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CD147 Expression in Advanced Cutaneous Squamous Cell Carcinoma

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Abstract

CD147 is upregulated in multiple cancer types, but its expression in advanced cutaneous squamous cell carcinoma (cSCC) is unknown. This study retrospectively evaluated the expression patterns of CD147 and related proteins monocarboxylate transporters (MCT1, MCT4) in patients with advanced stage cSCC (n=50). CD147, MCT1 and MCT4 expression levels were assessed in archived tumor samples and correlations were made with survival and clinicopathologic characteristics. The incidence of CD147 overexpression (93%, n=42) was higher than that of MCT1 (23%) or MCT4 (47%). Colocalization of MCT1 or MCT4 with CD147 was also observed. The two-year survival rate was 69% and the 5-year survival rate was 61%. A trend towards decreased survival was seen with overexpression of CD147 (p= .17), MCT1 (p= .11) and MCT4 (p= .15). Expression levels were not correlated with clinicopathologic characteristics. CD147 is overexpressed in the majority of advanced cSCC and may represent a biomarker or potential therapeutic target.

1. Introduction

With more than one million new cases reported each year, nonmelanoma skin cancer (NMSC) is the most common diagnosed malignancy in the United States [1]. Cutaneous squamous cell carcinoma (cSCC) is the second most common NMSC [2] and the majority are successfully treated by complete tumor excision. However, there is a small percentage of cSCCs which are more aggressive and refractory to standard surgical resection [1, 3]. Patients who present with recurrent disease are at increased risk for neck metastasis, poor local control and further cancer recurrence. High risk prognostic indicators include size, anatomic site, history of radiation, perineural invasion and recurrence [4]. Although these characteristics have been known for some time, the biology of aggressive cSCC is poorly understood.

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Extracellular matrix metalloproteinase inducer, CD147, is expressed in high levels in multiple squamous cell carcinomas arising from other anatomical sites including the head and neck, esophagus and lungs [5]. CD147 is a membrane bound glycoprotein known to promote tumor growth, lymphatic metastasis and drug resistance [5–9]. CD147 stimulates matrix metalloproteinase (MMP) expression from stromal tissue [6, 10] and upregulates VEGF [11, 12]. CD147 overexpression has previously been identified in squamous cell carcinoma of the aerodigestive tract [5, 7]. However, the association between CD147 expression and survival rates in cSCC has yet to be investigated.

Recently a relationship between CD147 and MCT1 or MCT4 has been investigated and a codependence upon each has been elucidated [13–15]. Monocarboxylate transporters (MCT), pump monocarboxylates out of the cell into the interstitium, preventing intracellular acidosis. The MCT family consists of at least 14 known members. Our focus is on the MCT1 and MCT4, the proton-coupled lactate transporters. MCT1 is the most ubiquitous family member and found to be associated with colorectal, gastric, cervical, breast and ovarian cancers [15–20]. MCT4 has a low affinity for lactate and tends to be found in tissues requiring glycolysis [21]. To prevent degradation and ensure trafficking of biologically active CD147 to the plasma membrane MCT1 and MCT4 are required [13–15]. Silencing of MCT4 RNA was found to prevent maturation of CD147, while silencing CD147 RNA lead to a decrease in MCT4. In addition, overexpression of MCT4 leads to increased mature CD147 on the plasma membrane [15]. CD147 was also found to form a heteromeric complex with MCT1 or MCT4, with MCT serving as the enzymatic, α -subunit and CD147 serving as the accessory, β -subunit [13–15]. Therefore, evaluation of CD147 expression should include evaluation of MCT expression. To this end, we evaluated aggressive cSCC for CD147, MCT1, and MCT4 expression levels.

2. Materials and Methods

Patient Selection

Following Institutional Review Board approval, a retrospective review of 56 patients who presented between June 1998 and December 2007 with recurrent, advanced stage (III or IV) cSCC of the head and neck was performed at the University of Alabama at Birmingham. Tumors were staged according to the American Joint Committee on Cancer (AJCC) [22] guidelines and histology was confirmed by pathology.

Each patient underwent aggressive surgical resection and the majority required neck dissection and postoperative radiation. The indication for neck dissection was suspicion of nodal metastasis on preoperative imaging or an advanced T classification of the lesion at presentation. Postoperative radiation was recommended for patients with large cutaneous malignancies, when more than one positive node was identified on neck dissection, when negative surgical margins could not be obtained or in the presence of perineural or lymphovascular invasion.

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was performed to determine CD147 expression. Samples were rehydrated in xylene, 95% ethanol, and 70% ethanol. Antigen retrieval was accomplished in 1 mM EDTA, pH 9.0, for 5 minutes at 100°C. Samples were then allowed to cool at room temperature and blocked with 5% BSA in TBST for 5 minutes at room temperature. Primary antibody, CD147 antibody (Millipore, clone 1S9-2A, mouse monoclonal IgG2ak), was applied at the concentrations recommended and allowed to incubate for 1 hour. Secondary antibody (Pierce, goat anti-mouse, horseradish peroxidase) was applied for one hour in a humidified chamber at room temperature. DAB substrate was

then applied to slides and allowed to incubate at room temperature until appropriate color developed. Samples were then counterstained with Harris Hematoxylin diluted 1:1 with tap water for 45 seconds. Finally, samples were dehydrated and counted with Permount and allowed to dry overnight.

Slides were analyzed in a blinded fashion for both staining intensity and quality. Scoring values were assigned as follows: 0 = no staining to < 10% of tumor cells, 1+ = light and incomplete staining in > 10% of tumor cells, 2+ = moderate and complete staining of > 10% of tumor cells and 3+ = intense and complete staining > 10% (Figure 1) [23].

Immunofluorescence Staining

Immunofluorescence analysis was performed to determine membrane expression levels of CD147, MCT1 and MCT4 as well as co-expression or colocalization of CD147 with either MCT1 or MCT4. Samples were rehydrated in xylene (3x), 95% ethanol, and 70% ethanol. Antigen retrieval was accomplished in 1mM EDTA, pH 9.0, for 10 minutes at 100°C. Samples were then allowed to cool at room temperature in EDTA solution and then blocked with 5% BSA in TBST for 10 minutes at room temperature. The sections were incubated for one hour at room temperature in a humidified chamber with antibody to both CD147 1:100 (Millipore, clone 1S9-2A, mouse monoclonal IgG2ak) and to MCT 1:100 (-4 Santa Cruz, sc-50329, polyclonal IgG; -1 sc-50324). Slides were then washed 3x in 0.05 M Tris-Buffer, pH 7.6 for 10 min. and then incubated in the dark for 1h using a mixture of fluorophore-linked secondary anti-bodies 1:1000 (AlexaFluor 568- conjugated goat anti-mouse IgG1 and AlexaFluor 488-conjugated goat anti-rabbit IgG). The slides were then washed 3x in .05 M Tris-Buffer, pH 7.6 for 10 min each and mounted using Gel Mount Aqueous Mounting Medium (Sigma, G0918). Fluorescent microscopy was performed for each field using Olympus IX70 fluorescent microscope with Olympus DP72 camera. Images were then processed using Olympus D2-Basic Imaging Software.

Slides were qualitatively analyzed in a blinded fashion. Staining intensity values were as follows: 0 = none (< 25% of tumors cells stain), 1 = light (25–50% of tumor cells stain), 2 = moderate (50–75% of tumor cells stain) and 3 = intense (>75% of tumor cells stain) (Figure 2) [16]. Colocalization was determined by comparing the level and intensity of expression for CD147 to MCT1 or MCT4 expression at the same location on the cell membrane.

Statistics

Descriptive variables were summarized by mean, median and standard deviation (SD) for continuous variables and n (%) for categorical variables. The relationship between EGFR expression levels and clinicopathologic characteristics was calculated using the Fisher's Exact Test.

Two groups were designated: low expressing tumors composed of scores 0 and 1+ and high expressing (over-expressing) tumors composed of scores 2+ and 3+. The relationship between patient, clinical and treatment factors, and cancer specific survival was calculated by using the Kaplan-Meier method. Survival time was calculated as the interval from date of surgery to date of death or date of last follow-up. Deaths due to other causes were censored for these analyses. A p-value of < .05 was considered statistically significant. All of the analyses were two-sided. Statistical analysis was performed using SAS® Ver. 9.2 software (SAS Institute, Inc., Cary, NC).

Results

The patients were elderly (mean age 72 ± 12) and the majority were male (86%, n=43). Common comorbidities were diabetes mellitus, hypertension, and hypercholesterolemia,

with 14% having had a previous malignancy. The majority of patients (92%, n=46) were stage III at initial diagnosis with 46% (n=23) having evidence of nodal disease and only 8% (n=4) with evidence of distant metastasis. Eighty-six percent (n=43) underwent radiation therapy during the course of treatment. A summary of patient characteristics can be found in Table 1.

Immunohistochemical analysis was performed to determine CD147 expression levels on cell membranes. Slides were scored semiquantitatively (0–3+) by two independent observers who were blinded to the clinical parameters. The majority of primary tumors (93%, n=42) demonstrated high expression of CD147 (2+ or 3+) while all nodal specimens positive for metastatic disease (n=11) demonstrated high expression of CD147 (Table 2).

Immunofluorescence analysis was also performed to determine CD147, MCT1 and MCT4 expression and colocalization. The majority of primary tumors (78%, n=35) and nodal metastasis (91%, n=10) demonstrated high CD147 reactivity (2+ or 3+, Table II). Only 23% (n=10) of primary tumors and 18% (n=2) of nodal metastasis analyzed for MCT1 demonstrated high expression (2+ or 3+) of MCT1. While 47% (n=20) of primary tumors and 50% (n=5) of nodal metastasis analyzed for MCT4 demonstrated high expression (2+ or 3+) of MCT4 (Figure 2). Baseline parameters, with the exception of nodal disease, were found to be similar when compared between the two groups, normal versus high expression of CD147, MCT1 and MCT 4 (Table 3). There was a statistically significant ($p = .048$) relationship between overexpression of CD147 and node positive disease (Table 3).

Colocalization of CD147/MCT1 and CD147/MCT4 was illustrated in the overlay of the green fluorescein isothiocyanate fluorescence of the anti-CD147 with the red tetramethylrhodamine isothiocyanate fluorescence of the anti-MCT forming a yellow hue. Colocalization was observed in each sample; however variations were seen among the different fields selected. Overall, scoring intensities were found to be the same for both CD147/MCT1 and CD147/MCT4 in 44% of cases. In the remaining cases, CD147 expression was higher compared to MCT1 (51%) and MCT4 (41%) and lower compared to MCT1 (5%) and MCT4 (15%).

Overall 2-year survival rate was 69% and the overall 5-year survival rate was 61%. When survival was compared to IHC and immunofluorescence reactivity, there was a trend of decreased survival in patients with tumors overexpressing CD147 ($p = .26$ and $p = .17$ respectively) (Figure 4a and 4b). In addition, CD147 membrane overexpression in primary lesions was associated with an increase in regional metastasis ($p = .048$) (Table 3). Although staining of nodal specimens found all lymph nodes (n=11) had high expression of CD147 by IHC and the majority (n=10) had high CD147 expression by immunofluorescence, the power was insufficient to delineate a statistically significant relationship (Figure 5a and 5b). For tumors overexpressing MCT1 or MCT4, there was also a trend towards decreased survival ($p = .11$ and $p = .15$ respectively) (Figure 4c and 4d). MCT1 and MCT4 overexpression, however, was not associated with an increase in regional metastasis ($p = .48$ and $p = .56$ respectively) (Table 5). In addition, there was no statistically significant relationship between nodal metastasis expression of MCT1 or MCT4 and survival (Figure 5b and 5c).

4. Discussion

To our knowledge, this is the first study to investigate the significance CD147, MCT1 and MCT4 expression in cSCC and its relationship to survival. Our results indicate CD147, MCT1 and MCT4 are highly expressed in cSCC and therefore may contribute to cancer cell survival mechanisms. CD147 allows for tumor invasion and MCT allows for increased

intracellular pH and prevention cell apoptosis. Furthermore, the efflux of lactate decreases interstitial pH, which has been found to promote metastasis and decrease disease-free survival [20, 24, 25]. In addition, CD147 and MCT create environments which promote VEGF expression, further promoting tumor growth, invasion, and metastasis [19, 26].

CD147 expression is elevated in a various forms of cancer, including dysplastic lesions; however its expression in normal tissue is low to absent [5, 27, 28]. Whereas MCT1 or MCT4 expression is found in the basal and parabasal levels of normal squamous and in all layers of neoplastic squamous cell carcinomas [19]. In the case of advanced cSCC, we found increased expression of plasma-membrane CD147, MCT4 and MCT1. Interestingly, MCT4 expression levels were greater than MCT1 expression levels. This pattern is consistent with what was found in cervical and colorectal carcinomas [19, 20]. Despite high expression levels of these plasma proteins, there was no association found with clinicopathologic parameters.

Several studies have demonstrated CD147 colocalizes with MCT on the cell membrane and that they are codependent on one another for expression, maturation, and transport to the cell membrane [14, 15]. The colocalization of CD147 with MCT1 or MCT4 demonstrated in this study supports the ancillary role of CD147 in MCT expression. CD147 and MCT overexpression have been linked to poor prognosis and advanced progression in several other cancers [16, 18–20, 29, 30]. While there were trends towards decreased survival in overexpressing tumors, these relationships were not statistically significant. Our inability to demonstrate statistical significance is likely due to a homogenous patient population (all advanced, recurrent cSCC), and lack of early stage or normal tissue comparisons. CD147 and MCT expression levels in normal tissue have been extensively investigated in previous studies [5, 27, 28, 31] and therefore normal tissue comparisons were not included in this study.

Given the small number of patients with distant metastatic disease (n=8) a comparison between the two groups proved to be futile. All patients had advanced, recurrent disease and were previously treated surgically. Therefore, stratifying by stage, recurrence, and surgical intervention were not possible. In addition, because advanced stage cSCCs are relatively rare, studies such as this one are limited by small numbers of patients which of course limits statistical power. Travel time and advanced age often limited patient's likelihood to return for routine visits; this likely contributed to our limited follow-up time. Although median follow-up time was 14 months, patients will continue to recur from this disease over the subsequent two to four years and as a result this study may not successfully capture all recurrent disease. Further limiting the power of this study was the absence of primary tumor samples in six patients.

5. Conclusion

It is becoming increasingly evident that targeting only one or even two molecular pathways is insufficient to halt cancer progression. The more knowledge there is about the molecular pathways associated with cancer cell survival and proliferation, the greater the understanding about their ability to resist targeted chemotherapeutics. Targeting CD147 and MCT could be a potential addition to the traditional chemotherapeutics thus eliminating another avenue cancer cells have for circumventing apoptosis.

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Figure 1.

Immunofluorescent staining of cutaneous squamous cell carcinoma primary lesions for (a) CD147 (*yellow*), (b) MCT 1 (*green*), (c) MCT 4 (*green*). Membrane reactivity was based on the following scores: 0 = no staining to < 10% of tumor cells staining, 1+ = light and incomplete staining in > 10% of tumor cells, 2+ = moderate and complete staining of > 10% of tumor cells and 3+ = intense and complete staining > 10%.

A

Antigen	CD 147			MCT 1		
Specimens	Low*	High [†]	<i>P</i> value	Low*	High [†]	<i>P</i> value
PT (n = 44)	23	77	0.43	77	23	1.0
ND (n = 11)	9	91		82	18	
PT primary tumors; ND nodal disease						

Antigen	CD 147			MCT 4		
Specimens	Low*	High [†]	<i>P</i> value	Low*	High [†]	<i>P</i> value
PT (n = 43)	23	77	0.43	53	47	1.0
ND (n = 10)	9	91		50	50	
PT primary tumors; ND nodal disease						

* % of no or low expressing tumors (≤ 1) in each subgroup
[†] % of overexpressing tumors (≥ 2) in each subgroup

Figure 2. Comparison of immunofluorescence staining of CD 147 and MCT in advanced cutaneous squamous cell carcinoma. (a) There was no statistical relationship between antigen expression levels and disease; (b) percentage of primary tumors or regional lymph nodes positive for metastatic disease with either low or high expression levels of the antigens.

Figure 3. Kaplan-Meier survival curves stratified by immunofluorescence staining of primary tumors for cell membrane expression of A) CD147, B) MCT1 and C) MCT4. Stratification is by low (score intensity 1+) vs. high expression (score intensity 2+) groups.

Figure 4.

Kaplan-Meier survival curves stratified by immunofluorescence staining of metastatic node disease for cell membrane expression of A) CD147, B) MCT1 and C) MCT4 in regional lymph node specimens positive for metastatic disease. Stratification is by low (score intensity 1+) vs. high expression (score intensity 2+) groups.

Table 1

Patient characteristics

Characteristic	n (%)
Age – years	
Mean (Range)	72 (42–91)
Gender	
Male	43 (86)
Comorbidities	
Diabetes mellitus	9 (18)
Hypertension	22 (44)
Hypercholesterolemia	9 (18)
Multiple malignancies	7 (14)
Congestive heart failure	1 (2)
Immunosuppressed	4 (8)
Hypothyroidism	2 (4)
Coronary artery disease	4 (8)
T classification	
T1	0 (0)
T2	5 (10)
T3	3 (6)
T4	41 (82)
Tx	1 (2)
TNM stage	
III	46 (92)
IV	4 (8)
Metastasis	
Nodal Disease	
No positive nodes	23 (46)
Positive nodes	27 (54)
Distant disease	
No metastasis	46 (92)
Positive metastasis	4 (8)
XRT	
Neoadjuvant	12 (24)
Adjuvant	30 (60)

Table 2

Clinicopathological characteristics associated with immunofluorescence staining intensity of CD 147, MCT 1 and MCT 4. The p-values in bold are considered significant ($p < 0.05$).

Variable	% of staining intensity									
	CD 147			MCT 1			MCT 4			
	Low*	High [†]	P-value	Low*	High [†]	P-value	Low*	High [†]	P-value	P-value
Tumor stage										
3	24	76	0.56	80	20	1	55	45		0.34
4	0	100		75	25		25	75		
Nodal disease										
N0	33	67	0.048	85	15	0.48	58	42		0.56
N+	9	91		74	26		45	55		
Metastatic disease										
M0	24	76	0.56	80	20	1	55	45		0.34
M+	0	100		75	25		25	75		
XRT										
Neoadjuvant	31	69	0.28	83	17	0.21	38	62		0.69
Adjuvant	23	77		74	26		57	43		

*. Percentage of no or low expressing tumors (1) in each subgroup.

[†] Percentage of overexpressing tumors (2) in each subgroup.