Cancer Stem Cell Markers in Eyelid Sebaceous Gland Carcinoma: High Expression of ALDH1, CD133, and ABCG2 Correlates With Poor Prognosis

Namju Kim,1,2 Ho-Kyung Choung,1,3 Min Joung Lee,4 Sang In Khwarg,1,5 and Ji Eun Kim6,7

1Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Korea
2Department of Ophthalmology, Seoul National University Bundang Hospital, Seongnam, Korea
3Department of Ophthalmology, Seoul Municipal Government-Seoul National University Boramae Hospital, Seoul, Korea
4Department of Ophthalmology, Hallym University Sacred Hospital, Pyongchon, Korea
5Department of Ophthalmology, Seoul National University Hospital, Seoul, Korea
6Department of Pathology, Seoul National University College of Medicine, Seoul, Korea
7Department of Pathology, Seoul Municipal Government-Seoul National University Boramae Hospital, Seoul, Korea

Correspondence: Ji Eun Kim, Department of Pathology, Seoul Municipal Government-Seoul National University Boramae Hospital, #20 Boramae-ro 5-gil, Dongjak-gu, Seoul 156-707, Korea; npol181@snu.ac.kr.
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PURPOSE. To investigate the expression of cancer stem cell (CSC) marker proteins in eyelid sebaceous gland carcinoma and evaluate the clinical significance.

METHODS. Archival tissue blocks from 50 cases of eyelid sebaceous gland carcinoma were tested via immunohistochemistry for 16 putative CSC markers. Levels of protein expression were analyzed alongside various clinicopathologic parameters such as metastasis-free survival time.

RESULTS. Ten patients (20%) showed nodal or distant metastasis during the follow-up period (median, 35.2 months; range, 1–128 months) without any mortality in our series. Among the 16 markers, ALDH1, CD44, CD133, ABCG2, Sox4, Sox9, and slug were selected for candidates of CSC markers because they were frequently and predominantly found in the tumor cells compared with control tarsus cells, which showed negative or very low expression. Univariate analysis revealed that ALDH1, CD133, and ABCG2 were significantly associated with metastasis; patients with ALDH1- or CD133-positive tumors developed metastasis more frequently than patients with tumors that were negative for these markers (log-rank test, \( P = 0.014 \), \( P = 0.015 \), respectively), and diffuse expression of ABCG2 was associated with significantly shorter metastasis-free survival (log-rank test, \( P = 0.010 \)). Multivariate Cox proportional hazard model revealed that ALDH1 (hazard ratio [HR] = 5.682, \( P = 0.038 \)) was significantly associated with metastasis.

CONCLUSIONS. Development of metastasis in eyelid sebaceous gland carcinoma might be attributed to increased number of CSCs or acquisition of dedifferentiated phenotype. Our findings suggest that CSCs are involved in the disease progression of eyelid sebaceous gland carcinoma, and in particular, expression of ALDH1 is a predictor of a poor outcome.

Keywords: cancer stem cell, sebaceous carcinoma, metastasis

Recently, cancer therapeutic strategies have focused on targeting a subpopulation of cells called cancer stem cells (CSCs), which are characterized by self-renewal capacity and multipotentiality. Whether the emergence of CSCs results from phenotypic switch toward dedifferentiation or clonal selection is unclear. These CSCs can lie quiescent after completion of treatment but may repopulate the tumor after a long period of time. Therefore, enrichment of CSCs induces chemotherapeutic resistance or metastasis, which ultimately results in an unfavorable outcome in many malignancies.

Various molecules have been investigated as markers of CSCs in human solid tumors, most notably CD44 and ALDH1 in breast cancer and CD133 in glioblastoma. Thereafter, researchers discovered numerous stem cell markers that are transiently expressed in developmental stages or in specialized cells. Early embryonic transcription factors such as Sox1, Sox2, Sox9, and Sox10 are involved in the morphogenesis of the skin and foregut, though aberrant expression of these proteins has been found to be a strongly negative prognostic predictor in some carcinomas. Additionally, chemoresistance proteins such as ATP binding cassette (ABC) G2 function as CSC markers because tumor cells expressing these proteins remain viable during treatment due to active transportation of chemotherapeutic drugs. Finally, proteins involved in epithelial-mesenchymal transition are also categorized as CSC markers.

In the field of CSC research, one of the most actively investigated tumors is breast carcinoma, in which CSC is not only a prognostic marker but also a target for therapies. However, to our knowledge, no study has been conducted regarding CSCs in sebaceous gland carcinoma. Although long-term survival rates have improved in recent years, sebaceous gland carcinoma is still considered to be a potentially aggressive tumor. Metastasis to regional lymph nodes or distant organs is relatively common and the recurrence rate reaches up to 10% to 25%. It is very likely that a population of chemoresistant CSCs participate in tumor regrowth or that remaining CSCs evolve into more aggressive subclones.
TABLE 1. Lists of Antibodies and Applications Used in the Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Classification Positive Controls or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALDH1</td>
<td>44/ALDH</td>
<td>BD Biosciences (San Diego, CA, USA)</td>
<td>Enzyme rich in progenitor cells</td>
</tr>
<tr>
<td>CD44</td>
<td>156-3C11</td>
<td>Thermo Scientific (Fremont, CA, USA)</td>
<td>Surface markers</td>
</tr>
<tr>
<td>CD133</td>
<td>NA</td>
<td>Spring Bioscience (Pleasanton, CA, USA)</td>
<td>Surface markers</td>
</tr>
<tr>
<td>ABCG2</td>
<td>BXP-21</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
<td>Chemosresistance protein</td>
</tr>
<tr>
<td>MRP1</td>
<td>MRP1</td>
<td>Kamiya Biomedical (Seattle, WA, USA)</td>
<td>Chemosresistance protein</td>
</tr>
<tr>
<td>LRP</td>
<td>LMR5</td>
<td>Kamiya Biomedical (Seattle, WA, USA)</td>
<td>Chemosresistance protein</td>
</tr>
<tr>
<td>Pglycoprotein</td>
<td>EPR4766</td>
<td>Diagnostic Biosciences (Pleasanton, CA, USA)</td>
<td>Chemosresistance protein</td>
</tr>
<tr>
<td>Sox1</td>
<td>5C10</td>
<td>Abnova (Taipei, Taiwan)</td>
<td>Developmental marker</td>
</tr>
<tr>
<td>Sox2</td>
<td>D6D9</td>
<td>Cell Signaling (Beverly, MA, USA)</td>
<td>Developmental marker</td>
</tr>
<tr>
<td>Sox4</td>
<td>NA</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
<td>Developmental marker</td>
</tr>
<tr>
<td>Sox9</td>
<td>M20</td>
<td>Novocastra, UK</td>
<td>Developmental marker</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>NCL-L-Gad</td>
<td>Novocastra, UK</td>
<td>Developmental marker</td>
</tr>
<tr>
<td>Snail</td>
<td>A-7</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
<td>Developmental marker</td>
</tr>
<tr>
<td>Slug</td>
<td>CE2C3</td>
<td>Santa Cruz (Santa Cruz, CA, USA)</td>
<td>Developmental marker</td>
</tr>
<tr>
<td>Oct4</td>
<td>52G6</td>
<td>Cell Signaling</td>
<td>Developmental marker</td>
</tr>
</tbody>
</table>

NA, not available; EMT, epithelial mesenchymal transition.

This study aims to elucidate the role of CSCs in the pathogenesis of eyelid sebaceous gland carcinoma by investigating the expression of CSC markers in tumor samples in relation to clinicopathologic features. Because there is no universally accepted CSC marker, we screened 16 proteins that had been identified in many solid cancers and finally extracted seven markers for potential candidates of CSCs of eyelid sebaceous gland carcinoma.

METHODS

Patients

Tissue from 50 cases of surgically resected eyelid sebaceous gland carcinoma from three different hospitals in Korea between January 1999 and June 2013 were enrolled through retrospective database analysis. Clinical information including demographic data, treatment, response, and follow-up data with survival periods were obtained from electronic medical records. TNM staging was estimated according to the 2010 American Joint Committee on Cancer (AJCC, seventh edition). This study was approved by the institutional review board of Seoul Municipal Government-Seoul National University Boramae Hospital. This study was conducted in compliance with the Declaration of Helsinki.

Immunohistochemical Analysis

Histologic diagnosis of sebaceous gland carcinoma was re-evaluated by experienced pathologist and histologic differentiation was assessed. Cases were classified as undifferentiated when marked pleomorphism, comedo-like necrosis, or brisk mitotic figures was found.

To select CSC markers for sebaceous gland carcinoma, we screened 16 CSC markers including surface markers (CD44, CD133), established CSC markers in breast cancer (CD44, ALDH1), CSC markers involved in epithelial-mesenchymal transition (Slug, Snail, N-cadherin, Sox4, Sox9), markers involved in early embryonic development (Oct4, Sox1, Sox2, Sox10), and chemoresistance proteins (ABCG2, P-glycoprotein, MRP1, LRP). The expression of these CSC markers was investigated by immunohistochemistry (IHC) on paraffin-embedded tissue sections using an automated immunostainer. The standard CC1 protocol and Ultraview detection kit were used in accordance with the manufacturer’s recommendation (Benchmark Ventana, Tuscan, AZ, USA). List of primary antibodies is shown in Table 1. Appropriate positive and negative controls were stained simultaneously.

At least three different representative high-power (>400) fields, each containing at least 50 tumor cells, were evaluated for immunostaining and histology by an experienced pathologist who was blinded to patient ID and clinical data. Immunoreactivity localized in the cytoplasm or cytoplasmic membranes (ALDH1, CD44, CD133, and ABCG2) was interpreted as ‘negative’ when the percentage of positive cells was less than 10%, ‘focal positive’ when the percentage was 10% to 50%, and ‘diffuse positive’ when it was more than 50%. For markers exhibiting nuclear positivity (Sox4, Sox9, and slug), IHC results were calculated by a semiquantitative H score; the percentages of positive tumor nuclei were assigned as 0 to 1 (0% for 0%, 0.1 for 1% to 9%, 0.5 for 10% to 49%, and 1.0 for ≥50%) and multiplied by the staining intensity on a scale of 0 to 3.

TABLE 2. Clinical Features of Patients With Eyelid Sebaceous Gland Carcinoma

<table>
<thead>
<tr>
<th>Sex, male:female</th>
<th>Age,‡ y</th>
<th>Follow-up,† mo</th>
<th>Stage (seventh AJCC)</th>
<th>Metastasis</th>
<th>Sebaceous Gland Carcinoma Without Metastasis, n = 40</th>
<th>Sebaceous Gland Carcinoma With Metastasis, n = 10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:28</td>
<td>64.4 ± 12.6 (37–88)</td>
<td>32.3 ± 22.4 (1–98)</td>
<td>T2N0/T3N0/T2N1/T3N1/T4N0</td>
<td>None</td>
<td>3.7</td>
<td>56.0 ± 17.1 (38–93)</td>
<td>46.6 ± 39.4 (1–127)</td>
</tr>
<tr>
<td>3.7</td>
<td>3.4:1:F:1</td>
<td>8 in LN, 2 in lung</td>
<td>3.7</td>
<td>1.000*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fisher’s exact test.
† Mean ± SD.
‡ Mann-Whitney U test.
Negative or infrequent expression of cancer stem cell marker proteins in normal eyelids ([A–G], all ×400). Expression of (A) ALDH1 or (B) CD133 was not found in normal sebaceous gland cells. Expression of (C) CD44 is restricted to peripheral basal cells, and (D) ABCG2 is positive in less than 10% of normal sebaceous gland cells. Nuclear positivity of (E) Sox4, (F) Sox9, and (G) slug was seen in a small subset of peripheral basal cells.
Figure 2. Frequent expression of cancer stem cell marker proteins in eyelid sebaceous gland carcinoma ([A–G], all ×400). Diffuse aberrant expression of (A) ALDH1, (B) CD133, (C) CD44, and (D) ABCG2 was found in the cytoplasm of cancer cells. (E) Sox4, (F) Sox9, and (G) slug were highly expressed in the nucleus of cancer cells.
Cancer Stem Cell Markers | Group | Negative | Focal Positive | Diffuse Positive | H-Score < 1 | H-Score ≥ 1
--- | --- | --- | --- | --- | --- | ---
ALDH1 | Total | 33 (73%) | 8 (18%) | 4 (7%) | --- | ---
  | M | 4 | 4 | 2 | --- | ---
  | NM | 29 | 5 | 2 | --- | ---
CD44 | Total | 12 (27%) | 8 (18%) | 24 (55%) | --- | ---
  | M | 3 | 3 | 4 | --- | ---
  | NM | 9 | 5 | 20 | --- | ---
CD133 | Total | 57 (82%) | 6 (13%) | 2 (5%) | --- | ---
  | M | 7 | 2 | 1 | --- | ---
  | NM | 30 | 4 | 1 | --- | ---
ABCG2 | Total | 34 (71%) | 11 (23%) | 3 (6%) | --- | ---
  | M | 6 | 1 | 2 | --- | ---
  | NM | 28 | 10 | 1 | --- | ---
Sox4 | Total | --- | 15 (65%) | 8 (35%) | --- | ---
  | M | --- | 4 | 0 | --- | ---
  | NM | --- | 11 | 8 | --- | ---
Sox9 | Total | --- | 14 (39%) | 22 (61%) | --- | ---
  | M | --- | 4 | 4 | --- | ---
  | NM | --- | 10 | 18 | --- | ---
Slug | Total | --- | 23 (68%) | 11 (32%) | --- | ---
  | M | --- | 5 | 3 | --- | ---
  | NM | --- | 18 | 8 | --- | ---

M: group with metastasis; NM: group without metastasis.

Statistical Analysis

Metastasis-free survival was calculated from the date of surgery until the first day of metastasis detection; overall survival could not be evaluated because no mortality occurred after curative resection. Patients lost to follow-up without the development of metastasis were censored at the date of the last follow-up. The study of the prognostic value of stem cell markers was based on a Kaplan-Meier method with log-rank tests and multivariate Cox proportional hazard model. All probable variables were entered in one single step to develop the final multivariate model and adjusted hazard ratios (HR) with 95% confidence interval (CI) were calculated. Statistical analyses were performed with SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). All reported P values are two sided, and P less than 0.05 was considered significant.

RESULTS

Patient Demographics and Clinical Features

Female patients (N = 35) predominated over males (N = 15) with a mean age of 63.2 years (range: 37–93 years). There were 36 patients with T2N0 tumors, 11 patients with T3N0 tumors, and one patient with T4N0, T2N1, T3N1, respectively. Three patients underwent exenteration, and 47 patients underwent wide surgical excision. All patients survived during the follow-up (median, 35.2 months; range, 1–128 months). Lymph node metastasis was detected in eight patients at the time of surgery or during follow-up, and distant metastasis was found in two patients late after surgery. There were no significant differences in sex, age, or follow-up periods between the group with metastasis and the group without metastasis (Table 2).

Immunohistochemistry Results

Among the 16 markers tested, we selected seven markers as candidates for clinically applicable CSC markers of eyelid sebaceous gland carcinoma, which are ALDH1, CD44, CD133, ABCG2, Sox4, Sox9, and slug. Many markers were excluded after comparison of expression pattern between the normal and carcinoma tissues. Snail and N-cadherin showed diffuse positivity even in the normal sebaceous glands. Most markers related to the early embryonic development, especially neural crest forming markers such as Sox1, Sox2, Sox10, and Oct4 that were uniformly negative in both normal and carcinoma tissues. Among chemoresistance proteins, only ABCG2 was expressed, whereas others were negative. Based on these findings, we finally selected seven CSC markers in this study. In normal tarsal tissue, these seven CSC markers were negative or were expressed in less than 10% of normal cell nuclei (Fig. 1). However, aberrant overexpression was noted in the carcinoma cells (Fig. 2). The other markers were excluded from CSC markers of eyelid sebaceous gland carcinoma because most of them were totally negative in both normal and malignant cells, and as for N-cadherin and snail, they were excluded because they revealed diffuse positivity even in normal sebaceous glands.

ALDH1 was positive in 25% of the tumors, CD44 in 73%, CD133 in 18%, and ABCG2 in 29%. High expression of Sox4, Sox9, and slug (H-scores exceeding 1.0) was found in 35%, 62%, and 32% (Table 3, Fig. 2). There were five cases of histologically undifferentiated cases; however, histologic types were not correlated with the CSC marker expression or development of metastasis.

In univariate analysis using log-rank tests, three CSC marker expressions were significantly associated with metastasis. Patients showing positive expression of ALDH1 or CD133 developed metastasis more frequently than patients showing negative expression of ALDH1 or CD133 (log-rank test, P = 0.014, P = 0.013, respectively). Patients showing diffuse positive expression of ABCG2 developed metastasis more frequently than patients showing negative or focal positive expression of ABCG2 (log-rank test, P = 0.010; Fig. 3). However, only ALDH1 (HR = 5.682, 95% CI: 1.103–29.266, P = 0.038) was significantly associated with metastasis after multivariate Cox proportional hazard model analysis.
DISCUSSION

The role of CSCs has been drawing attention based on the hypothesis of intratumoral heterogeneity and clonal evolution of carcinogenesis. Increased numbers of CSCs have been implicated in aggressive behavior in many cancers. This is the first study investigating the clinical significance of CSCs in sebaceous gland carcinoma of the eyelid. We chose six proteins as candidates for CSC markers of eyelid sebaceous gland carcinoma based on results from previous studies. We found that these six markers were selectively highly expressed in carcinoma cells in contrast to normal tarsus, which verifies the stem cell characteristics of these markers. This result also implies that CSCs could be involved, at least in part, in the development of eyelid sebaceous gland carcinoma.

We found that patients with positive ALDH1, positive CD133, or diffuse positive ABCG2 had shorter metastasis-free survival compared with ALDH1-negative, CD133-negative, or ABCG2-negative groups. After eliminating confounding effect, ALDH1 positivity was found to have positive correlation with metastasis, indicating that CSCs are significantly associated with the metastatic potential of eyelid sebaceous gland carcinoma. These results are consistent with previous reports that expression of CSC markers is upregulated in some solid carcinomas and was one of the significant risk factors for poor prognosis. For example, ALDH1 expression was associated with the development of early metastasis in inflammatory breast cancer, and CD133 expression strongly correlated with liver metastasis in colon cancer. Shen et al. reported that high levels of ABCG2 were correlated with lymph node metastasis in head and neck squamous cell carcinoma. In addition to these results, several studies have demonstrated the direct clonal relationship between CSCs in the primary origin and cancer cells in metastatic lesions. Pang et al. identified CD26 as a marker of migratory and metastasis-causing CSCs in colon cancer and reported that only patients with CD26-expressing cells in their primary tumor developed metastasis.

It is well established that CSCs are sources of tumor cells, responsible for cancer initiation, tumor growth, and relapse due to their ability to undergo multilineage differentiation and survive in adverse microenvironments. Increased motility, invasiveness, and resistance to DNA damage-induced apoptosis are characteristics of CSCs and are relevant to metastasis. Mortality of metastatic eyelid sebaceous gland carcinoma patients is relatively high, with a 5-year survival rate of 50% to 67%. Currently, there are no validated markers to predict metastasis or disease outcome. Sentinel lymph node biopsies have been used in an attempt to identify lymph node metastasis in eyelid sebaceous gland carcinoma patients; however, false-negative results are common. Therefore, identification of patients with high risk of metastasis is crucial to develop individualized therapeutic approaches and more sophisticated prognostication. Our findings suggest that CSC marker, especially ALDH1 is possible predictive factors for development of metastasis in eyelid sebaceous gland carcinoma. Expression of CSC marker ALDH1 is a useful prognostic indicator and may facilitate selection of patients who require more aggressive treatments, such as neck dissection, to reduce the chance of metastasis after primary resection.

Our study is the first to evaluate expression of CSC markers as a prognostic indicator in eyelid sebaceous gland carcinoma. We demonstrated that CSC markers are frequently overexpressed in eyelid sebaceous gland carcinoma and in particular, immunoreactivity of ALDH1 can be used as predictors of lymph node metastasis.

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References

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