Reduced Level of CD44 and Hyaluronan Associated with Unfavorable Prognosis in Clinical Stage I Cutaneous Melanoma

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The cell surface glycoprotein CD44 and its ligand, hyaluronan (HA), enhance growth and metastatic capacity of melanoma cells *in vitro*, but their clinical significance in primary cutaneous melanoma is still unclear. Therefore, we studied whether the levels of CD44 and HA associate with disease progression and survival of cutaneous melanoma. A series of 292 clinical stage I cutaneous melanomas was analyzed by immunohistochemistry using an anti-CD44H antibody (clone 2C5). HA was demonstrated histochemically using a biotinylated HA-specific affinity probe (bHABC). The reduced staining levels of CD44 and HA were associated with each other and indicators of progressive disease. Reduced CD44 and HA level, high tumor thickness, high pT category, high Clark's level, bleeding, and male gender predicted short univariate recurrence free survival (RFS) and overall survival (OS). In Cox's multivariate analysis ($N = 251$), the decreased level of CD44, high tumor thickness, and bleeding predicted independently short RFS. High tumor thickness and bleeding were associated with short OS. We conclude that the reduced cell surface CD44 and HA levels associate with poor prognosis in clinical stage I cutaneous melanoma. The notion that the decreased level of CD44 independently predicts short RFS suggests that reduced cell surface CD44 enhances the spreading potential in localized cutaneous melanoma and that quantification of CD44 offers a prognostic tool for its clinical evaluation. (Am J Pathol 2000, 157:957-965)

The incidence and mortality of cutaneous malignant melanoma has increased worldwide during past decades among white populations.¹ Local disease is in most cases curable by surgical excision.² However, the probability of development of disseminated disease is progressively higher as the depth of the primary melanoma increases. In

the worldwide multiinstitutional database of cutaneous melanoma patients, the average 5-year overall survival rate for patients with localized (AJCC stages I and II) melanoma was 79%.³ Those patients who later develop a metastatic disease have a 2-year survival rate less than 5%.⁴ As more effective adjuvant therapies, such as interferon- α , have become available it is even more important to identify the patients at high risk of developing metastatic disease.⁵ More research is also needed to improve our basic understanding on the biology of cutaneous melanoma.

CD44 is a structurally variable and multifunctional cell surface glycoprotein expressed on most cell types.^{6,7} Many functions of CD44 are mediated through interaction with its ligand hyaluronan (HA),⁸ a ubiquitous extracellular polysaccharide.⁹ HA is abundant in soft connective tissues, but also in epithelial and neural tissues.⁸ HA organizes certain proteoglycans in the extracellular matrix, and facilitates cell migration and proliferation during embryogenesis, inflammation, and wound healing.⁸⁻¹¹

The interaction of CD44 with HA contributes to tumor cell proliferation,^{8,12} migration,^{6,8,13-15} invasion,^{6,8,16} and formation of metastatic tumor emboli or peritoneal implants.^{8,17-21} Previous experimental data suggest that CD44 and HA enhance growth and metastatic capacity of melanoma cells,^{17,18,21-26} but the clinical significance of this suggestion has not been confirmed in the relatively small and divergent clinical materials reported so far.²⁷⁻³⁵ We demonstrate here that the reduced stainings of cell surface CD44 and tumor cell associated HA are associated with each other, and with progressive disease and poor prognosis in localized cutaneous melanoma.

Materials and Methods

Patients and Histology

This retrospective study consists of primary melanomas derived from a consecutive series 369 clinical stage I

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Table 1. Patient and Tumor Characteristics of 292 Patients with Stage I Cutaneous Malignant Melanoma

Characteristic	n	%
Total no. of patients	292	100
Age (years)		
Mean (SD)	55.9 (15.2)	
Range	19.0–89.7	
Anatomic site		
Head and neck	47	16
Trunk and perineum	145	50
Upper limbs	44	15
Lower limbs	56	19
Bleeding* of the primary tumor		
Yes	76	26
No	119	41
Unknown	97	33
Sex		
Male	143	49
Female	149	51
Disease recurrence		
Yes	84	29
No	208	71
Cause of death		
Malignant melanoma	52	18
Other	40	14
Alive	200	68
Clark's level		
I	12	4
II	54	18
III	82	28
IV	119	41
V	25	9
Tumor thickness		
≤0.75 mm	64	22
0.76–1.50 mm	69	23
1.51–4.0 mm	99	34
>4.0 mm	41	14
Not possible to analyze	19	6
TNM category		
pT1-T2, N0, M0	131	45
pT3, N0, M0	115	39
pT4, N0, M0	46	16

*Either as an anamnestic information or as a clinical sign observed by the treating physician.

cutaneous melanoma patients with sufficient clinicopathological and long-term follow-up data.³⁶ The patients were diagnosed and treated in the district of Kuopio University Hospital between 1974 and 1989. The histological diagnosis, Breslow thickness and Clark level were re-examined from 1 to 4 original sections of the primary tumor by the same pathologist (VMK), unaware of the clinical data. Of the original 369 cases, 292 had enough archival tumor material available for the present study (Table 1). The most representative block was cut into new 5- μ m-thick consecutive sections for CD44 and HA stainings. Depending on the availability of representative tumor material as compared with the original sections, 282 stainings with an antibody recognizing all forms of CD44 and 277 stainings for HA were eventually evaluable.

CD44 Immunostaining

CD44 was demonstrated by using a mouse IgG anti-CD44H antibody (clone 2C5) (R&D Systems, Abingdon, UK) which recognizes all forms of CD44.³⁷ Adjacent

5- μ m sections from tumors were deparaffinized and rehydrated using xylene and graded alcohols. The sections were microwaved in a 0.01 mol/L citrate buffer (pH 6.0) for 3 $\times$ 5 minutes, incubated in a citrate buffer for 18 minutes, and washed twice for 5 minutes with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by 5% hydrogen peroxide for 5 minutes, followed by a wash twice for 5 minutes with PBS. The sections were incubated with 1% bovine serum albumin (BSA) and PBS for 30 minutes at 37°C. The primary antibody was diluted with 1% BSA to 1:2000 and incubated on the slides overnight at 4°C. After another washing step, the bound antibody was localized using a biotinylated secondary antibody and an avidin-biotin-peroxidase detection kit (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA), and the slides were developed with diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO), counterstained with Mayer's hematoxylin, dehydrated, cleared and mounted with DePex (BDH Poole, UK). In each batch, a melanoma specimen processed without primary antibodies served as a negative control, and one CD44-positive melanoma block served as a source for positive control sections. In addition, the adjacent normal epidermis within the tumor served as positive internal control.

Preparation of bHABC and Staining of HA

The biotinylated complex of hyaluronan binding region and link protein (bHABC) was prepared from bovine articular cartilage as described previously.^{38,39} Briefly, the proteoglycans were extracted from the cartilage with 4 mol/L guanidinium chloride. The extract was dialyzed against distilled water in the presence of high molecular weight hyaluronan. The C-terminus of the proteoglycan molecule was cleaved off with trypsin, and the resultant complex of hyaluronan binding region and link protein (HABC) with HA was purified using hydroxylapatite chromatography and gel filtration. The complex was biotinylated, and the bHABC was separated from HA using gel filtration under dissociative conditions. The purity of the preparation was tested by polyacrylamide gel electrophoresis and Western blotting.

The sections were deparaffinized in xylene, rehydrated with graded alcohols and washed with PB. Endogenous peroxidase was blocked with 1% hydrogen peroxide for 5 minutes and nonspecific binding was blocked with 1% BSA in PB for 30 minutes. The sections were incubated in bHABC (2.5 μ g/ml, diluted in 1% BSA) overnight at 4°C. The slides were washed with PB and treated with avidin-biotin-peroxidase kit (1:200 dilution) for 1 h at room temperature. Following wash with PB the color was developed with 0.05% DAB and 0.03% hydrogen peroxide in PB at room temperature. The slides were counterstained with Mayer's hematoxylin for 2 minutes, washed, dehydrated, and mounted in Depex. The specificity of the staining was controlled by digesting some sections with 100 TRU/ml of *Streptomyces* hyaluronidase (Seikagaku Kogyo Co., Tokyo, Japan) in the presence of protease inhibitors before the staining, or preincubating the bHABC-probe with hyaluronan oligosaccharides to block the specific binding site (Figure 1A).³⁹

Figure 1. HA (A, C, E) and CD44 (B, D) stainings of cutaneous melanoma. **A: Left,** HA-positive tumor. Note HA-positive dermal stroma (asterisk). **Right,** a consecutive section digested by *Streptomyces* hyaluronidase before HA staining (negative control). Scale bar, 100 μm. **B and C:** Consecutive sections, strong CD44 and HA positivity. Asterisk, epidermis. Scale bars, 100 μm. **D and E:** Consecutive sections without cell surface CD44 or cellular HA signal. Tumor stroma (arrows) is HA positive. Scale bars, 50 μm.

Evaluation and Scoring of the CD44 and HA Stainings

Throughout the evaluations, the observers were unaware of the clinical data. In all stainings, scoring was performed with a dual head microscope (field diameter 490 μm) by two observers (JMK and VMK) in the whole tumor area on the slide. In both CD44 and HA stainings, the positivities were assessed qualitatively, ie, the tumor cells were considered as positive when there was a homogeneous and clearly visible signal present, and negative if the signal was absent. According to this principle, the

fraction of positively stained cancer cells in the entire slide was evaluated within each specimen. The frequency distribution and percentiles for the HA- and CD44-positive cancer cell fractions within each specimen were analyzed within the whole series (Figure 2). For both stainings, the tumors were eventually categorized as high (91 to 100% of positively stained cancer cells for CD44 and 71 to 100% of positively stained cancer cells for HA) or reduced (0 to 90% of positively stained cancer cells for CD44 and 0 to 70% of positively stained cancer cells for HA) expressors according to the median percentage of positively stained cancer cells.⁴⁰ The staining intensity of

Figure 2. Distributions of the cutaneous melanomas according to the percentage of CD44 (A) and HA (B) positivity among the malignant cells ($n = 282$ for CD44 and 277 for HA).

hyaluronan in the intratumoral stroma was compared qualitatively to the adjacent normal HA-positive epidermis and categorized as follows: +, weaker than epidermis; ++, as strong as epidermis; and +++, stronger than epidermis.

Statistical Analyses

The SPSS-Win 7.5 program package was used in a PC computer for basic statistical calculations. The χ^2 test was used to compare the frequency distributions of clinicopathological features between the original database ($n = 369$) and the population available for the current study. A Spearman correlation coefficient was used to test the relationships between continuous variables. Non-parametric tests (Kruskal-Wallis) were applied for the comparisons of staining levels between different clinicopathological categories. Frequency tables were analyzed using a 2-sided Fisher's exact test. In univariate survival analyses the EGRET statistical software package was

Table 2. Expression of CD44 and Hyaluronan in Clinical Stage I Cutaneous Malignant Melanoma

Staining	<i>n</i>	%
CD44		
0–90%	153	54
91–100%	129	46
Total	282	100
Cellular HA		
0–70%	140	51
71–100%	137	49
Total	277	100
Stromal HA intensity		
+	43	15
++	218	79
+++	16	6
Total	277	100

used for calculation of Kaplan-Meier estimates of survival rates and the log rank analysis⁴¹ to test the differences between the survival curves. Cox's multivariate survival analysis was done using the Log likelihood ratio significance test in a forward stepwise manner.⁴² The adequacy of the proportional hazards assumption was tested by logminlog plots. Overall survival (OS) analysis included as an event only the deaths due to malignant melanoma. Deaths due to postoperative complications within 30 days were excluded. Recurrence-free survival (RFS) was defined as the time elapsed between the primary treatment and the recurrent melanoma. For all statistical tests, probability values less than 0.05 were regarded as significant.

Results

Patient and Tumor Characteristics

The frequency distributions of the clinicopathological characteristics, as well as the follow-up and survival times between the original database ($N = 369$)³⁶ and the patients with valid material available for CD44 and HA stainings were almost identical (Table 1). The mean follow-up time of all 292 patients was 6.3 ± 3.3 (SD) years (median, 5.4 years; range, 0.5–18 years).

Staining Patterns of CD44 and HA

The CD44 positivity (median value 90%) was confined to tumor cell membranes (Figure 1B). Most tumors showed intense membranous and cytoplasmic HA-positivity (median value 70%), as did the consistently positive epidermis (Figure 1C). The dermal stroma within and outside the tumor was always HA positive (Figure 1A). The histograms of the cellular CD44 and HA levels are shown in Figure 2 and the cellular CD44 and HA levels and stromal HA intensity in different categories in Table 2.

Correlations of CD44 and HA Stainings with Each Other and Markers of Disease Progression

CD44 positivity was strongly associated with cellular HA according to Spearman's statistics ($r = 0.309$; $P <$

Figure 3. A: OS according to cell surface CD44 expression. *N* = 282. **B:** OS according to tumor cell associated HA expression. *N* = 277.

0.00005; *N* = 267) and a 2-sided Fisher's exact test (*P* = 0.005). Decreasing levels of cancer cell-associated CD44 (Figure 1D) and HA (Figure 1E) were both related to increasing Breslow thickness ($\chi^2 = 20.5$, *P* < 0.00005 for CD44 and $\chi^2 = 16.4$, *P* = 0.001 for HA), increasing Clark level ($\chi^2 = 35.1$, *P* < 0.00005 for CD44 and $\chi^2 = 26.5$, *P* < 0.00005 for HA), and increasing pT category ($\chi^2 = 34.8$, *P* < 0.00005 for CD44 and $\chi^2 = 19.1$, *P* < 0.00005 for HA), and this trend was evenly distributed within Breslow's thickness, Clark's level and pT categories. Decreasing CD44 and HA levels also associated with bleeding ($\chi^2 = 7.4$, *P* = 0.024 for CD44 and $\chi^2 = 9.0$, *P* = 0.011 for HA) and with recurrent disease ($\chi^2 = 18.2$, *P* < 0.00005 for CD44 and $\chi^2 = 6.7$, *P* = 0.01 for HA). Stromal HA intensity did not show statistically significant correlation with CD44 level or with any of the clinicopathological variables.

Univariate Survival Analysis

During the follow-up, 84/292 patients (29%) had a recurrence, 52 patients (18%) died of melanoma, and 40 pa-

Figure 4. A: RFS according to cell surface CD44 expression. *N* = 282. **B:** RFS according to tumor cell associated HA expression. *N* = 277.

tients (14%) died of other causes. The 5-year rates for crude, overall, and recurrence-free survivals were 78%, 86%, and 75%, respectively.

Reduced levels of CD44 (0 to 90% positively stained cancer cells) and cancer cell-associated HA (0 to 70% positively stained cancer cells) predicted short OS (*P* = 0.0077 and *P* = 0.0146, Figure 3) and short RFS (*P* = 0.0001 and *P* = 0.0141, Figure 4) (Table 3). Reduced CD44 level predicted poor RFS also within the low-risk (≤ 1.5 mm) subgroup, (*P* = 0.0147, *N* = 127, other data not shown). Stromal HA intensity was not significantly related to OS or RFS. The conventional parameters predicting poor RFS and OS were high tumor thickness and high pT category (*P* < 0.00005 for both RFS and OS, respectively), high Clark's level of invasion (*P* < 0.00005 for RFS and *P* = 0.0001 for OS), bleeding (*P* = 0.0001 for both RFS and OS) and male gender (*P* = 0.0292 for RFS and *P* = 0.0339 for OS) (Table 3). In addition, the reduction of CD44 and HA staining associated fairly well with unfavorable prognosis also by using the 33rd and 66th percentiles of frequency distribution as cut off points (RFS: *P* = 0.0027 for HA and *P* = 0.001 for CD44; OS:

Table 3. Clinical, Histological, Immunohistochemical, and Histochemical Factors Related to Survival in 292 Clinical Stage I Cutaneous Malignant Melanoma

Category (variable)	No. of patients	5-year RFS (95% CI)	<i>P</i> *	5-year OS (95% CI)	<i>P</i> *
Sex			0.0292		0.0339
Male	143	0.69 (0.60–0.76)		0.81 (0.73–0.87)	
Female	149	0.80 (0.73–0.86)		0.90 (0.83–0.94)	
Bleeding			0.0001		0.0001
Yes	76	0.65 (0.52–0.75)		0.80 (0.68–0.88)	
No	119	0.86 (0.78–0.91)		0.93 (0.86–0.97)	
Unknown	97	0.69 (0.59–0.78)		0.81 (0.71–0.88)	
Clark's level			<0.00005		0.0001
I	12	0.88 (0.39–0.98)		0.88 (0.39–0.98)	
II	54	0.98 (0.88–1.0)		0.98 (0.87–1.0)	
III	82	0.79 (0.68–0.87)		0.93 (0.84–0.97)	
IV	119	0.63 (0.54–0.71)		0.79 (0.70–0.86)	
V	25	0.61 (0.38–0.78)		0.63 (0.39–0.80)	
Tumor thickness (mm)			<0.00005		<0.00005
≤0.75	64	0.97 (0.87–0.99)		0.96 (0.86–0.99)	
0.76–1.50	69	0.79 (0.66–0.87)		0.93 (0.82–0.97)	
1.51–4.0	99	0.67 (0.57–0.76)		0.82 (0.72–0.88)	
>4.0	41	0.48 (0.31–0.63)		0.60 (0.40–0.75)	
TNM category			<0.00005		<0.00005
pT1–T2, NO, MO	131	0.91 (0.85–0.95)		0.96 (0.90–0.99)	
pT3, NO, MO	115	0.66 (0.56–0.74)		0.81 (0.73–0.88)	
pT4, NO, MO	46	0.52 (0.36–0.66)		0.63 (0.44–0.77)	
CD44			0.0001		0.0077
≤90%	153	0.67 (0.59–0.74)		0.81 (0.74–0.87)	
>90%	129	0.83 (0.75–0.88)		0.89 (0.82–0.94)	
Cellular HA			0.0141		0.0146
≤70%	140	0.69 (0.61–0.76)		0.82 (0.74–0.87)	
>70%	137	0.78 (0.70–0.84)		0.88 (0.80–0.93)	

RFS, recurrence free survival; OS, overall survival; CI, confidence interval; HA, hyaluronan.

*Log rank analysis.

$P = 0.0632$ for HA and $P = 0.0112$ for CD44, other data not shown).

Multivariate Survival Analysis

Cox's multivariate analysis of 251 patients with a complete set of data included variables that significantly predicted univariate survival, except pT category because it includes Clark's level and tumor thickness. Tumor thickness ($P = 0.0008$) and bleeding ($P = 0.0411$) were associated with short OS. Reduced CD44 level was an independent predictor of short RFS ($P = 0.0308$). Other independent predictors of short RFS were tumor thickness ($P = 0.001$) and bleeding ($P = 0.0346$) (Table 4).

Discussion

The clinical role of CD44 and HA in primary cutaneous melanoma has remained obscure in previous clinical reports due to divergent results and relatively small materials that do not enable multivariate analysis.^{27–35} As far as we know, the current series consisting of patients with localized disease is decisively larger than any previous report on the CD44 level and prognosis of cutaneous melanoma. Further, we have included novel data on the level of HA, the main ligand of CD44. Our findings demonstrate that a reduced level of cell surface CD44 and associated HA within the primary tumor correlates with subsequent disease progression and short survival. In

addition, reduced level of CD44 acts as an independent, unfavorable prognostic factor.

There are no presettled criteria for selecting the cut off points for CD44 and HA positivities in cutaneous melanoma. We chose the median values, because they can be used without introducing a statistical bias,⁴⁰ and because they easily divided the material into two groups of equivalent size. Dichotomizing continuous variables according to a single more or less arbitrary percentile may discard important relationships. However, using the 33rd and 66th percentiles of frequency distribution as HA and CD44 positivity cut off points separated the present series into significantly different prognostic groups almost as effectively as the median (data not shown), thus strengthening our results. The conventional parameters that predicted poor RFS and OS in this series have also been established in most prognostic studies worldwide,^{3,43–45} indicating that the current material was comparable with previous ones. Thus, we believe that the present findings significantly contribute to the current understanding in this area.

The present results on clinical tumors apparently contrast with those of previous experimental studies, in which the growth or metastatic capacity of melanoma cells is inhibited by breaking the CD44-HA interaction with CD44 antibodies,²³ soluble IgG fusion proteins²² or hyaluronan oligomers.²⁶ Considering that cutaneous melanomas evolve in basal epidermis, surrounded by keratinocytes with high levels of CD44^{46,47} and an environment rich in

Table 4. Independent Predictors of Overall Survival and Recurrence-Free Survival in Cox's Analysis

Category	Hazard rate	95% CI	P*
Overall survival			
Tumor thickness (>1.5 mm vs. ≤1.5 mm)	3.41	1.67–7.01	0.0008
Bleeding (yes vs. no)	2.45	1.04–5.78	0.0411
Recurrence-free survival			
Tumor thickness (>1.5 mm vs. ≤1.5 mm)	2.46	1.44–4.21	0.001
CD44 expression (≤90 vs. >90%)	1.74	1.05–2.87	0.0308
Bleeding (yes vs. no)	1.93	1.05–3.56	0.0346

Multivariate analysis included 251 patients with a complete set of data available. CI, confidence interval.

*Log-likelihood ratio significance test.

HA,⁴⁷ results on CD44 and HA metabolism in melanoma cell lines may not be comparable to the clinical behavior of human melanoma. The genetically labile and heterogeneous nature of cells within a single tumor and between tumors may further lead to selection of cell lines that do not represent the expression pattern *in vivo*.^{48,49}

Both high^{50–52} and reduced^{53–55} CD44 expression levels have been associated with cancer growth and adverse prognosis in various malignant tumors, supporting the concept that the growth regulation of cancer cells by CD44 is highly dependent on the cellular background.^{53,56} Supporting our findings, CD44 expression diminished with increasing invasiveness of primary tumors^{30,31,34} and in metastatic melanomas.³³ However, in the only prognostic study concerning CD44 expression in cutaneous melanoma, high CD44 level in primary malignant melanomas (n = 92) associated with progressive disease and poor univariate survival.³²

Although elevated concentrations of HA have been found in several human cancers,^{8,57} there are relatively few studies in which HA has been detected in specific locations of the cancer tissue architecture.^{58–63} Two categories have arisen among the malignancies studied with this kind of assay. In one of them, comprising malignancies of hyaluronan-poor monolayered epithelia such as that of colon,⁵⁸ breast⁵⁹ and ovary,⁶⁰ abnormally increased hyaluronan is strongly associated with unfavorable prognosis. The other category with normally hyaluronan positive squamous epithelia, like that in the esophageal,⁶¹ laryngeal⁶² and lung⁶³ carcinoma, show an opposite trend where loss of cell surface HA is associated with poor differentiation,^{61,63} metastasis and poor survival.⁶² The present study indicates that cutaneous melanoma also belongs to the latter category.

The mechanism by which CD44 (and HA) influence the clinical outcome of cutaneous melanoma patients in the present series remains to be solved. *In vitro*, hypoxia leads to down-regulation of CD44 and subsequent melanoma cell detachment, which, on reoxygenation, is followed by up-regulation of CD44, cell reattachment, and growth.⁶⁴ Melanoma cells may thus modulate their CD44/HA function to enable initial cell detachment (down-regulation)⁶⁴ and later formation of metastatic deposits (up-regulation),^{6,21–23} depending on environmental factors such as oxygen content.⁶⁴ Cell surface CD44 and HA maintain adhesive restraints between the cells in normal epidermis,^{46,47,65,66} and may mediate a tumor suppressive effect also in primary cutaneous melanoma

by the same mechanism. It should be noticed that the metastasis suppression by the standard CD44 isoform can be independent of its ability to bind to hyaluronate and may require also other ligands.⁶⁷ In addition, cell surface CD44, with its ligand HA, may suffer as victims of enhanced proteolysis, and diffuse out of the tissue or be subjects of premature endocytosis and degradation, as discussed earlier.⁶²

Restoration of CD44H expression in colon carcinomas has been observed to reduce tumorigenicity *in vitro* and *in vivo*.⁶⁸ Because of the possibility that the biological role of CD44 may change depending on the cellular environment, the use of CD44 in gene transfection treatments might be complicated in melanoma. Defining how the malignant cells regulate their CD44 expression and its affinity for HA or other ligands could provide alternative ways to interfere with tumor cell spreading. Meanwhile, quantification of CD44 in the primary cutaneous melanoma offers a reproducible and available prognostic tool for clinical practice, for instance to select patients for more aggressive therapy.

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