ARTICLE
Genetics and Genomics

Downregulation of ITGA6 confers to the invasion of multiple myeloma and promotes progression to plasma cell leukaemia

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BACKGROUND: Secondary plasma cell leukaemia (sPCL) is an aggressive form of multiple myeloma (MM), but the mechanism underlying MM progresses into PCL remains unknown.
METHODS: Gene expression profiling of MM patients and PCL patients was analysed to identify the molecular differences between the two diseases. Cox survival regression and Kaplan–Meier analysis were performed to illustrate the impact of integrin subunit alpha 6 (ITGA6) on prognosis of MM. Invasion assays were performed to assess whether ITGA6 regulated the progression of MM to PCL.
RESULTS: Gene expression profiling analyses showed that cell metastasis pathways were enriched in PCL and ITGA6 was differentially expressed between PCL and MM. ITGA6 expression was an independent prognostic factor for event-free survival (EFS) and overall survival (OS) of MM patients. Moreover, the stratification ability of the International Staging System (ISS) of MM was improved when including ITGA6 expression. Functional studies uncovered that increased ITGA6 reduced the myeloma cell invasion. Additionally, low expression of ITGA6 resulted from epigenetic downregulating of its anti-sense non-coding RNA, ITGA6-AS1.
CONCLUSION: Our data reveal that ITGA6 gradually decreases during plasma cell dyscrasias progression and low expression of ITGA6 contributes to myeloma metastasis. Moreover, ITGA6 abundance might help develop MM prognostic stratification.

BACKGROUND

Multiple myeloma is a bone-marrow-based malignancy characterised by proliferation of clonal plasma cells.[1] A characteristic of MM is its main localisation in the bone marrow and its tendency to progress from the original bone position to the new bone position in local and distal bones.[1,2] During the progression of MM, cancer cells develop the ability to proliferate at sites outside of the bone marrow, manifesting as plasma cell leukemia.[3] Plasma cell leukaemia can be divided into two types according to the source. One is primary plasma cell leukaemia (pPCL) in a newly diagnosed setting, and the other is secondary plasma cell leukaemia (sPCL) in a relapsed MM setting.[3]

Genomic differences between PCL and MM have been studied.[3] Hypodiploidy, 1q gains, 13q deletion and p53 deletion partially defined the progressive the plasma cell (PC) disease.[3] However, genetic characteristics could not completely explain PC disease progression.[4] Thus, the comprehensive assessment of transcriptional characterisation might provide fresh information about biological differences between MM and PCL.

A large part of the human genome is transcribed into lncRNAs longer than 200 nt that have no protein coding potential.[5,6] Recent studies indicate that lncRNAs have abundant functions in multiple biological processes.[7–9] In addition, emerging evidences support the notion that anti-sense lncRNAs play key roles in their associated protein coding genes and particularly affecting the latter expression.[10–12]

Current prognosis markers for MM is International Staging System (ISS), which may not reflect the molecular characteristics of the myeloma clone.[13] It is therefore essential to develop biomarkers that are specific to define the biological aspect of MM.

Herein, we analysed the gene expression profiling of MM patients and PCL patients using GEO databases and identified an integrin, ITGA6, whose expression was decreased (>threefold) in PCL samples *versus* its expression in MM samples in two datasets (GSE2113 and GSE39925). We also performed survival analysis to determine the impact of ITGA6 on patient outcomes and noticed that high level of ITGA6 was related to increased EFS and OS compared with lower expression levels of ITGA6 in MM. Meanwhile, functional studies revealed that the overexpression of ITGA6 reduced invasion in MM cell lines. Furthermore, we discovered that a lncRNA, ITGA6-AS1 formed an RNA duplex with ITGA6 pre-mRNA, which increased the level of ITGA6 pre-mRNA stability and reduced the invasion of MM cells. Therefore, our data indicate that downregulation of ITGA6 confers to the invasion of multiple myeloma, further suggesting that a potential therapy targeted ITGA6 might inhibit myeloma cell migration associated with progression of MM to PCL.

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METHODS

Collection of patient samples
All samples were collected with ethical permission from the Second Affiliated Hospital of Soochow University (characteristics of patients in Table S1). The study was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from each subject or each subject's guardian.

RNA extraction and real-time PCR Analysis
Total RNA was isolated using TRIZOL (Invitrogen, USA) according to the manufacturer's instruction. QPCR performed with the Real-Time PCR System (7500). The expression of indicated genes was normalised to endogenous reference control (GAPDH) by using $2^{-\Delta\Delta Ct}$ method (primer sequences in Table S2).

Cell proliferation
The cell proliferation rate was determined using Cell Counting Kit-8 (CCK-8, Dojindo Corporation) according to the instructions. Assays were performed at 24, 48 and 72 h, and each sample was assayed in triplicate.

Transwell assay
The invasion ability of the MM cells was assessed using the transwell assays by means of 8-μm pore-size Matrigel-coated transwell chambers (Corning). After starvation in serum-free RPMI 1640 medium for 12 h, the cells (2×10^5) were placed on Matrigel (BD Biosciences)-coated transwell chambers (Corning). After 24 h incubation in an incubator at 37 °C and 5% CO_2, non-invasive cells were removed from the upper surface. The cells invading through the Matrigel were fixed using 4% paraformaldehyde, stained by haematoxylin, imaged using the microscope and counted in the upper surface and the lower medium. These experiments were performed in triplicate for each condition.

Statistical analysis
Survival analysis was performed to investigate the associations between the censored outcomes, the clinical variables and the expression of ITGA6 using the R package survival. All clinical variables and the expression of ITGA6 were numeric and standardised to facilitate the comparison of hazards ratios (HR).

We first used univariate Cox survival regression to separately analyse predictor variables. To illustrate the impact of ITGA6, we partitioned the patients into two groups based on the medians of ITGA6 expression, estimated the survival curves of these two groups of patients using the Kaplan–Meier method and compared their differences using the log-rank test. We then used multivariate Cox survival regression to jointly fit all the clinical variables with ITGA6, which was adjusted for the potential confounding effects of the clinical variables on ITGA6. By fitting these Cox models, we obtained estimates of the hazards ratios (HR), their 95% confidence intervals (CIs), corresponding p-values, and Harrell's concordance indices (C-indices). We also validated the prognostic values of these models using leave-one-out cross-validation.

All results were listed as the mean ± SD (standard deviation). Student's t-test was used when the variance between groups was similar. The difference in mean values between two groups was analysed by Student's t-test. Pearson correlation analysis was used as mentioned above. A value of $P < 0.05$ indicated significance.

Other methods are detailed in supporting information.

RESULTS

### ITGA6 may regulate the progression of MM to PCL
To clarify the underlying mechanisms that lead to extramedullary myeloma (PCL) from MM, the gene expression profiling of MM patients and PCL patients was analysed using GEO datasets (GSE39925 and GSE2113). GSEA indicated that gene signatures for the positive regulation of cell metastasis (the gene sets: LIAO_METASTASIS, NAKAMURA_METASTASIS_MODEL_UP, BIDUS_METASTASIS_UP and WINNEPENNINCKX_MELANOMA_METASTASIS_UP) were significantly enriched in PCL samples (Fig. 1a). Furthermore, the distribution of principal component analysis (PCA) showed a clear distinction between MM samples and PCL samples in the four pathways (Fig. 1b). To identify the specific mRNAs involved in cell metastasis, the differentially expressed genes between PCL and MM were analysed (Fig. 1c). Both datasets (GSE39925 and GSE2113) identified the same seven genes which were increased or decreased (>threefold) in PCL versus MM, probably reflecting their distinct oncogenic programs and genetic profiling (Fig. 1d). Among them, we noted integrin subunit alpha 6 (ITGA6, GSE39925: fold change = 4.88, $P = 0.01$, GSE2113: fold change = 3.51, $P = 0.004$), which was shown to be responsible for cell adhesion and invasion.[14] Integrins are a class of glycoproteins that are widely found in many cell types, and have pivotal roles in regulating cell adhesion, migration, homing, invasion and drug resistance.[15–17] A wide range of integrins is also detected in MM cells including α4, α5, β1, β2, β3 and β7.[18–21] ITGA6 is a member of the integrin alpha chain family of proteins, which are responsible for mediating epithelial-basement membrane interactions.[14] Recent evidence suggests that signalling events mediated by the α6 subunit also regulate the processes involved in tumorigenesis, including proliferation and metastasis.[22–27] It is not yet clear what role ITGA6 plays in the progression from MM to PCL and whether its functions are essential for the invasion of myeloma extramedullary sites.

ITGA6 could be used as a biomarker of prognosis in MM patients
To further explore the role of ITGA6 in plasma cell tumour transformation, we analysed the ITGA6 expression in patients with plasma cell disease at different stages (MGUS, SMM, MM and PCL) and PCs of healthy donor (NC). As shown in Fig. 2a, the expression level of ITGA6 in patients with MGUS, SMM and MM was lower than that of healthy donors, but there was no significant difference among MGUS, SMM and MM. And ITGA6 level in PCL was lower than that of MM patients. Together, ITGA6 expression was reduced by the progression of plasma cell dyscrasias. We subsequently sought to explore the differences in ITGA6 expression between pPCL and sPCL samples. Furthermore, comparison of pPCL samples to sPCL samples showed that the expression of ITGA6 was no significant difference (Fig. 2a GSE66291). To investigate whether ITGA6 expression was related to the prognosis of MM, we analysed two datasets obtained from the GEO databases (GSE24080 and GSE4581). The effect of ITGA6 on the EFS and OS was illustrated by Kaplan–Meier curves dichotomised expression of ITGA6 at the median. We observed that reduced expression of ITGA6 consistently associated with poor survival (Fig. 2b). Consistent with this, univariate and multivariate analyses were performed using the Cox risk proportion model and ITGA6 was significantly associated with OS (cut-off = 10.8116) (Table 1, GSE24080). To further assess the prognostic value of ITGA6, we tested the c-indices of the models fit with ISS alone and ISS with ITGA6. Leave-one-out cross-validation was performed to validate the prognostic value of ITGA6. The c-indices of model fit with the combination of ISS and ITGA6 were higher than those of models fit without ITGA6, suggesting that ITGA6 improved the prognostic stratification of patients with MM (Table 2). To further assess the prognostic significance for ITGA6, we validated this risk stratification in another cohort of patients. All samples were collected from the Second Affiliated Hospital of Soochow University. The detailed clinical features of these patients were described in our previous publication.[28] In the univariate (HR = 0.2534, 95% CI: 0.0963–0.6666, $P = 0.0054$) and multivariate (HR = 0.3033, 95% CI: 0.1002–0.9183, $P = 0.0348$) Cox analyses, ITGA6 was an independent predictor for OS (cut-off = 1.3600) (Table S3). Together, the above results suggested that ITGA6 probably has an important effect on the progression from MM to PCL.

Downregulation of ITGA6 confers to the invasion of multiple myeloma and...
S Song et al.

1845
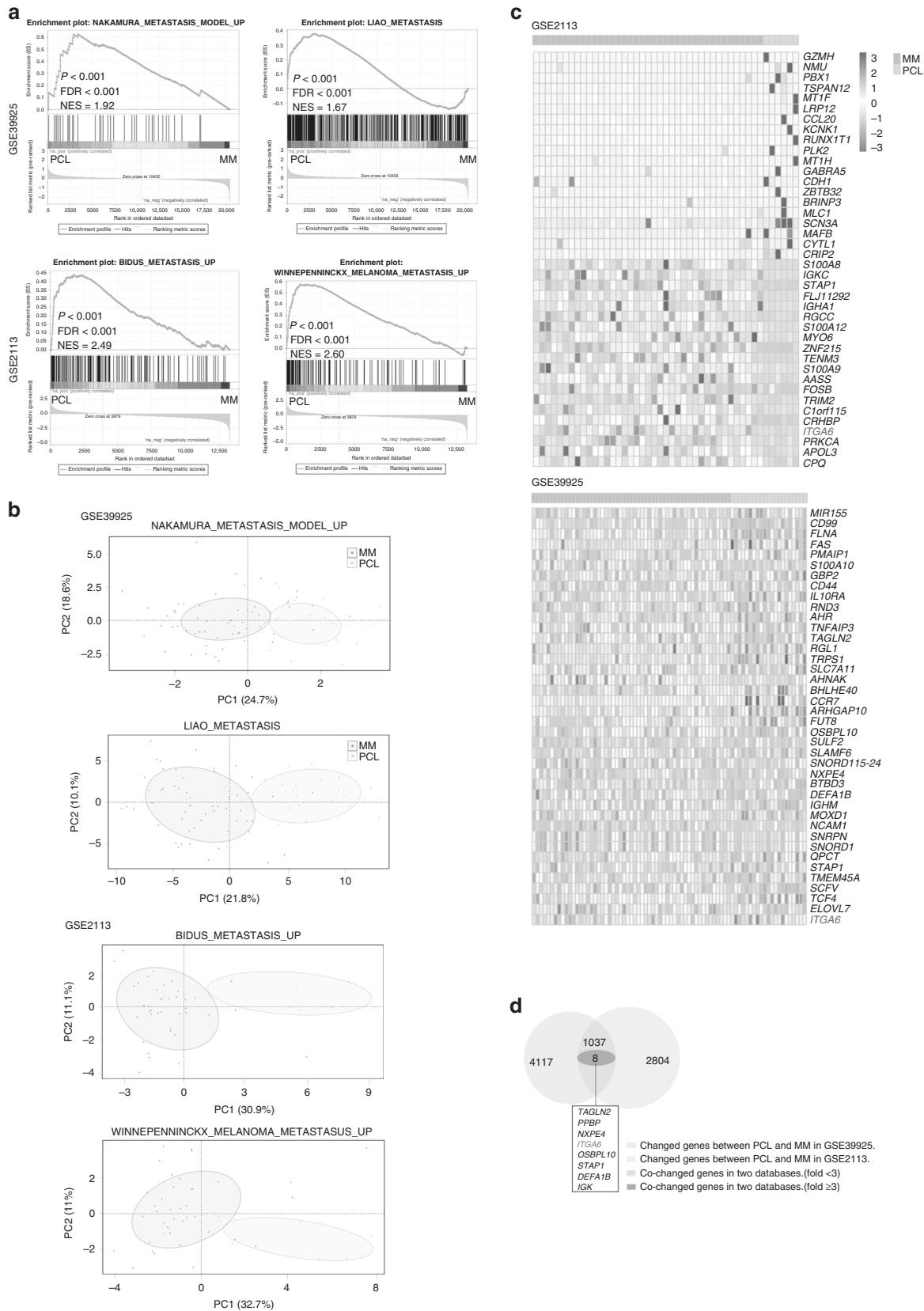
Fig. 1 *ITGA6* **may regulate the progression of MM to PCL. a** Specific gene expression signatures were associated with cell metastasis. Representative GSEA plots illustrating the enrichment of genes involved in positive regulation in PCL samples. FDR false discovery rate, NES normalised enrichment score. GSE2113 (MM $n = 39$, PCL $n = 6$), GSE39925 (MM $n = 55$, PCL $n = 21$). **b** Principal component analysis (PCA) plot showed unsupervised clustering, demonstrating a clear distinction between MM and PCL samples in each pathway. **c** The 30 up- and downregulated genes in PCL *versus* MM were presented in heat maps. **d** Venn diagram showing the overlapping differentially expressed genes identified in the two groups.

Downregulation of ITGA6 confers to the invasion of multiple myeloma and…
S Song et al.

1846
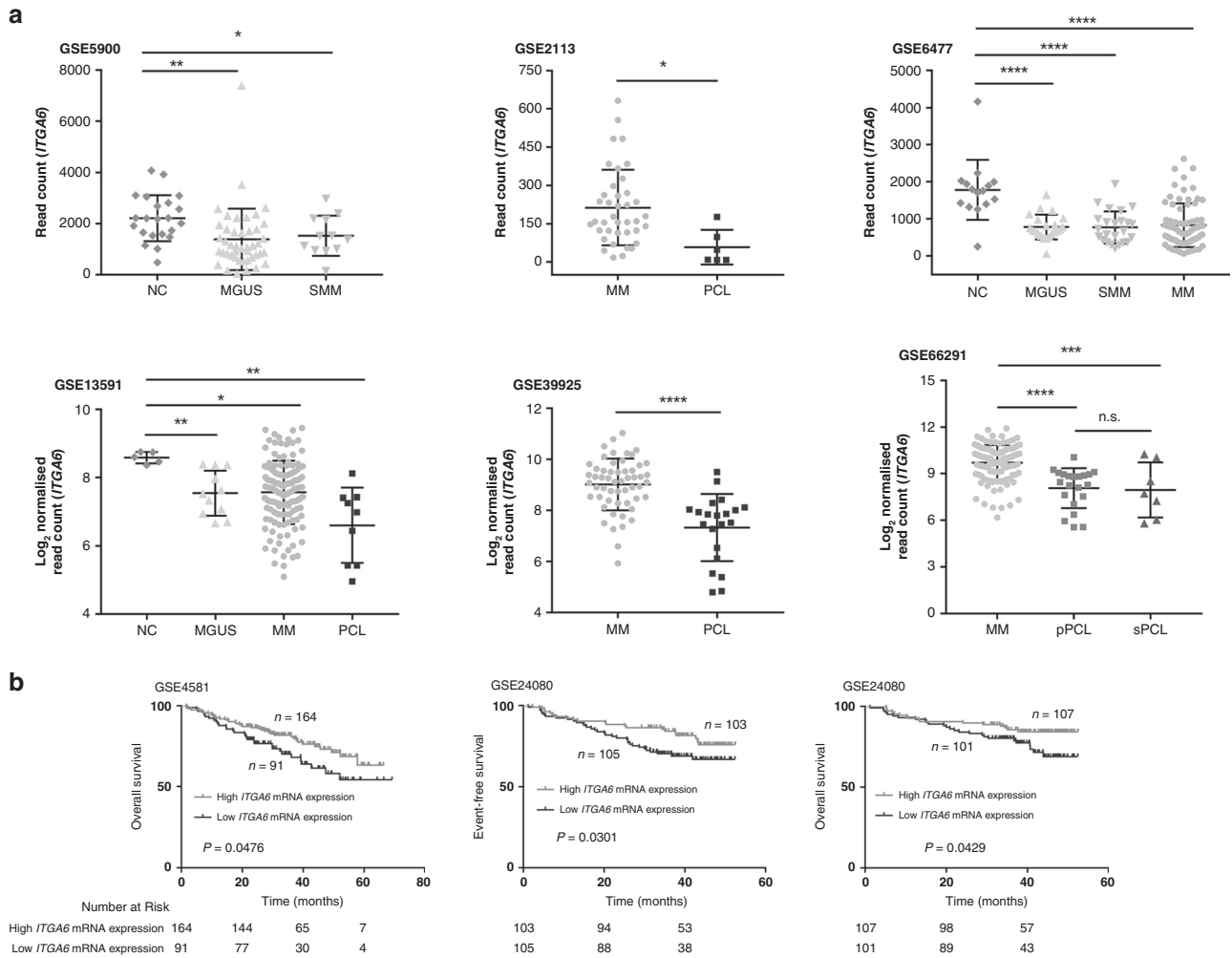
Fig. 2   *ITGA6* could be used as a biomarker of prognosis in MM patients. a The expression of *ITGA6* in plasma cell dyscrasias (MGUS, SMM, MM and PCL) and PCs of healthy donor (NC). The expression level of ITGA6 in patients with MGUS, SMM and MM was lower than that of healthy donors. ITGA6 level in PCL was lower than that of MM patients. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns not significant versus NC. **b** Kaplan–Meier plots indicating the prognostic relevance of *ITGA6* expression in the event-free and overall survival of MM patients (GSE24080 and GSE4581).

ITGA6 mediated MM cell invasion

To confirm this hypothesis, we transfected the ITGA6 expression vector (pcDNA3.1-ITGA6) or negative control (pcDNA3.1) into the MM cell lines to enhance the expression of ITGA6 (Fig. 3a). To investigate whether ITGA6 altered cell invasion, transwell assays were performed. As shown in Fig. 3b, the overexpression of ITGA6 greatly reduced the invasion of U266 and LP-1 cells compared to the control. Moreover, we performed Cell Counting Kit-8 (CCK-8) assays to further elucidate the effects of ITGA6 expression on tumour cell proliferation. However, there were no differences in cell proliferation between the two groups (Fig. 3c). Overall, these data indicated that ITGA6 could regulate the invasion of MM cells, suggesting a potential role of ITGA6 in the progression of MM to PCL.

Characterisation of the lncRNA ITGA6-AS1

To further elucidate the underlying mechanisms of invasion in MM, we sought to identify the elements that might contribute to the preferential regulation of ITGA6. Considering that lncRNAs regulate the expression of neighbouring genes by diverse mechanisms,[29] we concentrated on intergenic lncRNAs located near the *ITGA6* gene. To this end, we focused on a long non-coding RNA, *ITGA6-AS1*, located on chromosome 2 (chr2: 172464262-172466022, GRCh38.p13). *ITGA6-AS1* is transcribed in

the opposite direction as *ITGA6* and contains 521 nucleotides complementary to the *ITGA6* pre-mRNA (Fig. 4a). Based on the sequence, we speculated that overlapped region was likely to mediate the interaction between *ITGA6-AS1* and *ITGA6*. We further examined the expression levels of *ITGA6* and *ITGA6-AS1* in CD138+ MM cells from 30 MM subjects by qPCR. We found that *ITGA6-AS1* expression was positively correlated with the expression of *ITGA6* (Pearson $r^2 = 0.537$, $P < 0.0001$) (Fig. 4b). To gain insights into the lncRNA *ITGA6-AS1*, we characterised it. *ITGA6-AS1* was detected in both the cytoplasm and the nucleus by qPCR (Fig. 4c). Northern blot was performed to demonstrate the presence of *ITGA6-AS1* in U266 and LP-1 cells (Fig. 4d). Based on these results, only one transcript variant of *ITGA6-AS1* existed in MM cells. Furthermore, Coding Potential Calculator (CPC)[30] and Coding Potential Assessment Tool (CPAT)[31] analyses displayed that *ITGA6-AS1* had no coding potentiality (Fig. 4e). Collectively, these data indicated that *ITGA6-AS1*, as an lncRNA, was present in MM cells and positively correlated with its anti-sense coding RNA, ITGA6.

ITGA6-AS1 and ITGA6 pre-mRNA may form an RNA duplex

To investigate whether *ITGA6-AS1* regulate ITGA6, *ITGA6-AS1* expression vector (OE-*ITGA6-AS1*) were transfected into U266 and LP-1 cells (Fig. 5a). Notably, the upregulation of *ITGA6-AS1* led to a significant increase in *ITGA6* pre-mRNA and ITGA6 at both the

Downregulation of ITGA6 confers to the invasion of multiple myeloma and…
S Song et al.

1847

Table 1. Summary for the results of univariate and multivariable analysis.

Factors	Number of Patients	OS (overall survival)					EFS (event-free survival)				
		Univariate		Multivariate			Univariate		Multivariate		
		HR	p-value	HR	95% CI	p-value	HR	p-value	HR	95% CI	p-value
Age	345	1.0309	0.1081	1.0385	0.9960–1.0828	0.0764	1.0245	0.1421	1.0380	1.0008–1.0767	0.0453
Beta-2 microglobulin	345	1.0819	0.0000	1.0734	1.0212–1.1282	0.0053	1.0770	0.0000	1.0530	1.0055–1.1027	0.0283
C-reactive protein	345	1.0041	0.4113	0.9882	0.9710–1.0057	0.1841	1.0096	0.0045	1.0013	0.9917–1.0109	0.7958
Creatinine	345	1.2250	0.0035	1.0848	0.8580–1.3717	0.4964	1.2504	0.0001	1.1106	0.9120–1.3523	0.2967
Lactate dehydrogenase	345	1.0083	0.0000	1.0103	1.0057–1.0150	0.0000	1.0081	0.0000	1.0077	1.0037–1.0118	0.0002
Albumin	345	0.5633	0.0005	0.6611	0.4469–0.9780	0.0383	0.5826	0.0003	0.7157	0.5016–1.0211	0.0651
Haemoglobin	345	0.8082	0.0216	0.9508	0.7418–1.2187	0.6906	0.7604	0.0013	0.8868	0.7130–1.1030	0.2807
Aspirate plasma cells	345	1.0069	0.2831	1.0050	0.9896–1.0206	0.5272	1.0079	0.1653	1.0081	0.9947–1.0217	0.2361
Bone marrow biopsy plasma cells	345	1.0126	0.0366	1.0138	0.9979–1.0300	0.0894	1.0131	0.0141	1.0083	0.9947–1.0221	0.2342
Number of foal lesions under MRI	345	1.0191	0.0247	1.0238	1.0042–1.0438	0.0173	1.0146	0.0669	1.0171	0.9995–1.0351	0.0570
ITGA6	345	0.7652	0.0057	0.7270	0.5829–0.9068	0.0047	0.8005	0.0136	0.7946	0.6507–0.9703	0.0241
ISS		1.6141	0.0099	0.5505	0.2998–1.0109	0.0542	1.6166	0.0092	0.6503	0.3905–1.0828	0.0981
ISS II	272										
ISS III	73										

MRI magnetic resonance imaging, *EFS* the length of time after primary treatment for a cancer ends that the patient remains free of certain complications or events that the treatment was intended to prevent or delay.

Table 2. C-index of ISS alone, ISS with ITGA6 (GSE24080).

	OS		EFS	
	C-index	C-index (cross-validated)	C-index	C-index (cross-validated)
ISS	0.7588	0.6940	0.7515	0.6858
ISS+ITGA6	0.7647	0.7396	0.7524	0.6972

mRNA and protein levels (Fig. 5b, c). Based on their sequence complementarity, we hypothesised that *ITGA6-AS1* might regulate ITGA6 expression through base-pairing interactions with single strands. To test the hypothesis, we used RNase protection assays to assess the interaction between *ITGA6-AS1* and *ITGA6*. RNase A digested single-stranded RNA, and double-stranded RNA remained that was analysed by qRCR using specific primers (Fig. 5d). Notably, *ITGA6* pre-mRNA and *ITGA6-AS1* indeed formed an RNA duplex at the overlapping part of both transcripts that was protected from degradation (Fig. 5e). It has been reported that an RNA duplex could protect the RNA from RNase degradation and enhance RNA stability.[32] Next, we set out to investigate whether *ITGA6-AS1* affected the *ITGA6* pre-mRNA stability. Our data demonstrated that overexpression of *ITGA6-AS1* elongated the half-life of *ITGA6* pre-mRNA, suggesting that *ITGA6-AS1* is required for the regulation of *ITGA6* pre-mRNA stability (Fig. 5f). These data suggest that an RNA duplex formed between *ITGA6* pre-mRNA and *ITGA6-AS1* affected on the *ITGA6* transcript at the post-transcriptional level.

*ITGA6-AS1* reduced invasion by regulating ITGA6 expression in MM cells

Having shown that *ITGA6-AS1* specifically targets ITGA6 and leads to its increased expression, we decided to investigate the ability of *ITGA6-AS1* to interfere with cell invasion. Towards this end, transwell invasion assays were performed. As expected, the overexpression of *ITGA6-AS1* dramatically downregulated the invasion of U266 and LP-1 cells compared to that of control cells (Fig. 6a). To further evaluate the possibility that *ITGA6-AS1* contributes to the invasion of MM cells by regulating ITGA6, we sought to determine whether the downregulation of ITGA6 reversed the biological activity altered by *ITGA6-AS1*. Our data demonstrated that the downregulation of *ITGA6* in *ITGA6-AS1* overexpressed cells significantly abrogated the impeded invasion of MM cells (Fig. 6b, c, Table S4). Taken together, these data demonstrated that *ITGA6-AS1* affects invasion by regulating ITGA6 expression in MM cells.

DISCUSSION

Multiple myeloma is a neoplasia of plasma cells, hallmarked by clonal proliferation and immunoglobulin overproduction. It may progress from an intramedullary form to PCL.[33] Whole-genome sequencing of multiple myeloma and plasma cell leukaemia patients reveals that seven single-nucleotide variants (SNVs) unique to sPCL samples may contribute to the leukemic transformation from myeloma to sPCL.[34] Global miRNA expression profiling shows significantly different miRNA expression patterns between pPCL and multiple myeloma.[35] Little, however, has been verified about the unique biological roles of the differentially expressed genes between the two diseases. We collected PC data from multicentre, descriptive, cross-sectional studies and conducted the gene expression difference analysis. Particularly, our study aims to identify critical genes that may mediate the MM migration, and further progression to PCL.

The interaction of plasma cells with bone marrow extracellular matrix (BM-ECM) components depends on adhesion molecules.[23] As such, integrins play an important role in the development of malignant plasma cells, including their invasion, proliferation and survival.[22,23,26,36] Accordingly, we focused on the integrin α6 subunit, which presented significant *p*-values and was among the top 8 candidate genes. ITGA6 forms α6β1 and α6β4 integrin complexes with other integrin subunits, and then mediate several biological activities such as embryogenesis, organogenesis, and the invasion of carcinoma cells.[24,25,27,37] In previous studies on
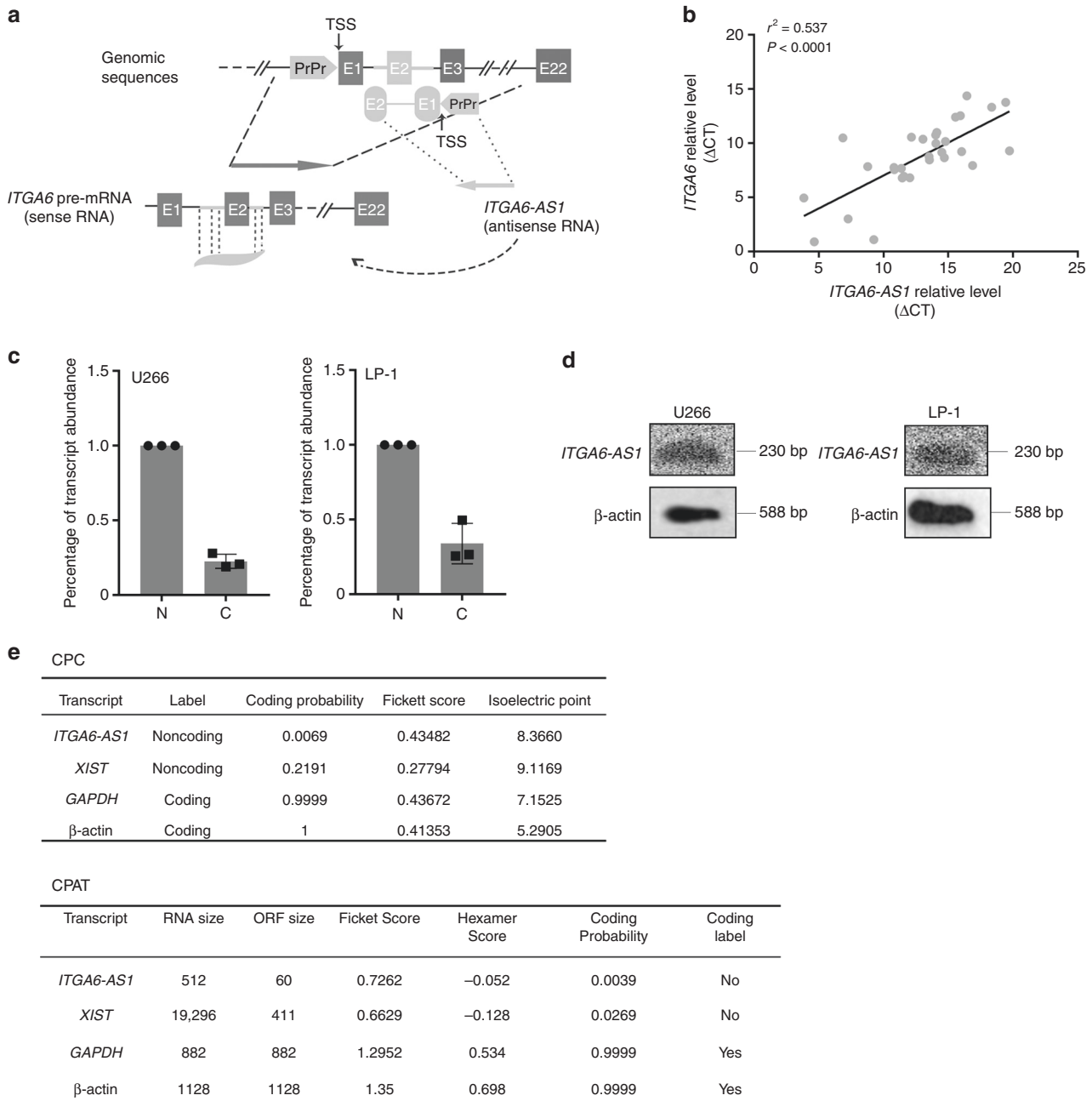
Downregulation of ITGA6 confers to the invasion of multiple myeloma and...
S Song et al.

1848
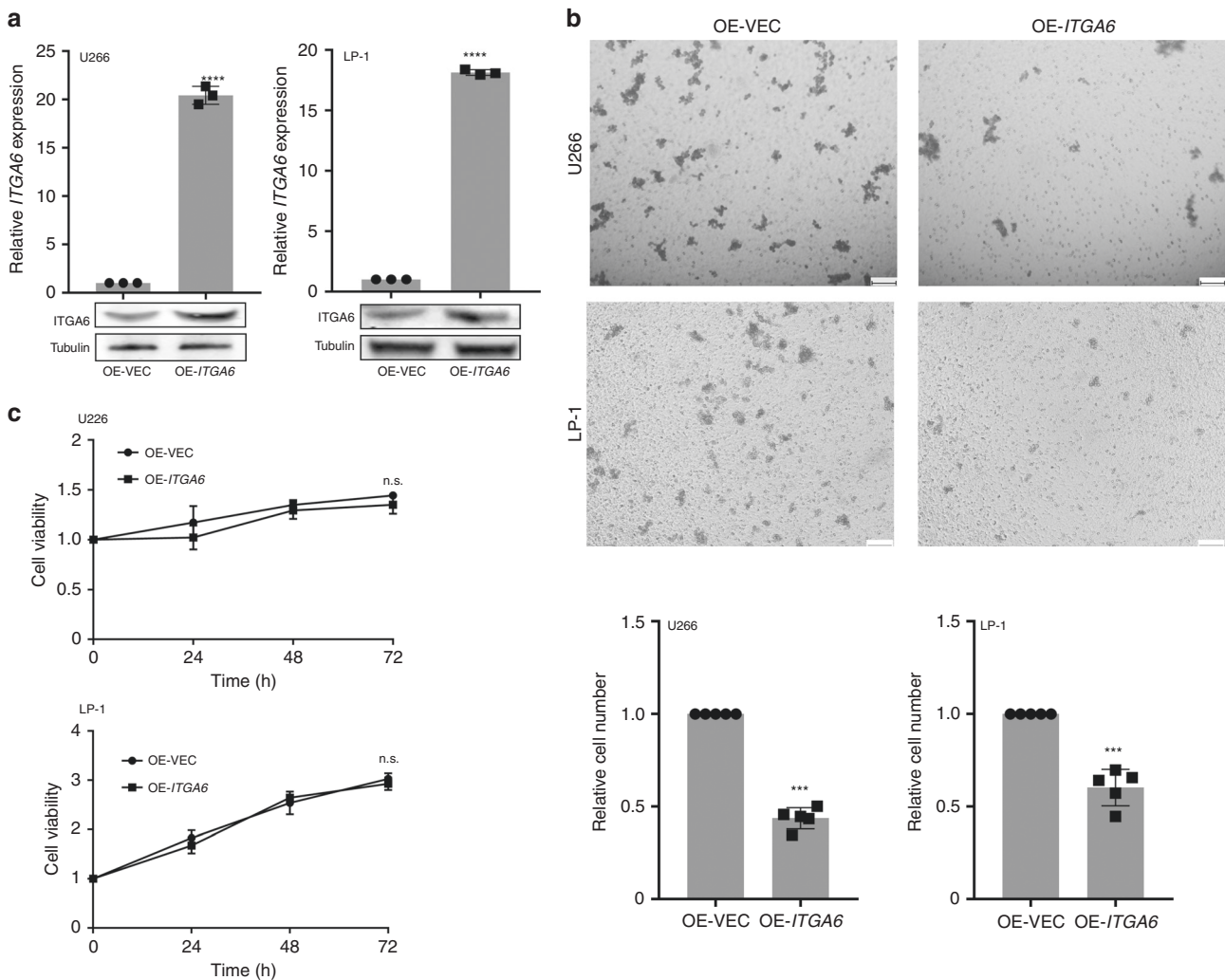
Fig. 3 ITGA6 mediated MM cell invasion. a MM cells were transiently transfected with ITGA6 overexpression vector or empty vector. The levels of ITGA6 were analysed by qPCR and western blot. OE-VEC overexpression empty vector, OE-ITGA6, overexpression of ITGA6. qPCR results are shown as the means ± SDs from three independent experiments ($n = 3$). **b** Upper: Transwell assays were performed to evaluate the invasion of U266 and LP-1 cells overexpressing ITGA6. Scale bar = 100 μm. Lower: statistical graph indicating the number of cells averaged from 5 random high-power fields. The results are presented as the mean ± SD from three independent experiments ($n = 3$). **c** Cell proliferation in U266 and LP-1 cells overexpressing ITGA6 was measured using CCK-8 assays. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns not significant versus control cells ($n = 3$).

breast cancer, ITGA6 was necessary for cell tumourigenicity.[14,26] A recent report indicated that adhesion molecules and extracellular matrix proteins became increasingly downregulated in pPCL compared to non-pPCL MM.[38] Consistent with this research, we found that ITGA6 was expressed at high levels in MM samples and low levels in PCL samples. Our functional experiments demonstrated that ITGA6 acts as a pivotal player in the invasion of MM cells. We overexpressed ITGA6 and identified that it was sufficient to reduce MM cell invasion.

The International Staging System (ISS) are the most extensively used prognostic factors in MM.[13] However, MM patients in the same prognostic group exhibited genetically heterogeneous outcomes. Therefore, combining ISS with molecular markers might develop the prognostic value for MM. Using the Kaplan–Meier analysis with the log-rank test of MM patients, high expression levels of ITGA6 were correlated with good outcomes in regards to EFS in univariate and multivariate analyses, suggesting that ITGA6 influenced MM progression. ITGA6 also predicted EFS in MM patients in an independent manner, improving the predictive value of the ISS for MM. Our study provides evidence of an association between ITGA6 and outcome in MM patients.

Interestingly, patients with high ITGA6 expression in GSE4581 appears poorer prognosis than patients with low ITGA6 expression in GSE24080, suggesting that the prognostic effect can be partially abrogated by treatment. And according to our present study, ITGA6 should be dichotomised at the median based on a low-versus-high expression. The Cox model need to be employed to compute the cut-off values for ITGA6. Together, combined with GEO data and our clinical sample data, we identified the prognostic significance of ITGA6 in MM patients, which requires further validation in other independent prospective MM cohorts.

LncRNAs have been implicated in gene regulation and many cellular processes, such as transcriptional regulation, epigenetic modulation through chromatin modification, scaffolding of nuclear or cytoplasmic complexes, and pairing with other RNAs.[6,39] Here we identified a 521bp lncRNA, ITGA6-AS1, which corresponds to the intron of the ITGA6 gene. Gene expression analysis by qPCR of 30 MM specimens showed a positive correlation between ITGA6 and ITGA6-AS1 expression. Here, we hypothesised that ITGA6-AS1 affected ITGA6 expression through a direct cis-mediated mechanism. The localisation of ITGA6-AS1 transcripts in both the cytoplasm and the nucleus, where this

Downregulation of ITGA6 confers to the invasion of multiple myeloma and...
S Song et al.

1849

a

b

c

d

e

CPC

Transcript	Label	Coding probability	Fickett score	Isoelectric point
ITGA6-AS1	Noncoding	0.0069	0.43482	8.3660
XIST	Noncoding	0.2191	0.27794	9.1169
GAPDH	Coding	0.9999	0.43672	7.1525
β-actin	Coding	1	0.41353	5.2905

CPAT

Transcript	RNA size	ORF size	Ficket Score	Hexamer Score	Coding Probability	Coding label
ITGA6-AS1	512	60	0.7262	−0.052	0.0039	No
XIST	19,296	411	0.6629	−0.128	0.0269	No
GAPDH	882	882	1.2952	0.534	0.9999	Yes
β-actin	1128	1128	1.35	0.698	0.9999	Yes

Fig. 4   Characterisation of the lncRNA *ITGA6-AS1*. a Genomic organisation of *ITGA6* and *ITGA6-AS1*. *ITGA6* and *ITGA6-AS1* are transcribed from opposite strands of the same region on chromosome 2. **b** The expression levels of *ITGA6* and *ITGA6-AS1* were detected in 30 MM samples and subjected to correlation analysis. Data were normalised to *GAPDH* expression and were shown as ∆CT (threshold cycle). **c** qPCR analysis of *ITGA6-AS1* purified from nuclear and cytoplasmic compartments in U266 and LP-1 cells. Data are shown as the means ± SDs. **d** Northern blot analysis with in vitro transcribed strand-specific RNA probes for *ITGA6-AS1* was performed with U266 and LP-1 cells. β-Actin served as a loading control. **e** In silico analysis of *ITGA6-AS1* coding potential using the Coding Potential Calculator (CPC) and the Coding Potential Assessment Tool (CPAT). As shown by the CPC Coding probability, the score indicates the quality of a predicted ORF, and the higher the score is, the higher the quality is. In the CPAT, analysis was performed using the default settings for human sequences and coding probabilities below 0.364 were considered to indicate non-coding sequences.

lncRNA can affect transcription and pre-mRNA processing, lend support to this hypothesis. Remarkably, our data indicated that *ITGA6-AS1* formed an RNA duplex with *ITGA6* pre-mRNA, which affected the *ITGA6* pre-mRNA stability and the invasion of MM cells.

Even though the overall expression of *ITGA6* in MM is high, there are still 7.7% of MM patients (GSE2113) and 5.4% of MM patients (GSE39925) whose *ITGA6* expression level is lower than the mean level of PCL. And this suggested that low *ITGA6*
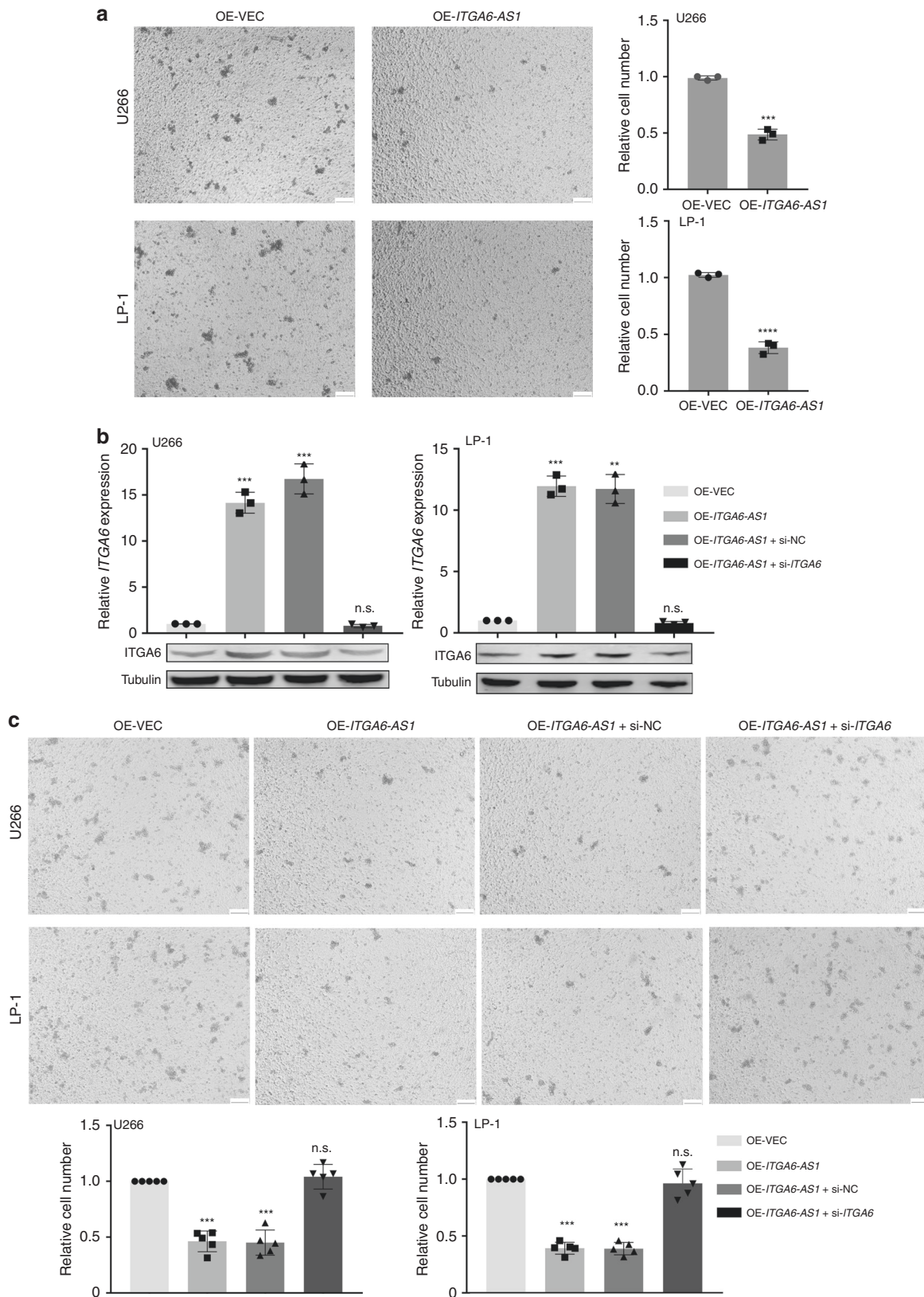
expression might not be the unique factor contributing to PCL transformation. As shown in Fig. 1d, besides *ITGA6*, significant changes in *TAGLN2*, *PPBP*, *NXPE4*, *OSBPL10*, *STAP1*, *DEFA1B*, *IGK* level were identified. Further studies are needed to investigate the significance of those genes in the process of PCL transformation.

In summary, the findings from this study supported essential roles of the integrin, ITGA6 in mediating MM cell metastasis. Our study elucidated a new mechanism underlying the correlation between ITGA6 and *ITGA6-AS1* that provided new insights into the

Downregulation of ITGA6 confers to the invasion of multiple myeloma and…
S Song et al.

1850
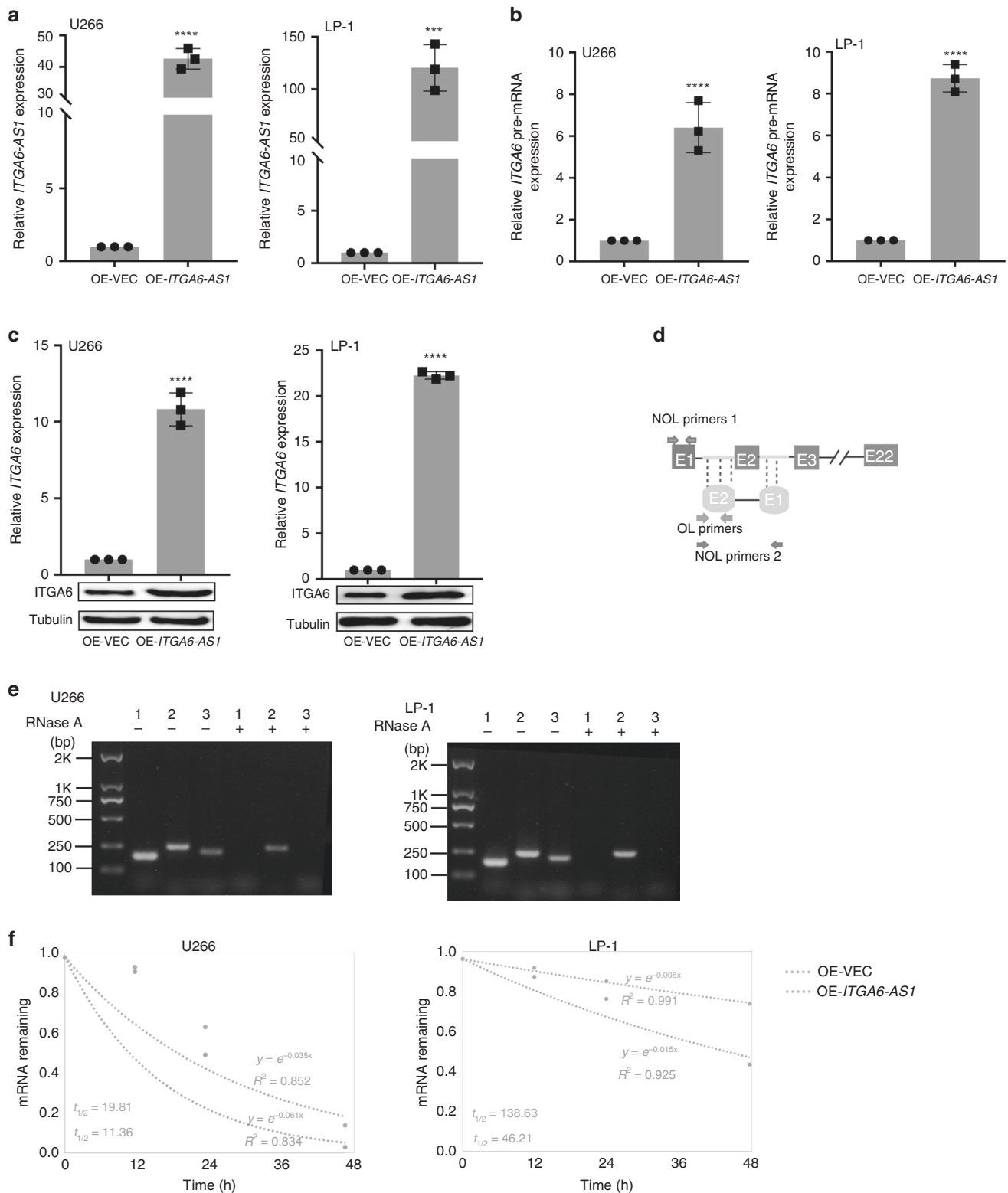
Fig. 5 ITGA6-AS1 and ITGA6 pre-mRNA may form an RNA duplex. a Cells were treated with *ITGA6-AS1* overexpression vector (OE-*ITGA6-AS1*) or empty vector, and then the expression of *ITGA6-AS1* was analysed by qPCR. Data are shown as the means ± SD and normalised to *GAPDH* expression. **b** *ITGA6* pre-mRNA expression was analysed by qPCR in cells treated with *ITGA6-AS1* overexpression vector or empty vector. **c** The expression of ITGA6 was analysed by qPCR and western blotting in cells treated with *ITGA6-AS1* overexpression vector or empty vector. **d** The primers used with RPA assays in RNA samples. NOL primers non-overlapping primers, OL primers overlapping primers. **e** The qPCR results from three sets of primers covering overlapping or non-overlapping regions of *ITGA6* pre-mRNA and *ITGA6-AS1* are shown. The overlapping regions of *ITGA6* pre-mRNA and *ITGA6-AS1* were protected from degradation by RNase A, suggesting that an RNA duplex was formed. (1) non-overlapping region in *ITGA6* pre-mRNA; (2) overlapping region; (3) non-overlapping region in *ITGA6-AS1*. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control cells. **f** U266 or LP-1 cells was transiently transfected with vector control or overexpression of *ITGA6-AS1*. The stability of *ITGA6* pre-mRNA was measured. $t_{1/2}$, the half-life.

Downregulation of ITGA6 confers to the invasion of multiple myeloma and...
S Song et al.

1851

Fig. 6 *ITGA6-AS1* reduced invasion by regulating ITGA6 expression in MM cells. a The invasion of cells in transiently transfected with vector control or overexpression of *ITGA6-AS1* was detected by transwell invasion assays. Representative images are shown, and invasive cells were counted. Scale bar, 100 μm. The results are presented as the mean ± SD from three independent experiments ($n = 3$). **b** The expression of ITGA6 was detected in each treatment group by qPCR and western blot. The results are presented as the mean ± SD from three independent experiments ($n = 3$). **c** The changes in invasion following transfection with *ITGA6-AS1* overexpression vector alone or in combination with *ITGA6* siRNA were investigated through transwell assays. Cells were counted, and representative images was shown ($n = 3$). Scale bar, 100 μm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns not significant versus control cells.

Downregulation of ITGA6 confers to the invasion of multiple myeloma and...
S Song et al.

1852

efficient prevention of and therapeutic strategies for MM. More importantly, our results suggested that ITGA6 may improve survival prediction and enhance the risk factor-based stratification provided by ISS in MM patients.

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Downregulation of ITGA6 confers to the invasion of multiple myeloma and...
S Song et al.

1853

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