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Original Article

Immunohistochemistry for Histone H3 Lysine 9 Methyltransferase and Demethylase Proteins in Human Malignant Melanomas

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Running title: EHMT2, SETDB1 and LSD1 in melanomas

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Abstract: Methylation and demethylation of histone H3 lysine 9 (H3K9) play a role in the transcriptional regulation of several cancer-related genes, and are closely associated with malignant tumor behavior. A novel study has recently demonstrated that SETDB1, a member of the H3K9 methyltransferases, accelerates tumor formation significantly in a zebrafish-melanoma model. However, the expression of H3K9 methyltransferases including SETDB1 and demethylases has not been systematically examined in samples of human melanoma. Here we used immunohistochemistry to examine the expression of the H3K9 methyltransferases, EHMT2 and SETDB1, and a H3K9 demethylase, LSD1, in 67 patients with malignant melanoma. Overexpression of EHMT2, SETDB1 and LSD1 was observed in 14 (21%), 38 (57%) and 53 (79%) of the 67 patients, respectively. A significant relationship was observed between overexpression of EHMT2 or SETDB1 and aggressive tumor behavior such as lymph node metastasis and/or distant metastasis ($P<0.05$), whereas no significant relationship was evident for LSD1 immunoreactivity. Univariate log-rank tests demonstrated that patients with malignant melanoma overexpressing EHMT2 had a poorer outcome ($P<0.001$), whereas overexpression of SETDB1 or LSD1 had no prognostic impact. These results suggest that overexpression of EHMT2 might be a prognostic marker in patients with malignant melanoma.

Key Words: EHMT2, H3K9, LSD1, malignant melanoma, SETDB1

INTRODUCTION

Although relatively rare, malignant melanoma is a leading cause of death among skin cancers.[1] Although the 5-year survival rate of patients with early-stage melanoma (stage 0/I) is higher than 90%, that of patients with distant metastasis (stage IV) is less than 20%.[2] Moreover, the incidence of melanoma has recently been increasing at a greater rate than that of any other skin cancers.[2] In general, early diagnosis of malignant tumors greatly improves disease outcome. For the management of patients with malignant melanoma, the development of effective biomarkers for determining the most appropriate therapeutic modality has been anticipated.

Histone modification is a primary epigenetic mechanism for regulation of gene expression.[3] In particular, histone lysine methylation is important for maintenance of chromatin structure, and plays a pivotal role in the regulation of gene expression.[3, 4] Histone lysine methylation occurs predominantly within histones H3 and H4, and is associated with a distinct transcriptional outcome.[5] Methylation of lysine 9 of histone H3 (H3K9) is typically associated with heterochromatin and a transcriptionally repressed state.[6, 7]

A notion that aberrant histone modifications are the possible cause of aberrant gene expression in human cancers has recently been attracting attention. Several studies have suggested that changes in global levels of individual histone modifications are independently predictive of the clinical outcome of certain types of cancer.[8-10] Among them, the methylation status of H3K9 is suspected to have a strong association with several forms of tumorigenesis.[11-15]

Methylation of H3K9 is catalyzed by members of the SET domain-containing family of histone methyltransferases (HMTs).[16] So far, five mammalian enzymes involved in the methylation of H3K9 have been identified, including G9a (gene symbol of homo sapiens, EHMT2) and SETDB1.[6,16] Gene knockout of G9a in the mouse results in embryonic lethality, suggesting that its function is critical for mammalian development.[17] Intriguingly, both EHMT2 and SETDB1 are highly expressed in some cancers including malignant melanoma.[11,13] Recently, through a global genetic analysis of 101 human melanoma cell lines, Ceol *et al.*[11] demonstrated that the copy number of amplification on chromosome 1q21 is closely associated with malignant melanoma. To determine which amplified genes within this region contribute to melanoma, they examined gene overexpression in a zebrafish-melanoma model and found that SETDB1 was an amplification target. SETDB1 overexpression was associated with increased melanoma aggressiveness and invasiveness, compared with overexpression of a control protein, suggesting that downstream genes regulated by SETDB1-HMT activity might be involved in tumor behavior in zebrafish, as well as in human melanomas.

The methylation status of H3K9 may depend on a delicate balance between the activities of HMTs and histone demethylases (HDMs). Lysine specific demethylase 1 (LSD1), the first of several enzymes discovered to be involved in the demethylation of H3K9, removes the methyl groups from mono- and dimethylated H3K4 and H3K9.[18] A recent study has indicated that LSD1 might promote cell proliferation, suggesting a possible link between its overexpression and tumorigenesis.[14,15] No previously reported study has determined the status of LSD1 expression in human melanomas.

In the present study, we retrieved specimens of malignant melanoma that had been archived at our institution, and evaluated the expression of HMT and HDM

proteins responsible for H3K9 methylation using immunohistochemistry (IHC). We also examined the relationship between the immunohistochemical results and the clinicopathological data.

MATERIALS AND METHODS

Patients

The subjects for whom specimens were available comprised 67 patients (43 female and 24 male) with malignant melanoma who had been treated at the Department of Dermatology, Iwate Medical University School of Medicine, between 1997 and 2009. Permission for the study was obtained from the Institutional Review Board (School of Medicine, Iwate Medical University, Morioka, Japan) and written consent had been obtained from all patients before surgery.

The mean age of the patients was 67 years (range, 34 to 88 years). The clinical data for the patients are summarized in Table 1. Forty-three of the lesions were acral, 9 were located on the head and neck, 8 on the trunk, and 7 on the extremities. The subtypes of the primary tumors were categorized according to Clark's classification: ALM, acral lentiginous melanoma; NM, nodular melanoma; LMN, lentigo malignant melanoma; and SSM, superficial spreading melanoma. Forty-two (63%) of the patients had ALM tumors, 14 (20%) had NM, 6 (9%) had LMM, and 5 (8%) had SSM. Tumor thickness (TT) was measured according to the criteria of Breslow. The TT was 2.0 mm or less in 28 patients (42%), and more than 2.0 mm in 39 (58%). We defined the extent of lymph node metastasis as follows: N0, no metastasis to lymph nodes; N1, metastasis to one node; N2, metastasis to 2 to 3 nodes; N3, metastasis to $\geq$4 nodes. Twenty-five (37%) of the 67 patients had metastatic disease. We defined the extent of distant

metastasis as follows: M0, no distant metastasis; M1, metastasis to distant organs. Eight (12%) of the 67 patients had distant metastasis. Clinical stage was defined according to the melanoma TNM classification.[19]

Immunohistochemistry

Surgical specimens were fixed in 10% buffered formalin solution and embedded in paraffin wax, and two or more blocks were prepared for immunohistochemistry. Sections (4 μm thick) were cut, and stained with hematoxylin and eosin. Serial sections were stained using the avidin-biotin system and antigen retrieval methods on a Ventana automated immunostainer employing the Ventana immunohistochemistry detection system (Ventana Medical Systems, Tucson, AZ, USA), in accordance with the manufacturer's manual. A mouse monoclonal anti-EHMT2 antibody (diluted 1:50; A8620A, Perseus Proteomics, Tokyo, Japan), a rabbit monoclonal anti-SETDB1 antibody (diluted 1:60; HPA018142, Sigma Aldrich, Irvine, UK), and a rabbit monoclonal anti-LSD1 antibody (diluted 1:60; #2184S, Cell Signaling Technology, Beverly, MA, USA) were used. The extent of immunohistochemical reactivity was estimated by light microscopy and graded according to the number of immunoreactive cells (proportion score) and staining intensity (intensity score), as described previously.[20] In brief, proportion scores were graded as follows: 0, no immunoreactive cells evident; 1, immunoreactive cells accounting for less than 10% of the total; 2, less than two-thirds; and 3, more than two-thirds. Intensity scores were graded as follows: 0, no immunoreactivity; 1, representative staining intensity weak; 2, intermediate; and 3, strong. The final evaluation of immunoreactivity was assigned as follows: negative/faint,

if the sum of the proportion score and intensity score ranged from 0 to 2; medium, if the sum ranged from 3 to 4; and strong, if the sum ranged from 5 to 6.

Statistical analysis

Correlations between immunoreactivity for EHMT2, SETDB1 and LSD1, and clinicopathological data were analyzed by Fisher's exact test or Kruskal-Wallis test. To correlate the results for EHMT2, SETDB1 and LSD1 status with the overall survival of patients, Kaplan-Meier survival analysis and log-rank test were performed. Multivariate analysis of the clinicopathological factors associated with prognosis was conducted using the Cox proportional hazards model. The level of significance was considered to be $P<0.05$.

RESULTS

IHC for HMT2, SETDB1 and LSD1

We immunohistochemically examined the expression of the EHMT2, SETDB1 and LSD1 proteins in 67 patients with malignant melanoma. Immunoreactivity for all three proteins was evident in the nucleus (Fig. 1). As described previously,[20] two pathologists evaluated each case independently, and scores were determined on the basis of quantitative comparative analysis (negative/faint, medium and strong; see Materials and Methods section). Staining intensity and proportion scores varied among the tumors, and individual intratumoral heterogeneity was often observed.

We finally assigned melanomas graded negative/faint or medium as negative, and those graded strong as positive. Tumors positive for EHMT2, SETDB1 and LSD1 were observed in 14 (21%; negative/faint: medium: strong=30: 23: 14), 38 (57%;

negative/faint: medium: strong=16: 13: 38) and 53 (79%; negative/faint: medium: strong=6: 8: 53) of the 67 patients, respectively. Expression of EHMT2 was inversely correlated with LSD1 expression (P<0.05), but that of SETDB1 was significantly correlated with LSD1 expression (P<0.01) (Table 2).

Relationship between EHMT2, SETDB1 or LSD1 status and clinicopathological variables

Table 3 summarizes the relationships between EHMT2, SETDB1 and LSD1 immunoreactivity in the primary tumor and clinicopathological variables in all cases. HEMT2 was significantly correlated with the presence of both lymph node and distant metastases (P<0.001). Overexpression of SETDB1 was significantly increased in patients with lymph node metastasis (P=0.013), but showed no significant relationship with distant metastasis (Table 3). Immunoreactivity for LSD1 showed no relationship with any of the clinicopathological variables in the 67 patients (Table 3).

We then carried out univariate analyses of factors (including immunohistochemistry for EHMT2, SETDB1 and LSD1 proteins, and clinicopathological variables) affecting the overall survival of patients. After a median follow-up of 54 months (range, 5 to 147 months), 25 patients (37%) had died due to disease relapse. Kaplan-Meier curves showed a trend towards a worse outcome in patients with EHMT2-immunoreactive tumors compared with those whose tumors were EHMT2-negative, and the difference was statistically significant (P<0.001, log-rank test, Fig. 2, Table 4). Immunoreactivity for neither SETDB1 nor LSD1 was significantly related to survival. Besides EHMT2 immunoreactivity, survival rates were significantly

correlated with TT (P<0.001), lymph node status (P<0.001) and distant metastasis (P<0.001) (Table 4).

We then performed multivariate analysis using TT, presence of lymph node metastasis, distant metastasis, and EHMT2-immunopositivity. TT and distant metastasis were each independently associated with a poor outcome, but EHMT2-immunopositivity was not (Table 5).

DISCUSSION

Unexpectedly, our immunohistochemical study of HMT and HDM proteins revealed that overexpression of only EHMT2, and not SETDB1, was of prognostic significance in patients with malignant melanoma. Ceol *et al.*[11] examined not only a zebrafish-melanoma model but also human tissue samples. They observed high levels of SETDB1 expression in 5% of normal melanocytes (n=20), 15% of benign nevi (n=20), and 70% of malignant melanomas (n=91), and speculated that human nevi harboring SETDB1 overexpression might have a higher likelihood of oncogenic progression than nevi showing a basal level of expression. They also demonstrated that SETDB1 overexpression downregulated the target genes of methyltransferase, including transcriptional regulators and homeobox genes. Overexpression of SETDB1 seems to represent an important early stage of melanoma development, rather than melanoma cell aggressiveness. In fact, few reports have indicated that SETDB1 is able to regulate cancer-associated genes involved in tumor cell invasion and metastasis. Moreover, our immunohistochemical study demonstrated an inverse correlation between the expression of EHMT2 and that of LSD1, whereas there was a positive correlation between the expression of SETDB1 and that of LSD1. If the methylation status of

H3K9 is delicately regulated by both HMT and HDM, co-overexpression of LSD1 might disturb the methyltransferase activity of SETDB1. The inverse correlation between EHMT2 and LSD1 expression in melanoma cells may serve to repress the activity of suppressors of invasion and metastasis.

It has already been shown that EHMT2 is able to repress molecules related to tumor cell invasion and metastasis. In breast cancer, Snail directly interacts with EHMT2 and sequentially recruits DNA methyltransferases to the E-cadherin promoter.[21] Knockdown of EHMT2 restores E-cadherin expression by suppressing H3K9 methylation and blocking DNA methylation. Moreover, EHMT2 knockdown in highly invasive lung cancer cells has been shown to inhibit cell migration and invasion *in vitro* and metastasis *in vivo*.[12] Also in lung cancer, EHMT2 knockdown reduces the levels of H3K9 dimethylation and decreases recruitment of the transcriptional cofactors HP1, DNMT1, and HDAC1 to the cell adhesion molecule, Ep-CAM.[12] Furthermore, in a study of 119 patients with non-small cell lung cancer, an inverse correlation was demonstrated between EHMT2 and Ep-CAM expression, and overexpression of EHMT2 was significantly correlated with low rates of overall and disease-free survival[12]. Thus, overexpressed EHMT2 might directly regulate metastasis-associated genes, and be involved in aggressive tumor behavior.

The present study did not simultaneously investigate Snail, E-cadherin or Ep-CAM, whereas numerous reports have indicated that disruption of these molecules contributes to acquisition of metastatic potency in melanoma cells.[22, 23] Macgregor *et al.*[24] and *Ceol et al.*[11] have suggested that amplification of 1q21 targeting SETDB1 is a relatively early event in melanoma development. However, our present immunohistochemical study demonstrated that gain of another type of H3K9 HMT,

EHMT2, was significantly associated with late events such as invasion and metastasis. Both HMTs might play an important role in malignant melanoma tumorigensis, and overexpression of EHMT2 appears to be a prognostic biomarker that would be potentially useful for the management of melanoma patients.

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Figure legends

FIGURE 1. Immunohistochemistry for EHMT2, SETDB1 and LSD1 protein in malignant melanomas. Staining intensity was graded into 3 categories: negative, medium and strong. Representative pictures are shown. Bar = 100 μm.

FIGURE 2. Survival curves of 67 melanoma patients according to EHMT2 immunoreactivity. Kaplan-Meier estimates of the survival of 67 patients with malignant melanomas (EHMT2-positive, n=14; -negative n=53). Disease outcome shows a tendency to be worse for patients whose tumors overexpressed EHMT2 in comparison with those who were EHMT2-negative (P<0.001, log-rank test).

TABLE 1. Characteristics of the 67 Patients with Malignant Melanoma

Factor		**No. of Patients (%)**	
Gender	Female	43	(64)
	Male	24	(36)
Age (years)	Mean (range)	67	(34 – 88)
	<65	22	(33)
	≥65	45	(67)
Ulcer	Absent	47	(70)
	Present	20	(30)
Tumor thickness(mm)	<2.0	28	(42)
	≥2.0	39	(58)
Lymph node status	N0	42	(63)
	N1	10	(15)
	N2	4	(6)
	N3	11	(16)
Clinical stage	M0	59	(88)
	M1	8	(12)
Clinical stage	I	21	(31)
	II	21	(31)
	III	17	(26)
	IV	8	(12)
Histological type	ALM	42	(63)
	NM	14	(21)
	LMM	6	(9)
	SSM	5	(7)
Outcome	Alive	42	(63)
	Dead	25	(37)

N0, no metastasis to lymph nodes; N1, metastasis to one node; N2, metastasis to 2 to 3 nodes; N3, metastasis to ≥4 nodes; M0, no distant metastasis; M1, metastasis to distant organs; ALM, acral lentiginous melanoma; NM, nodular melanoma; LMM, lentigo malignant melanoma; SSM, superficial spreading melanoma.

Clinical stage was defined according to the melanoma TNM classification (Balch *et al.*, 2009).

TABLE 2. Immunohistochemical Relationships among EHMT2, SETDB1 and LSD1

		SETDB1 (%)			LSD1 (%)		
		Positive	Negative	*P*	Positive	Negative	*P*
EHMT2	Positive	11 (79)	3 (21)	0.06	12 (86)	2 (14)	0.05
	Negative	27 (51)	26 (49)		41 (77)	12 (23)	
SETDB1	Positive				35 (92)	3 (8)	0.002
	Negative				18 (62)	11 (38)	

P, *P-value* by Fisher's exact test.

TABLE 3. Relationships of EHMT2, SETDB1 and LSD1 Statuses with Clinicopathological Factors in 67 Patients with Malignant Melanoma

Factor		EHMT2 (%)					SETDB1 (%)					LSD1 (%)				
(No. of patients)		Positive		Negative		*P*	Positive		Negative		*P*	Positive		Negative		*P*
Gender																
Female	(43)	10	(23)	33	(77)		25	(58)	18	(42)		33	(77)	10	(23)	
						0.52					0.66					0.52
Male	(24)	4	(17)	20	(83)		13	(54)	11	(46)		20	(83)	4	(17)	
Age (years)																
<65	(22)	8	(36)	14	(64)		11	(50)	11	(50)		17	(77)	5	(23)	
						0.06					0.46					0.79
≥65	(45)	6	(13)	39	(87)		27	(60)	18	(40)		36	(80)	9	(20)	
Ulcer																
Absent	(47)	8	(17)	39	(83)		29	(62)	18	(38)		36	(77)	11	(23)	
						0.21					0.21					0.49
present	(20)	6	(30)	14	(70)		9	(45)	11	(55)		17	(85)	3	(15)	
Tumor thickness(mm)																
<2.0	(28)	3	(11)	25	(89)		16	(57)	12	(43)		23	(82)	5	(18)	
						0.08					0.95					0.6
≥2.0	(39)	11	(28)	28	(72)		22	(56)	17	(44)		30	(77)	9	(23)	
Lymph node status																
N0	(42)	2	(5)	40	(95)		19	(45)	23	(55)		32	(76)	10	(24)	
						<0.001					0.013					0.44
N1,N2,N3	(25)	12	(48)	13	(52)		19	(76)	6	(24)		21	(84)	4	(16)	
Clinical stage																
M0	(59)	8	(14)	51	(86)		32	(54)	27	(46)		46	(78)	13	(22)	
						<0.001					0.29					0.53
M1	(8)	6	(75)	2	(25)		6	(75)	2	(25)		7	(88)	1	(12)	
Outcome																
Alive	(42)	5	(12)	37	(88)		24	(57)	18	(43)		33	(79)	9	(21)	
						0.07					0.92					0.88
Dead	(25)	9	(36)	16	(64)		14	(56)	11	(44)		20	(80)	5	(20)	

N0, no metastasis to lymph nodes; N1, metastasis to one node; N2, metastasis to 2 to 3 nodes; N3, metastasis to ≥4; nodes M0, no distant metastasis; M1, metastasis to distant organs.

P, *P-value* by Fisher's exact test.

Clinical stage was defined according to the melanoma TNM classification (Balch *et al*., 2009).

TABLE 4. Univariate Analysis of Factors Affecting Overall Survival

Factor	No. of Patients	(%)	5-Year Survival Rate (%)	*P*
Gender				
Female	43	(64)	77.6	0.028
Male	24	(36)	47.6	
Age				
<65	22	(33)	54.5	0.26
≥65	45	(67)	73.4	
Ulcer				
Absent	47	(70)	70.8	0.54
Present	20	(30)	58.3	
Tumor thickness (mm)				
<2.0	28	(42)	96.4	< 0.001
≥2.0	39	(58)	46.3	
Lymph node status				
N0	42	(63)	83.0	<0.001
N1, N2, N3	25	(37)	40.0	
Clinical stage				
M0	59	(88)	75.3	<0.001
M1	8	(12)	12.5	
EHMT2				
Negative	53	(79)	74.0	<0.001
Positive	14	(21)	42.8	
SETDB1				
Negative	29	(43)	69.6	0.67
Positive	38	(57)	66.3	
LSD1				
Negative	14	(21)	61.3	0.73
Positive	53	(79)	69.7	

P, *P*-value by log-rank test.

Clinical stage was defined according to the melanoma TNM classification (Balch *et al.*, 2009).

TABLE 5. Multivariate Analysis of Clinicopathological Factors Associated with Outcome

Factor	**Hazard Ratio**	**95% CI**	***P***
Tumor thickness	5.47	1.52-19.6	<0.01
Lymph node status	2.11	0.72-6.12	0.17
Clinical stage	3.86	1.28-11.5	<0.01
Immunoreactivity for EHMT2	1.75	0.6-4.61	0.73

P, *P*-value by Cox regression analysis.

Clinical stage was defined according to the melanoma TNM classification (Balch *et al.*, 2009).

Figure 1
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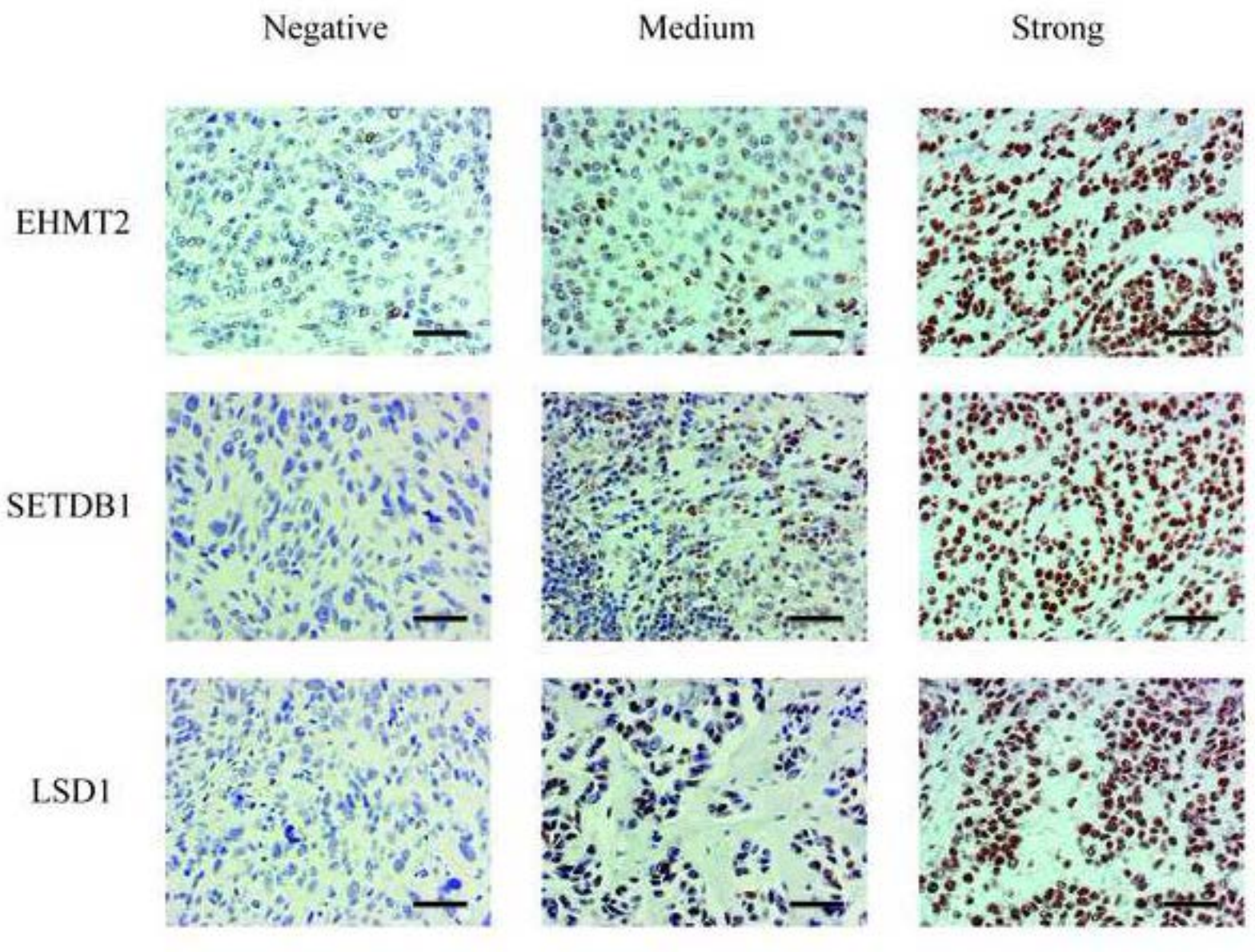

FIGURE 1.

Figure 2
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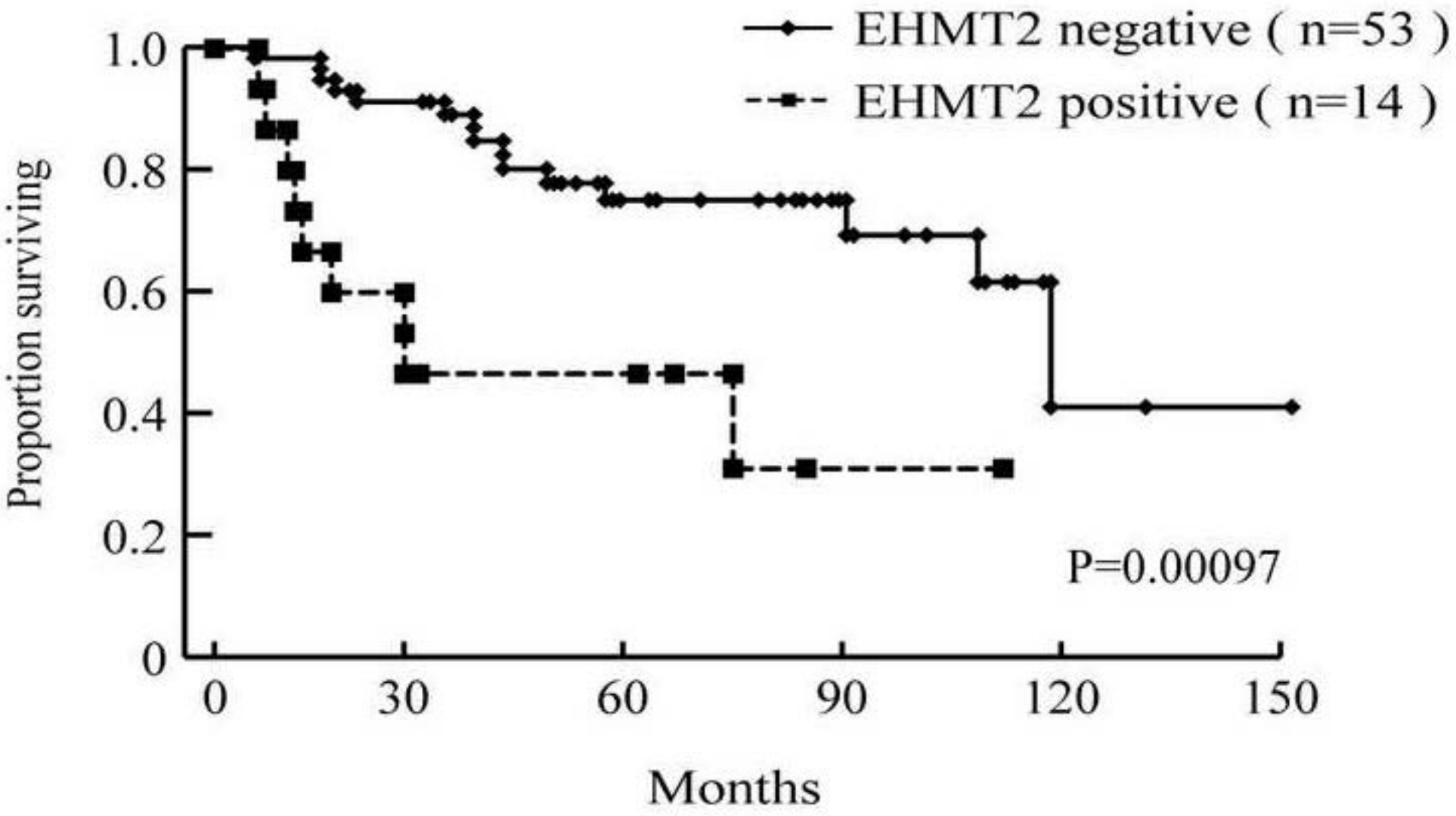

FIGURE 2.