 36 | 26 | 33 (19–46) | |

Note: *Out of 72 patients, clinical follow-up data were available in 51 patients. Abbreviations: PD-poorly differentiated carcinoma; AD-adenocarcinoma; SC-squamous cell carcinoma; UD-undifferentiated carcinoma

IL-10, PTGS2, IRF5, CD163, VSIG4, and MS4A4A [43]. These findings suggest the potential expression of EMP 1 in tumor stromal cells, including fibroblasts and immune cells, and its association with tumor biology. Therefore, further research is needed to explore this relationship.

In this study, among the CUP cases with lymph node involvement, a low EMP 2 H-score was associated with poor prognosis (p=0.016). Previous studies have reported associations between EMP 2 expression and prognosis in various types of tumors. For instance, increased EMP 2 expression in estrogen receptor-negative breast cancer was associated with shorter relapse-free survival [44], while low EMP 2 expression in urinary bladder urothelial carcinoma was an independent prognostic factor for poor disease-specific survival [45]. In nasopharyngeal carcinoma, loss of EMP 2 expression was an independent prognostic factor for worse disease-specific survival and local recurrence-free survival [29]. These findings indicate that the role of EMP 2 can either be a tumor suppressor or tumor promoter depending on the type of the tumor, leading to different functions as a prognostic factor. Therefore, further research is needed to investigate the role of EMP2 as a prognostic factor in CUP.

Based on the results of this study, EMPs show potential as therapeutic targets in CUP. Previous research has demonstrated that anti-EMP2 recombinant bivalent antibody fragments (diabodies) can inhibit proliferation and increase apoptosis in uterine endometrial cancer and ovarian cancer [33], while anti-EMP2 IgG1 promotes cell death and inhibits cell invasion in breast cancer [46]. Therefore, EMP inhibitors can be proposed as one of the therapeutic agents for CUP. However, the development of monoclonal antibodies targeting EMP faces several obstacles. One significant challenge is the complex and context-dependent role of EMP in tumors, as mentioned earlier, where it exhibits different roles either as a tumor suppressor or tumor promoter depending on the type of the tumor. Consequently, further preclinical and clinical studies targeting CUP are necessary.

In conclusion, CUP exhibits EMP 1, EMP 2, and EMP 3 protein levels and their protein levels are different according to the clinical subtype

**Supplementary information** is available in the online version of the paper.

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# Protein level of epithelial membrane protein (EMP) 1, EMP 2, and EMP 3 in carcinoma of unknown primary

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## Supplementary Information

**Supplementary Table S1. Clone, dilution, and source of antibodies used.**

| Antibody | Clone | Antibody number | Dilution | Source |
|---|---|---|---|---|
| EMP related | | | | |
| EMP1 | Polyclonal | ab230445 | 1:100 | Abcam, Cambridge, UK |
| EMP2 | Polyclonal | ab174699 | 1:100 | Abcam, Cambridge, UK |
| EMP3 | SW-5 | sc-81797 | 1:100 | Santa Cruz Biotechnology, CA, USA |
| CK related | | | | |
| CK7 | OV-TL12.30 | M7018 | 1:500 | DAKO, Carpinteria, CA, USA |
| CK20 | Ks20.8 | M7019 | 1:100 | DAKO, Carpinteria, CA, USA |

**Supplementary Table S2. Clinicopathologic characteristics of patients according to the histologic subtype.**

| Clinical parameters | Total N=72 (%) | Histologic subtype | | | | p-value |
|---|---|---|---|---|---|---|
| | | ADC (n=22) (%) | PDC (n=15) (%) | SCC (n=19) (%) | UDC (n=16) (%) | |
| Age (years, mean±SD) | 54.8±11.8 | 59.3±12.5 | 53.6±12.6 | 56.6±8.6 | 47.3±10.2 | 0.013 |
| Sex | | | | | | 0.617 |
| Female | 24 (33.3) | 9 (40.9) | 6 (40.0) | 5 (26.3) | 4 (25.0) | |
| Male | 48 (66.7) | 13 (59.1) | 9 (60.0) | 14 (73.7) | 12 (75.0) | |
| Clinical subtype | | | | | | 0.003 |
| Favorable type | 17 (23.6) | 2 (9.1) | 4 (26.7) | 10 (52.6) | 1 (6.3) | |
| Unfavorable type | 55 (76.4) | 20 (90.9) | 11 (73.3) | 9 (47.4) | 15 (93.8) | |
| Organs involved | | | | | | 0.160 |
| Lymph node | 49 (68.1) | 10 (45.5) | 11 (73.3) | 18 (94.7) | 10 (62.5) | |
| Bone | 8 (11.1) | 5 (22.7) | 1 (6.7) | 0 (0.0) | 2 (12.5) | |
| Brain | 7 (9.7) | 3 (13.6) | 1 (6.7) | 1 (5.3) | 2 (12.5) | |
| Other | 8 (11.1) | 4 (18.2) | 2 (13.3) | 0 (0.0) | 2 (12.5) | |
| Postoperative treatment | | | | | | 0.007 |
| None | 18 (25.0) | 6 (27.3) | 2 (13.3) | 4 (21.1) | 6 (37.5) | |
| Chemotherapy | 25 (34.7) | 11 (50.0) | 5 (33.3) | 3 (15.8) | 6 (37.5) | |
| Radiation therapy | 12 (16.7) | 5 (22.7) | 0 (0.0) | 5 (26.3) | 2 (12.5) | |
| Chemo-radiation therapy | 17 (23.6) | 0 (0.0) | 8 (53.3) | 7 (36.8) | 2 (12.5) | |
| CK7 | | | | | | 0.372 |
| Negative | 32 (44.4) | 7 (31.8) | 9 (60.0) | 8 (42.1) | 8 (50.0) | |
| Positive | 40 (55.6) | 15 (68.2) | 6 (40.0) | 11 (57.9) | 8 (50.0) | |
| CK20 | | | | | | 0.428 |
| Negative | 66 (91.7) | 19 (86.4) | 15 (100.0) | 18 (94.7) | 14 (87.5) | |
| Positive | 6 (8.3) | 3 (13.6) | 0 (0.0) | 1 (5.3) | 2 (12.5) | |
| CK7/CK20 pattern | | | | | | 0.522 |
| CK7 (+)/CK20 (–) | 37 (51.4) | 14 (63.6) | 6 (40.0) | 10 (52.6) | 7 (43.8) | |
| CK7 (+)/CK20 (+) | 3 (4.2) | 1 (4.5) | 0 (0.0) | 1 (5.3) | 1 (6.3) | |
| CK7(–)/CK20(+) | 3 (4.2) | 2 (9.1) | 0 (0.0) | 0 (0.0) | 1 (6.3) | |
| CK7(–)/CK20(–) | 29 (40.3) | 5 (22.7) | 9 (60.0) | 8 (42.1) | 7 (43.8) | |

Abbreviations: PD-poorly differentiated carcinoma; AD-adenocarcinoma; SQ-squamous cell carcinoma; UD-undifferentiated carcinoma

**Supplementary Table S3. Clinicopathologic characteristics of patients according to the clinical subtype.**

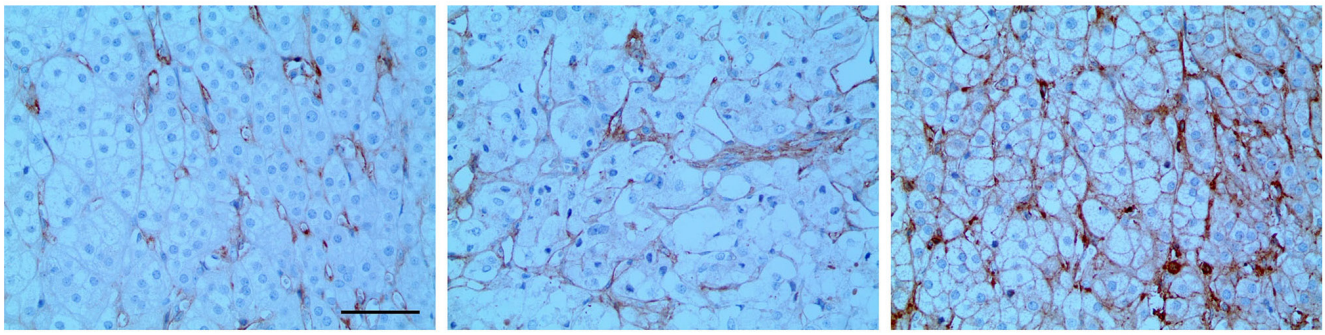
| Clinical parameters | Total (n=72) (%) | Clinical subtype | | p-value |
|---|---|---|---|---|
| | | Favorable type (n=17) (%) | Unfavorable type (n=55) (%) | |
| Age (years, mean±SD) | 54.8±11.8 | 50.4±8.1 | 56.1±12.4 | 0.084 |
| Sex | | | | 0.844 |
| Female | 24 (33.3) | 6 (35.3) | 18 (32.7) | |
| Male | 48 (66.7) | 11 (64.7) | 37 (67.3) | |
| Organs involved | | | | 0.336 |
| Lymph node | 49 (68.1) | 14 (82.4) | 35 (63.6) | |
| Bone | 8 (11.1) | 2 (11.8) | 6 (10.9) | |
| Brain | 7 (9.7) | 0 (0.0) | 7 (12.7) | |
| Other | 8 (11.1) | 1 (5.9) | 7 (12.7) | |
| Postoperative treatment | | | | 0.329 |
| None | 18 (25.0) | 6 (35.3) | 12 (21.8) | |
| Chemotherapy | 25 (34.7) | 3 (17.6) | 22 (40.0) | |
| Radiation therapy | 12 (16.7) | 4 (23.5) | 8 (14.5) | |
| Chemo-radiation therapy | 17 (23.6) | 4 (23.5) | 13 (23.6) | |
| CK7 | | | | |
| Negative | 32 (44.4) | 5 (29.4) | 27 (49.1) | |
| Positive | 40 (55.6) | 12 (70.6) | 28 (50.9) | |
| CK20 | | | | 0.676 |
| Negative | 66 (91.7) | 16 (94.1) | 50 (90.9) | |
| Positive | 6 (8.3) | 1 (5.9) | 5 (9.1) | |
| CK7/CK20 pattern | | | | 0.474 |
| CK7 (+)/CK20 (-) | 37 (51.4) | 11 (64.7) | 26 (47.3) | |
| CK7 (+)/CK20 (+) | 3 (4.2) | 1 (5.9) | 2 (3.6) | |
| CK7(-)/CK20(+) | 3 (4.2) | 0 (0.0) | 3 (5.5) | |
| CK7(-)/CK20(-) | 29 (40.3) | 5 (29.4) | 24 (43.6) | |

**Supplementary Table S4. H-scores of EMP 1, 2, and 3 in CUP.**

| Parameters | CUP (N=72) | | |
|---|---|---|---|
| | H-score (mean±SD) | H-score (range) | H-score median |
| EMP1 | 61.4±70.2 | 0-270 | 25 |
| EMP2 | 18.6±47.3 | 0-240 | 0 |
| EMP3 | 79.5±73.0 | 0-300 | 65 |

**Supplementary Table S5. H-scores of EMP 1, 2, and 3 according to the histologic type and clinical type in CUP.**

| Parameters | EMP1 | | EMP2 | | EMP3 | |
|---|---|---|---|---|---|---|
| | H-score (mean±SD) | p-value | H-score (mean±SD) | p-value | H-score (mean±SD) | p-value |
| Histologic subtype | | 0.997 | | 0.209 | | 0.829 |
| ADC (n=22) | 60.4±74.8 | | 5.0±15.2 | | 78.6±88.4 | |
| PDC (n=15) | 65.0±70.3 | | 32.0±72.8 | | 66.6±63.4 | |
| SCC (n=19) | 61.3±77.8 | | 30.2±57.8 | | 80.5±59.9 | |
| UDC (n=16) | 59.6±60.1 | | 11.2±25.1 | | 91.8±76.7 | |
| Clinical subtype | | 0.134 | | 0.013 | | 0.246 |
| Favorable type | 83.8±82.5 | | 43.2±73.3 | | 97.6±42.3 | |
| Unfavorable type | 54.5±65.3 | | 11.0±33.2 | | 74.0±79.6 | |




**Supplementary Figure S1. Immunohistochemical staining of EMP1, 2 and 3 in adrenal gland tissue as positive control (Scale bar=500μm).**