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AIM1 and LINE-1 Epigenetic Aberrations In Tumor and Serum Relate to Melanoma Progression and Disease Outcome

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Abstract

Aberrations in the methylation status of non-coding genomic repeat DNA sequences and specific gene promoter region are important epigenetic events in melanoma progression. Promoter methylation status in LINE-1 and *Absent in melanoma-1(AIM1;*6q21) associated with melanoma progression and disease outcome was assessed. LINE-1 and *AIM1* methylation status was assessed in paraffin-embedded archival tissues(PEAT)(n=133) and melanoma patients' serum(n=56). LINE-1 U-Index(hypomethylation) and *AIM1* were analyzed in microdissected melanoma PEAT sections. The LINE-1 U-Index of melanoma(n=100) was significantly higher than that of normal skin(n=14) and nevi(n=12)(*P*=0.0004). LINE-1 U-Index level was elevated with increasing AJCC stage(*P*<0.0001). *AIM1* promoter hypermethylation was found in higher frequency(*P*=0.005) in metastatic melanoma(65%) than in primary melanomas(38%). When analyzed, high LINE-1 U-Index and/or *AIM1* methylation in melanomas were associated with disease-free survival(DFS) and overall survival(OS) in Stage I/II patients (*P*=0.017, 0.027; respectively). In multivariate analysis, melanoma *AIM1* methylation status was a significant prognostic factor of OS(*P*=0.032). Furthermore, serum unmethylated LINE-1 was at higher levels in both stage III(n=20) and stage IV(n=36) patients compared to healthy donors(n=14)(*P*=0.022). Circulating methylated *AIM1* was detected in patients' serum and was predictive of OS in Stage IV patients (*P*=0.009). LINE-1 hypomethylation and *AIM1* hypermethylation have prognostic utility in both melanoma patients' tumors and serum.

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Keywords

LINE-1; AIM1; Melanoma; Methylation; Prognosis

INTRODUCTION

Epigenetic studies have shown that methylation status of CpG islands in the promoter region of tumor-related genes as well as non-coding repeat genomic sequences are associated with progression of cancers(Tanemura *et al.*, 2009; van Hoesel *et al.*, 2011). The former has been linked to transcriptional silencing(Jones and Baylin, 2007), but the latter is poorly understood in cancer progression(de Maat *et al.*, 2010a; de Maat *et al.*, 2010b; Eden *et al.*, 2003; Gaudet *et al.*, 2003; Sunami *et al.*, 2011).

Long interspersed nucleotide elements-1(LINE-1) represent a non-coding repeat sequence in humans. They are 6-8 kb long, GC-poor sequences with two open reading frames(ORF1 and ORF2), encode a reverse-transcriptase, endonuclease, and make up about 17% of the human genome(Lander *et al.*, 2001; Ostertag and Kazazian, 2001; Ovchinnikov *et al.*, 2002). The genome contains about 5000 full-length 6.0 kb LINE-1 elements, 60-100 of which are still capable of retrotransposition(Brouha *et al.*, 2003; Minakami *et al.*, 1992; Scott *et al.*, 1987). LINE-1 is hypermethylated in normal cells and hypomethylated in several malignancies(Estecio *et al.*, 2007; Sunami *et al.*, 2009; Yang *et al.*, 2004). LINE-1 hypomethylation level in tumor has been correlated to tumor progression(Chalitchagorn *et al.*, 2004; Cho *et al.*, 2007; Daskalos *et al.*, 2009; Lee *et al.*, 2009; Ogino *et al.*, 2008a; Pattamadilok *et al.*, 2008; Tellez *et al.*, 2009). The association of LINE-1 hypomethylation status has been assessed in human cutaneous melanoma cell lines(Tellez *et al.*, 2009), and the activation of LINE-1 transcript has also been observed in metastatic melanomas compared to primary melanomas(Haqq *et al.*, 2005). In this study, we assessed melanoma tumor cells microdissected from PEAT of different stages of primary and metastatic melanoma to investigate whether LINE-1 methylation status during tumor progression.

Methylation profiling of melanoma has demonstrated inactivation of tumor-related genes by hypermethylation of CpG islands in the promoter region(Furuta *et al.*, 2004; Hoon *et al.*, 2004; Mirmohammadsadegh *et al.*, 2006; Patino and Susa, 2008; Tanemura *et al.*, 2009; Worm *et al.*, 2004). These epigenetic changes are significant factors in melanoma progression as we have recently shown(Tanemura *et al.*, 2009). A multidomain non-lens member of βγ-crystallin superfamily, with six structural domains formed from 12 βγ-crystallin motifs, *Absent in melanoma-1*(*AIM1*), not to be confused with SLC45A2(melanoma antigen *Aim1*) on chromosome 5p13.2. was originally identified as a candidate tumor suppressor gene(chromosome 6q21)(Aravind *et al.*, 2008; Ray *et al.*, 1997). However, few studies have been carried out on *AIM1* in melanoma since then.

Higher prevalence of LINE-1 and tumor suppressor genes silenced by epigenetic aberration have been found on chromosome 6(Mungall *et al.*, 2003) where *AIM1* is also located. Previous studies have demonstrated correlation between LINE-1 hypomethylation and CpG island hypermethylation of tumor suppressor genes(Estecio *et al.*, 2010; Poage *et al.*, 2011).

As the genetic locus of *AIM1* is proximal to the binding site of several known transcription factors, LINE-1 and *AIM1* methylation status may be associated.

Studies have shown epigenetic tumor tissue biomarkers can have utility as biomarkers for cell-free circulating DNA(cf-cDNA)(Schwarzenbach *et al.*, 2011). Specific cf-cDNA have demonstrated prognostic utility in melanoma patients(Mori *et al.*, 2005). In this study, we assessed the level of circulating unmethylated LINE-1 and methylated *AIM1* DNA in serum of patients with melanoma metastasis. We demonstrated prognostic utility of unmethylated LINE-1 and *AIM1* methylation in both melanoma tissue and serum. These studies demonstrate that epigenetic aberrations of both coding and non-coding regions can have potential utility as biomarkers in tumors and serum of melanoma patients.

RESULTS

LINE-1 Hypomethylation in Melanomas

Using Absolute Quantitative Assessment of Methylated Alleles (AQAMA), microdissected melanoma cells were assessed for their LINE-1 U-Index which is calculated by dividing the copy number of unmethylated templates by the total of both unmethylated and methylated templates. The average LINE-1 U-Index of five melanoma cell lines was 0.79, whereas for normal PBL DNA LINE-1 U-Index ranged from <0.1 to 0.2 similar to normal skin and nevi. The LINE-1 U-Index of melanomas, including both primary and metastatic tissues(n=100, mean±SD; 0.32±0.26), was significantly higher than that of normal skin(n=14) and nevi(n=12)(mean±SD; 0.14±0.07)(T-test, P=0.0004)(Figure 1A). The LINE-1 U-Index level of metastatic melanomas(AJCC stage IIIm and IV: n=56, mean±SD; 0.40±0.30) was significantly higher than that of normal skin/nevi or primary melanomas(AJCC stage Ip, IIp, and IIIp: n=44, mean±SD; 0.22±0.14)(Tukey's HSD P<0.0001 and P=0.0001, respectively) (Figure 1B). Furthermore, LINE-1 U-Index in melanomas showed a significant elevation with increasing stage of disease(mean±SD; I: 0.20±0.05, II: 0.24±0.11, IIIp:0.23±0.19, IIIm: 0.27±0.23, and IV:0.50±0.31, respectively)(ANOVA P<0.0001)(Figure 1C). Most notably, LINE-1 U-Index level of stage IV melanomas(mean±SD; 0.50±0.31) was significantly higher than other stages(Newman-Keuls test, P<0.05). LINE-1 U-Index was not correlated with patient age or other histopathological factors including tumor thickness, ulceration, or mitotic index.

LINE-1 U-Index was further compared in 13 autologous pairs of primary(2 stage II and 11 stage IIIp) and metastatic(9 stage IIIm, 4 stage IVm) tumors from13 patients. In 11 pairs, LINE-1 U-Index values were higher in metastatic than respective paired primary tumors(Figure 1D). Signed rank test for the difference of LINE-1 U-Index between primary and metastasis was significant(mean±SD; primary: 0.08±0.11, metastasis: 0.20±0.22, P=0.024). These results suggested hypomethylation of LINE-1 was a progressive factor in melanomas.

AIM1 Promoter Methylation Status in Melanoma Cell Lines

Unlike LINE-1, *AIM1* is a protein encoding gene, therefore the evaluation of promoter methylation should cover regions or CpG sites relevant to the downstream expression level.

Four melanoma lines(M-15, M-24, M-101, and LF-0023) and normal PBL were subjected to MALDITOF MS quantitative analysis of the entire *AIM1* promoter region to profile CpG site methylation status. CpG sites of M-15 and M-101 were highly methylated across the whole promoter region compared to M-24, LF-0023, and normal PBL**(**Figure 2A**)**. The transcription of *AIM1* was analyzed by RT-PCR in the same melanoma lines**(**Figure 2B**)**. *AIM1* mRNA was not detected in the two highly *AIM1*methylated melanoma lines(M-15, M-101). The two *AIM1* unmethylated melanoma lines(M-24, LF-0023) and normal PBL showed high expression of *AIM1*. Specific CpG sites most related to mRNA transcription activity**(**Figure 2C**)** were identified for methylation specific PCR(MSP) study in PEAT and cf-cDNA. The average percentage of methylation in the selected region(CpG8-11) in M-15(77%) and M-101(81%) is higher than those of two *AIM1* mRNA positive cell lines, M-24 and LF-0023, and donor PBL (<12%).

AIM1 expression was significantly increased in the two methylated lines after treatment with 5Aza-dC(Supplementary Figure 1**)**. This demonstrated that demethylation of *AIM1* promoter region can re-activate *AIM1* mRNA expression, suggesting promoter region methylation regulates *AIM1* expression.

AIM1 Methylation Status and mRNA Expression in PEAT

To evaluate the *AIM1* methylation status in melanoma tissues, we designed MSP primers to the specific CpG sites(CpG8-11) that were related to transcription levels of *AIM1*. The *AIM1* promoter was found hypermethylated in 57 of 112(51%) melanoma tissues, as compared with none of histopathologically normal skin(n=14) and nevus tissues(n=12)(*P*<0.0001). *AIM1* promoter hypermethylation was identified in 35 of 54(65%) metastatic melanomas, as compared to 22 of 58(38%) primary melanomas(*P*=0.005); frequency of hypermethylation progressively increased with stage(Table 1**)**.

AIM1 mRNA expression was assessed in four hypomethylated stage I melanoma primary tumors and in five hypermethylated stage IV metastatic tumors. *AIM1* mRNA was found in all four hypomethylated melanomas but showed limited expression in the five hypermethylated melanomas(Supplementary Figure 2**)**.

Survival Analyses of LINE-1 and *AIM1* Methylation Status

We next evaluated the PEAT *AIM1* and LINE-1 methylation as biomarkers for prognostic significance. In early stage primary melanoma patients(n=23), stepwise multivariate Cox regression(variables age, gender, ulceration, Breslow, primary site) demonstrated that *AIM1* hypermethylation was a significant prognostic predictor of OS in Stage I/II patients when age is included in the model(*P*=0.032, HR:22.62, 95%CI:1.30-392.24). LINE-1, however, it did not reach significance as an individual biomarker. Univariate analysis of AIM1 or LINE1 alone showed no significance in prediction of OS or DFS. The relatively small sample size of the survival analysis cohort may have contributed to the differences in our survival cohort between univariate and multivariate survival analysis results. In combination, patients were categorized into two groups: patients who had presence of either hypermethylated *AIM1* or hypomethylated LINE-1, and patients who did not have either event. The presence of either hypermethylated *AIM1* or hypomethylated LINE-1 correlated

to significantly poorer patient OS and DFS in univariate analysis of Stage I/II melanoma (*P*=0.027 and 0.017, respectively)(Figure 3A&B). In multivariate analysis, the combination was a significant prognostic predictor of both OS and DFS in Stage I/II patients(OS: *P*=0.028, HR:6.46, 95%CI:1.22-34.15; DFS:*P*=0.046, HR:8.65, 95%CI:1.04-72.06). Table 2 lists clinicopathological factors for patients included in survival analysis along with biomarker prediction of OS results from Cox regression model.

Serum cf-cDNA

To determine whether LINE-1 and/or *AIM1* could be used for prognostic assessment of serum(Mori *et al.*, 2005), we examined levels of circulating unmethylated LINE-1 and methylated *AIM1* in stage III and IV melanoma patients. Patient serum samples had significantly higher unmethylated LINE-1 than healthy donor serum(T-test; *P*=0.022) (Supplementary Figure 3), and none of the normal healthy donor serum had detectable unmethylated LINE-1 before 25 PCR cycles. The serum MSP assay detected *AIM1* circulating methylated DNA in 15%(3 of 20) of stage III patients and 22%(8 of 36) of stage IV patients. Unmethylated LINE-1 status in patients' serum was limited in prognostic utility in this set of patients. However, serum methylated *AIM1* DNA was correlated to OS in Stage IV patients(*P*=0.0085, Log-Rank; Figure 4) and not associated with M stage. Patients found to have either serum unmethylated LINE-1 or methylated *AIM1* DNA have worse prognosis compared to patients with neither(*P*=0.0009, Log-Rank)(Figure 4).

DISCUSSION

The progressive demethylation of LINE-1 in malignancies has been correlated to increased aberrant genomic and epigenomic events(Sunami *et al.*, 2011). We demonstrated that LINE-1 became progressively hypomethylated during melanoma progression. LINE-1 hypomethylation level was higher in stage IV melanomas compared to other stages. These results suggested that LINE-1 hypomethylation may be a significant factor in melanoma progression. Sigalotti previously demonstrated within stage IIIC melanomas that higher methylation percentage of LINE-1 is correlated to worse OS(Sigalotti *et al.*, 2011); in this study, we further demonstrated that increasing hypomethylation of LINE-1 in advancing stages of melanomas and in metastasis of autologous metastasis-primary paired tissues. The observation of LINE-1 hypomethylation during tumor progression is in accordance with that found in gastrointestinal cancers(Ogino *et al.*, 2008a; Ogino *et al.*, 2008b; Sunami *et al.*, 2011). These LINE-1 hypomethylation findings support the pattern of genomic instability occurring during melanoma progression(Fujiwara *et al.*, 1999). LINE-1 hypomethylation has been suggested to facilitate genomic instability in cancer cells(Sunami *et al.*, 2011), and may have a role in the activation of LINE-1 transcription in metastatic melanoma(Haqq *et al.*, 2005).

Chromosome 6q21-22 has been long presumed as a tumor suppressor region in melanoma, and several candidate tumor suppressor genes located at 6q21-23 have been proposed. Trent et al. demonstrated that melanoma lines directly introduced with a normal copy of chromosome 6 lost their ability to form tumors in nude mice, and the loss of chromosome 6 from melanoma microcell hybrids resulted in reversion to tumorigenicity of these cells(Trent

et al., 1990). High frequency of loss of heterozygosity(LOH) of chromosome 6q has also been reported in melanoma lines and tissues(Bahrami *et al.*, 2007; Fujiwara *et al.*, 1999; Stark and Hayward, 2007). However, it still remains unclear which key genes are specifically involved in melanoma progression in the 6q21-22 region of melanomas. Recently, lower levels of *AIM1* expression was shown to be correlated with 6q21 deletion, and promoter hypermethylation of *AIM1* down-regulated *AIM1* expression in natural killer(NK)-cell malignancies(Iqbal *et al.*, 2009). We found *AIM1* to be significantly suppressed during melanoma progression.

Our study showed a significant association between LINE-1 hypomethylation or *AIM1* hypermethylation and poorer survival. The combination analysis of LINE-1(U) and/or *AIM1*(M) in melanomas was a significant predictor for OS and DFS, suggesting that the combination analysis of LINE-1 hypomethylation and *AIM1* hypermethylation can improve their sensitivity as prognostic biomarkers. Studies have demonstrated that genomic methylation status is dynamic during tumor progression(Tanemura *et al.*, 2009) where genomic repeats and specific gene promoter region can, independently or concurrently, become aberrantly methylated during tumor progression(de Maat *et al.*, 2007b; de Maat *et al.*, 2008).

cf-cDNA has been shown as a promising clinical biomarker for various malignancies(Mori *et al.*, 2005; Schwarzenbach *et al.*, 2011; Umetani *et al.*, 2006). We have reported the prognostic utility of specific circulating methylated DNA; RASSF1A, and RAR-β2 in stage IV melanoma patients(Mori *et al.*, 2005). Although serum unmethylated LINE-1 was higher in advanced melanoma patients, it was not correlated to disease outcome. A larger defined cohort of patients may be needed to demonstrate its potential prognostic utility. Patients with circulating methylated *AIM1* DNA, on the other hand, had worse OS than those who did not. The combination of *AIM1* or LINE-1 cf-cDNA was significantly correlated to OS. The analysis indicated that both unmethylated and methylated cf-cDNA can be utilized for prognostic utility.

In conclusion, we demonstrated that LINE-1 hypomethylation and *AIM1* hypermethylation status are related to tumor progression. Together, these two factors have prognostic value as shown with detection of cf-cDNA in melanoma patients' serum for predicting outcome in tissue and in detecting serum cf-cDNA for realtime disease status.

MATERIALS AND METHODS

Specimens

Melanoma patients treated at Saint John's Health Center (SJHC) between 1993-2008 were reviewed for inclusion in this study. PEAT melanomas from the patients were obtained under an IRB protocol that was approved by the SJHC/JWCI Joint IRB and Western IRB(WIRB).

The LINE-1 study included 126 PEAT(113 patients). Among these were 13 pairs of autologous tissues from a primary tumor and its synchronous regional metastasis(5 pairs) or metachronous regional/distant metastasis(8 pairs). Of the remaining 100 specimens, 44 were

from the primary tumors of patients with AJCC melanoma stage I(n=13), stage II(n=11) or stage III(n=20); 56 were from metastases of patients with stage III(n=23) or stage IV(n=33).

The *AIM1* study included 112 PEAT from 92 patients with AJCC stage I(n=14), stage II(n=14), stage III(n=30 primaries and 23 lymph node metastases), and stage IV(n=31 distant metastases) melanoma. Seventy-two patients from the *AIM1* study overlapped with the LINE-1 study, with AJCC stage I(n=13), stage II(n=10), stage III(n=15 primary tumors and 11 lymph node metastases), and stage IV(n=23 distant metastases) melanoma. Twenty-Six negative controls were obtained from histopathologically negative normal skin(n=14) and nevi tissue(n=12).

Serum from 56 AJCC stage III(n=20) and IV(n=36) melanoma patients, and 14 healthy donors with serum separator tubes, filtered, cryopreserved(-80°C)(Koyanagi *et al.*, 2010; Mori *et al.*, 2005). All patients' sera were obtained after informed consent.

Melanoma Cell Lines and Treatment

Melanoma cell lines(M-12, M-15, M-24, M-101, LF-0023) established from metastatic tumors at JWCI were cultured and harvested for the study and as assay controls. The melanoma lines were cultured as previously described(Narita *et al.*, 2009). Three melanoma cell lines, M-15, M-101, and M-24 were treated with 5-aza-2′ deoxycytidine(5Aza-dC; Sigma-Aldrich) for *AIM1* reactivation. Melanoma cells were cultured, and then treated with culture medium containing 5 μM 5Aza-dC or dimethyl sulfoxide as a vehicle control. 5Aza-dC containing medium was administered daily, and the cells were collected for assessment after 48 h.

DNA Isolation From PEAT and Serum Samples

Tissue sections were cut for H&E and microdissected(de Maat *et al.*, 2010a; de Maat *et al.*, 2007b). Microdissected tissues were incubated at 50°C overnight in lysis buffer(50 mM Tris, 1mM EDTA, 2.5% Tween 20, 6.0 mAU proteinase K) followed by 95°C(10min) incubation. DNA was extracted by phenol-chloroform isoamyl extraction and ethanol precipitation. DNA concentration was determined by both spectrophotometer reading at 260/280 nm, and Quant-iT™ PicoGreen® dsDNA assay kit(Invitrogen, Carlsbad, CA).

Serum samples(500μL) were diluted with 0.9% NaCl and mixed with a premix consisting of proteinase K and 10% sodium docecylsulfate. Samples were incubated at 50°C(3 hrs). After incubation, phenol-chloroform isoamyl(25:24:1, pH 8.0)(Sigma-Aldrich, St. Louis, MO) was added to each sample. DNA was extracted and quantified; up to 300 ng of DNA was subjected to sodium bisulfite modification using EpiTect bisulfite kit(Qiagen, Valencia, CA) (de Maat *et al.*, 2010b).

AQAMA

We previously reported accurate, targeted methylation analysis of PEAT using AQAMA, an assay based on bisulfite modification of DNA and real-time PCR using two hydrolysis minor groove binder(MGB) probes specific for the methylated or unmethylated templates labeled with fluorophores(de Maat *et al.*, 2007a; Tanemura *et al.*, 2009). Assays were in

triplicates and the average copy number was used. LINE-1 hypomethylated melanoma line DNA and LINE-1 methylated PBL of healthy donors were included as controls. The LINE-1 hypomethylation level was a continuous variable defined as the copy number of unmethylated LINE-1(U) relative to the total LINE-1 copy number which is the sum of the copy number of methylated LINE-1(M) and U(U/[U+M]), hereafter referred to as LINE-1 U-Index. The LINE-1 U-Index of each sample was compared for hypomethylation status. For assessment of cf-cDNA, modifications of the assay included the use of 4μL of bisulfite-treated DNA as template and PerfeCTa qPCR Supermix(Quanta BioScience). For serum, qPCR quantification cycle(C_q) cut-off at 25 was used to qualify whether there is sufficient level of unmethylated LINE-1 DNA present above that of a healthy donor serum.

RNA Isolation and RT-PCR

Total RNA from cultured cells was extracted using TRI-Reagent(Molecular Research Center, Cincinnati, OH). For melanomas, 5x10-μm sections were cut from PEAT blocks on a microtome. Apart from deparaffinization, RNA was extracted from PEAT using RNAwiz(Ambion, Austin, TX) after proteinase K digestion as previously described(Koyanagi *et al.*, 2006b; Takeuchi *et al.*, 2004). RNA quantity and quality were accessed by spectrophotometry and Quant-iT RiboGreen RNA assay(Invitrogen).

Reverse transcription of total RNA(1 g) and PCR with 5 ul of cDNA was performed as previously described(Koyanagi *et al.*, 2006a). *AIM1*-specific primers are 5′-TTAGTTTTAGACATTAAAGGGGG-3′ and 5′-TGGAAGGACCTCCAGAAGAT-3′. GAPDH was assessed by RT-PCR as reference and for normalization. Specimens were amplified with a pre-cycling hold at 95°C(10min), followed by 25 cycles of 95°C, 55°C for *GAPDH*/58°C for *AIM1*, and 72°C(1min).

MALDI-TOF Mass Spectrometry

EpiTYPER assay(Sequenom, San Diego, CA) is a tool for detection and quantification of DNA methylation using MALDI-TOF MS and MassCLEAVE analysis based on a base-specific cleavage reaction(Radpour *et al.*, 2008). This assay enables accurate quantification of DNA methylation(Coolen *et al.*, 2007). Each CpG site within an amplicon is assessed for methylation status. Three pairs of primers were designed to cover *AIM1*'s promoter region as predicted by Promoter Scan Web(http://www-bimas.cit.nih.gov/molbio/proscan/) using EpiDesigner (Sequenom)(Supplementary Figure 4**)**. Bisulfite-treated DNA PCR amplification was performed with a pre-cycling hold at 94°C(15min) followed by 45 cycles of 94°C(20s), 58°C(30s), and at 72°C(1min) and a final extension at 72°C(2min). Post-PCR amplicons are assessed as previously reported using MassARRAY(Yoshimura *et al.*, 2011).

MSP

Bisulfite-conversion based PCR primers were designed to cover the region where methylation percentage was well-correlated with mRNA expression. For MSP on PEAT, the forward and reverse methylation-specific were 5′-D4-TTTGTTTTTTCGTTTTTTTAGGTTC-3′, and 5′-ACTAACATCCAATACCCGCG-3′, respectively. Forward and reverse unmethylated-specific primers were 5′-D3-TGTTTTTTTGTTTTTTTAGGTTTGT-3′, and 5′-ACTAACATCCAATACCCACAC-3′,

respectively. For serum DNA MSP, different reverse primers were selected to reduce product size for optimal detection: methylation, 5′-CGCGATAACGCTCCG-3′and unmethylation, 5′-CAATAAACACAATAACACTCCA-3′.

For PEAT, PCR were performed with Accustart *Taq* DNA polymerase(0.5U)(Quanta BioSciences), 1xPCR buffer, 4.5mM $MgCl_2$, 0.2μmol/L each of forward and reverse primer, 800μmol/L deoxynucleotide triphosphates, and 1μL bisulfite-treated DNA. Amplifications were carried out: 95°C(3min) followed by 40 cycles of 95°C, 61°C, and 72°C(30s) for both methylated and unmethylated reaction, and a final extension at 72°C(7min). For circulating *AIM1* DNA in serum, 3μL of bisulfite-treated DNA was used as template in the PCR reaction, and PerfeCTa qPCR Supermix is used in place of Accustart *Taq* DNA polymerase cocktail. Amplifications were carried out: 95°C(3min) followed by 40 cycles of 95°C, 59°C, and 72°C(30s). PCR products were analyzed by capillary array electrophoresis(Beckman Coulter CEQ 8000X; Beckman Coulter, Brea, CA) as previously reported (Tanemura *et al.*, 2009). Methylation assay control includes PBL DNA methylated *in vitro* with excess *Sss*I methyltransferase(New England BioLabs, Ipswich, MA)(Umetani *et al.*, 2005). Unmethylated assay control includes leukocyte DNA that was amplified by *phi*-29 DNA polymerase to produce universally unmethylated control(UUC).

Biostatistical Analysis

All data were analyzed using SAS software(version 9.1.3, SAS Institute, Cary, NC). Chi-square test or Fisher's exact test were used to analyze categorical variables. ANOVA, Student's t-test, Kruskal Wallis test, or Tukey's HSD test was used to compare independent continuous variables across different strata. The Cochran Armitage trend test analyzed LINE-1 hypomethylation across AJCC stages. LINE-1 U indices were compared at each AJCC stage using the Student-Newman-Keuls test. Signed rank test and McNemar's test were used to compare LINE-1 U-Index in primary and metastatic paired tissues. Correlation between LINE-1 U-Index and patients' age was analyzed using Spearman's rank test correlation efficient. Association between methylation status of *AIM1* and AJCC stage was assessed by the *Chi*-square test, while trend analysis was conducted using the Cochran Armitage trend test. Cox proportional hazards(PH) regression models for OS and DFS were built incorporating methylation status of LINE-1 and *AIM1* along with other clinical variables such as Breslow, age, gender, and number of metastases. To convert LINE-1 U-Index into a categorical value for analysis, a cutoff of 0.297 was established using the average of LINE-1 U-Indices from normal skin samples plus 2 S.D. A sample with LINE-1 U-Index ≥0.297was considered hypomethylated in LINE-1. When combining both *AIM1* and LINE-1 status, patients with either *AIM1* hypermethylation or LINE-1 hypomethylation were compared to patients who had neither event. The PH assumption was tested in building the Cox PH regression model. Survival curves were generated using the Kaplan-Meier method. A p-value <0.05 was considered statistically significant. These analyses were performed in compliance with REMARK(McShane *et al.*, 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIM1	Absent in melanoma-1
AQAMA	Absolute Quantitative Assessment of Methylated Alleles
cf-cDNA	circulating cell-free DNA
LINE-1	long interspersed nucleotide elements-1
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
M	methylated
MSP	methylation specific PCR
MGB	minor groove binder
NK	natural killer
ORF	open reading frames
PEAT	paraffin-embedded archival tissues
PH	proportional hazards
PBL	peripheral blood lymphocyte
UMC	universally methylated control
UUC	universally unmethylated control
U	unmethylated
WIRB	Western Institutional Review Board

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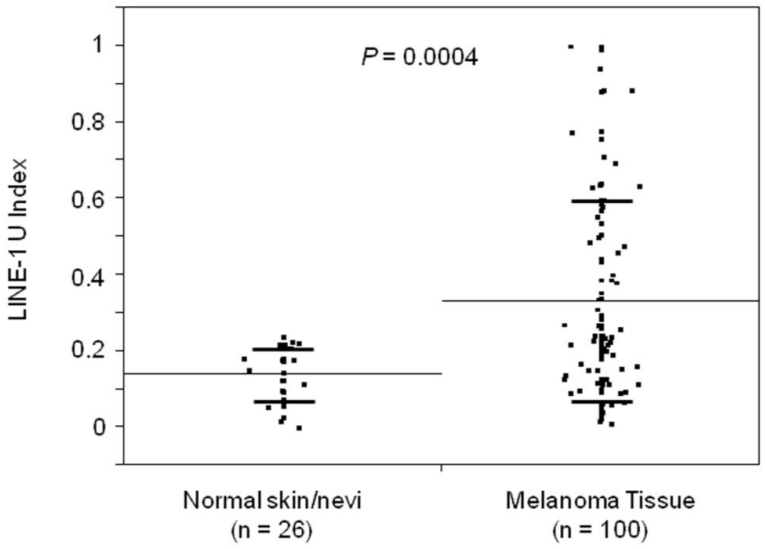

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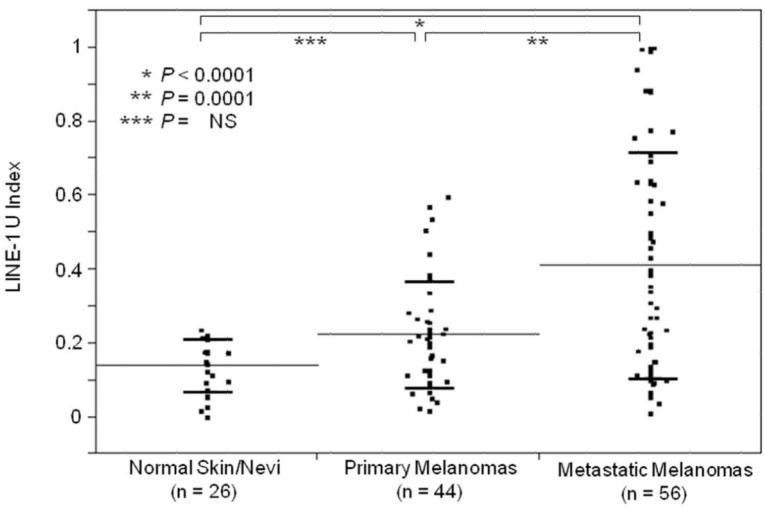

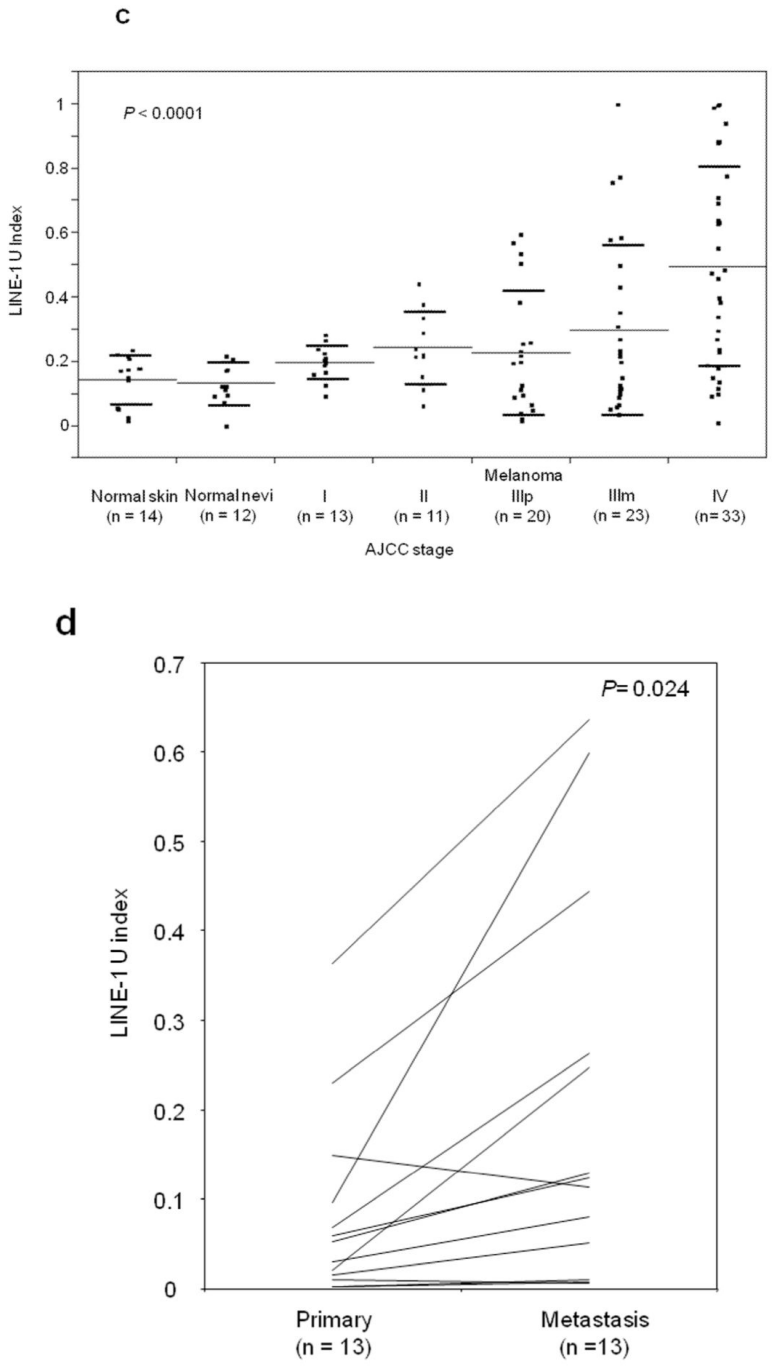

Figure 1.

Individual and mean (*solid horizontal line*) LINE-1 U-Index values for tissue specimens of normal skin vs. primary/metastatic melanoma (**A**), normal skin vs. primary melanoma vs. metastatic melanoma (**B**) and according to the AJCC stage (**C**) and primary and metastatic paired samples (**D**). *P* values were obtained by Student's t-test (**A**, **D**), Tukey's HSD (**B**) and ANOVA (**C**).

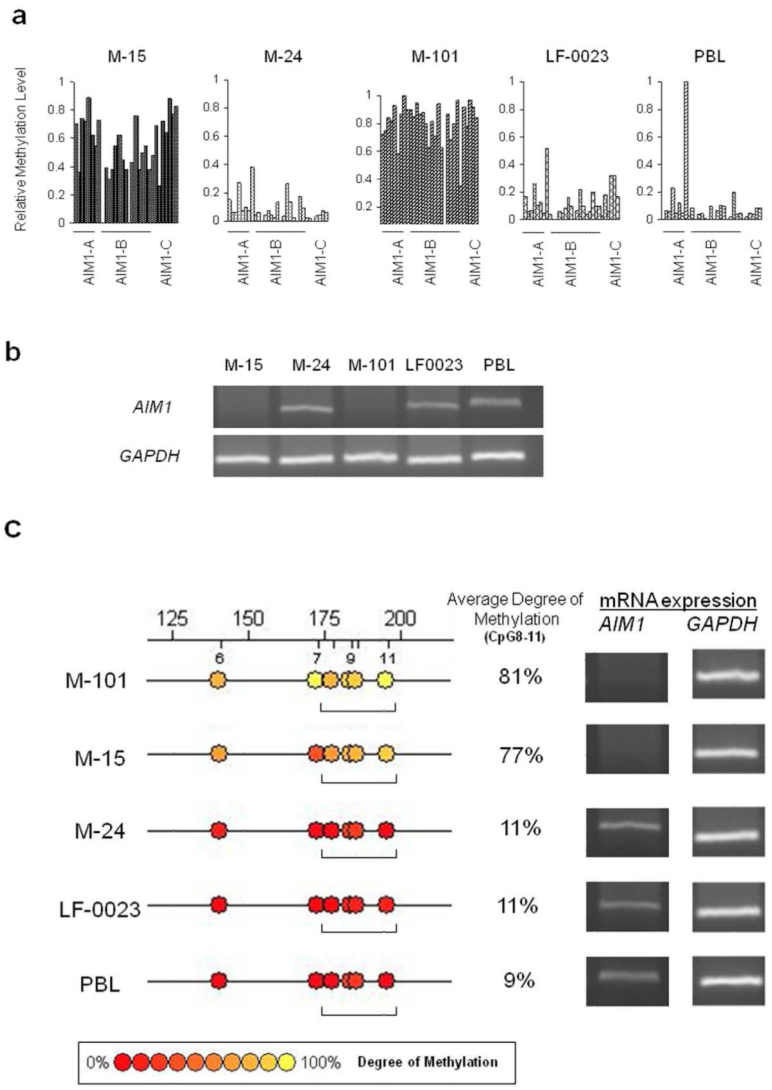

Figure 2.

AIM1 promoter methylation and gene expression. **A**: CpG methylation in the promoter region of *AIM1* gene as assessed by PCR of three amplicons (A, B, and C) covering over 1000bp of promoter region. **B**: *AIM1* mRNA expression in melanoma cell lines. **C**: MALDITOF MassARRAY analysis of specific CpG sites as target region of MSP primers for *AIM1*.

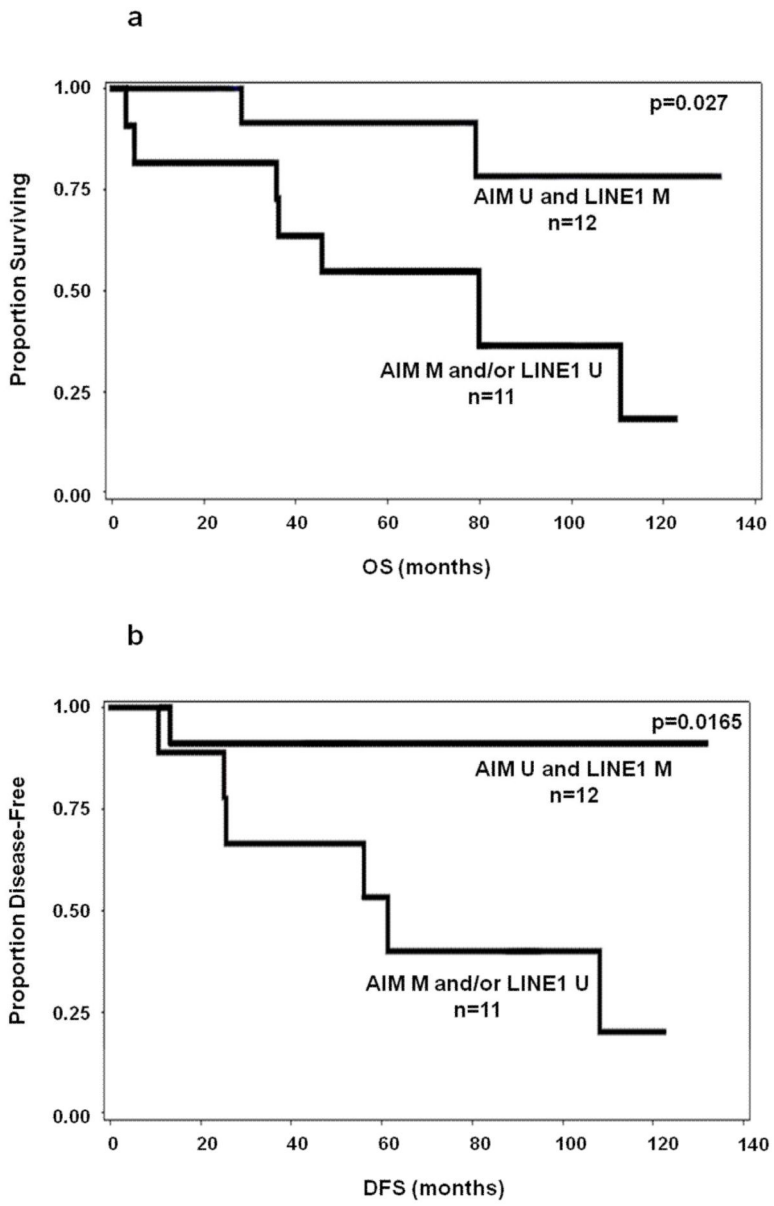

Figure 3.

Kaplan-Meier survival curves for melanoma patients using combination of LINE-1 and *AIM1* methylation status in PEAT. M=hypermethylated, U=hypomethylated. **A**: OS of patients with LINE-1 hypomethylation and/or *AIM1* hypermethylation versus LINE-1 hypermethylation and *AIM1* hypomethylation. **B**: DFS of stage I-II melanoma patients with LINE-1 hypomethylation and/or *AIM1* hypermethylation vs. LINE-1 hypermethylation and *AIM1* hypomethylation.

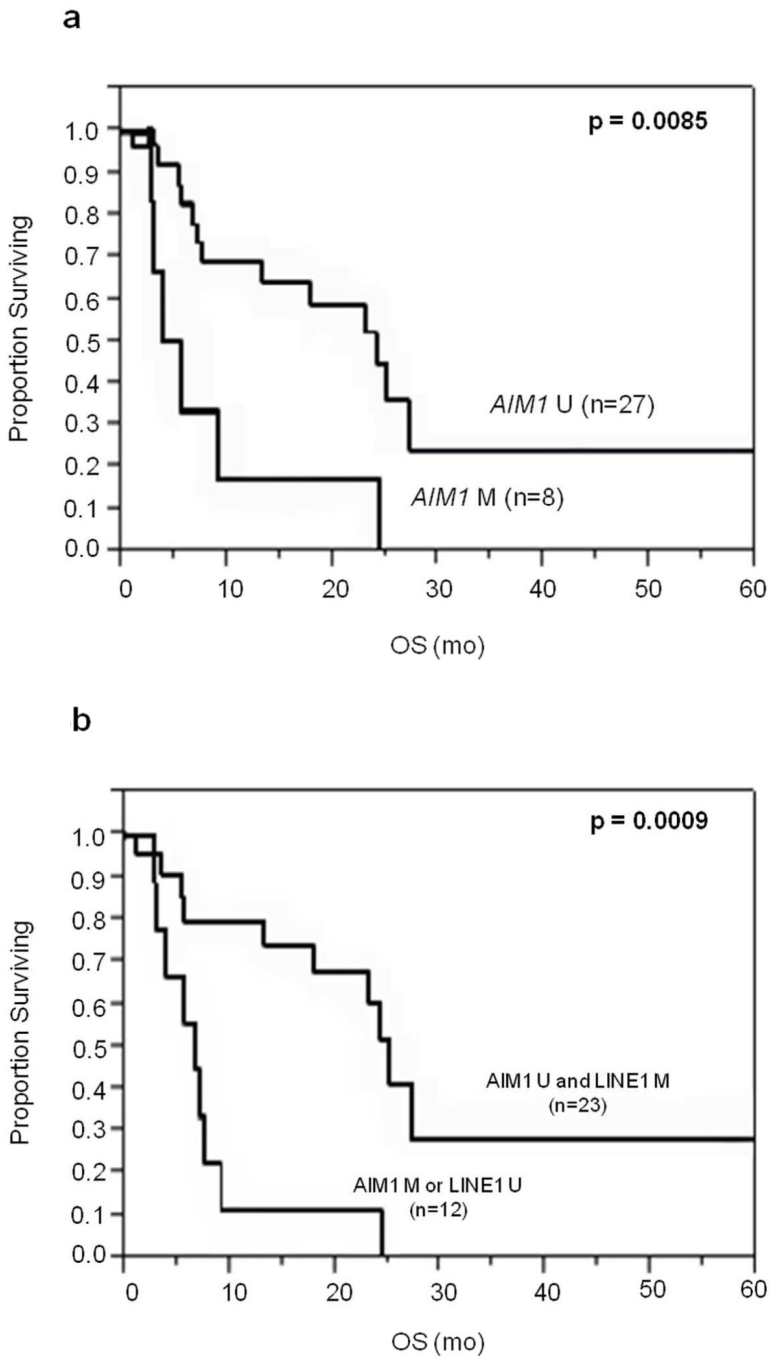

Figure 4.

Kaplan-Meier survival curves for stage IV patients in serum studies. **A**: OS of stage IV patients with methylated vs. unmethylated *AIM1* cf-cDNA. **B**: OS of stage IV patients with LINE1 unmethylated and/or *AIM1* methylated cf-cDNA vs. those with LINE-1 methylated and *AIM1* unmethylated cf-cDNA.

Table 1

Methylation Status of *AIM1*_Promoter Region in Melanoma Tissue

AJCC stage	Hypermethylation
Normal Tissue Total (n=26)	0 (0%)
Skin (n=14)	0 (0%)
Nevi (n=12)	0 (0%)
Primary Melanoma Total (n=58)	22 (38%)
Stage I (n=14)	4 (29%)
Stage II (n=14)	8 (57%)
Stage III Primary Tumor (m=30)	10 (33%)
Metastasis Tumor Total (n=54)	35 (65%)
Stage III Nodal Metastasis (n=23)	11 (48%)
Stage IV (n=31)	24 (77%)

Table 2
Clinicopathological Factors of AIM1/LINE1 Survival Analysis set (n=72)

Clinicopathological Factors				
	Stage I/II (n = 23)	**Cox OS**	**Stage III (n=26)**	**Stage IV (n=23)**
Gender		NS		
Male	12		15	15
Female	11		11	8
Age (years)				
Mean (SD)	68.4 (±8.9)	p=0.0203, HR:1.15, 95%CI(1.02-1.30)	62.4 (±17.1)	59.5 (±14.7)
Primary Site		NS		
Extremities	12			
Head/Neck	6			
Trunk	5			
# Distant Sites				
1				3
2				8
3+				12
Breslow Thickness		NS		
≤ 1.00mm	8		3	
1.01-2.00mm	6		5	
2.01-4.00mm	6		8	
>4.00mm	1		10	
Unknown	2		0	
Ulceration		NS		
Present	7		13	
Absent	13		11	
Unknown	3		2	
Mitotic Index		NS		
High ≥11/mm^2	2			
Intermediate 5-10/mm^2	1			
Low ≤4/mm^2	17			
Unknown	3			
# Lymph Nodes (+)				
1			7	
2-3			13	
4+			1	
0 (Skin Met)			2	

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Clinicopathological Factors				
	Stage I/II (n = 23)	**Cox OS**	**Stage III (n=26)**	**Stage IV (n=23)**
Unknown			3	
LINE1 or AIM1		p=0.0282, HR:6.46, 95%CI(1.22-34.15)		
LINE1/AIM1	11		12	22
Neither	12		14	1