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Correlation of *DLC1* gene methylation with oncogenic *PIK3CA* mutations in extramammary Paget's disease

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Extramammary Paget's disease is a rare cutaneous malignant neoplasm. The genetic and epigenetic mechanisms underlying its pathology remain unknown. In this study, we investigated the expression levels, and mutation and methylation status of a common tumor suppressor gene, deleted in liver cancer 1 (*DLC1*), and an oncogene, *PIK3CA*, in tumor ($n=132$) and normal tissues ($n=20$) from unrelated patients. The presence of epigenetic and genetic lesions was then correlated to the patient pathology data to determine the potential role of these genes in extramammary Paget's disease etiology and progression. The *DLC1* gene was found to be downregulated in 43 (33%) tumors, as compared with immunohistochemistry results from normal tissues. Methylation-sensitive, high-resolution melting analysis indicated that the *DLC1* promoter was hypermethylated in 51 (39%) extramammary Paget's disease tumors. This hypermethylation was associated with significantly decreased DLC1 levels ($P=0.011$), and had a strong positive correlation with advanced age ($P=0.002$). *PIK3CA* mutations were detected by direct sequencing in 32 (24%) tumors, the majority of which were invasive. Furthermore, *PIK3CA* mutations significantly correlated with *DLC1* hypermethylation. Thus, aberrant *DLC1* methylation and *PIK3CA* mutations may have important roles in extramammary Paget's disease pathogenesis, and may represent potential molecular targets for therapy.
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Extramammary Paget's disease is a relatively rare cutaneous malignant neoplasm, which was first described by Crocker in 1889.[1] Since then, many additional cases have been reported, providing general insights into the clinical and pathological characteristics of this rare condition. Extramammary Paget's disease most frequently involves skin with a high density of apocrine sweat glands, such as the genitoperineal region and axilla, but cases have also been reported for other skin regions and mucosa.[2] Clinical presentation often includes pruritus and an eczema-like lesion on the surface of the involved skin region.[3] Epidemiological studies have reported that the majority of extramammary Paget's disease patients are older adults, ranging in age from 50 to 80 years old and having an average age of 65 years.[4] Caucasian female patients are the most frequently reported population; however, in Asian populations, there is a male predominance for this disease.[4,5] Although some familial extramammary Paget's disease cases have been reported,[6] the epigenetic and genetic abnormalities that contribute to the pathogenesis of this rare disease remain poorly understood.

The deleted in liver cancer 1 (*DLC1*) gene, located on chromosome 8p21–8p22, encodes a regulator of

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the Rho family of small GTPases.[7] The principal function of DLC1 is to catalyze the conversion of active guanosine triphosphate-bound RhoA to the inactive guanosine biphosphate-bound form. As this enzymatic mechanism results in a negative regulatory effect on cell growth and tumorigenesis, *DLC1* is considered a tumor suppressor gene.[8,9] Loss of DLC1 expression, due to chromosomal deletion or promoter hypermethylation, has been detected in 50% of human hepatocellular carcinomas, and in many other human malignancies, including breast, prostate, colon, and lung.[10–13] Accordingly, therapeutic upregulation of DLC1 expression in cancer cells has been proposed as a novel method to induce apoptosis, and inhibit cell proliferation and colony formation,[14,15] and to restrain cell migration and suppress metastasis.[16–18]

A key factor in the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway is PIK3CA. The *PIK3CA* gene mutations have been implicated in perturbed events of cell metabolism, growth, and survival.[19–22] Several somatic mutations in the *PIK3CA* locus have recently been identified and found to occur at significant frequencies in various types of human cancer.[23–26] More than 80% of these mutations are clustered in exon 9, which encodes the protein's helical domain, and in exon 20, which encodes the kinase domain.[27] However, correlation analysis of the *PIK3CA* genetic alterations to determine their clinicopathological significance for extramammary Paget's disease has not yet been reported in the literature.

A recent study identified a statistically significant association between increased *PIK3CA* levels in tumors and promoter methylation of some tumor suppressor genes.[28] In addition, previous evidence revealed that the activated PI3K/AKT pathway could negatively regulate the expression of DLC1 protein in tumors derived from hepatocyte-specific *PIK3CA* transgenic mice.[29] Thus, a functional interaction likely exists between DLC1 silencing and PI3K/AKT signaling activation in tumor cells.

The aim of our study, therefore, was to investigate the methylation status of *DLC1* and the presence of *PIK3CA* mutations in extramammary Paget's disease patients. The results of our study not only provide novel insights into the pathogenic roles of epigenetic and genetic events in extramammary Paget's disease, but also correlate such events with the clinicopathological features of the disease.

Materials and methods

Clinical Samples

Tumor tissues were biopsied from 132 unrelated patients diagnosed with extramammary Paget's disease, and treated in the Dermatology Department of Huashan Hospital (Shanghai, China) between 2005 and 2010. The patient population consisted of 110 males and 22 females, with an average age of 69.1 years (range: 47–92). The study protocol was approved by the Ethics Committee of Huashan Hospital, and all study participants provided written informed consent before study inclusion. The clinical and demographic data of all patients were reviewed by two dermatologists. To confirm the pre-surgery extramammary Paget's disease diagnosis, the histological sections from all cases were evaluated by hematoxylin and eosin staining to detect Paget cells, which are large, irregularly shaped cells with clear cytoplasm that are the key feature of extramammary Paget's disease.[30] In addition, histological examination of sequential tumor sections was used to confirm that each specimen contained more than 50% tumor cells. The histological features of invasion were used to stratify the extramammary Paget's disease cases as noninvasive ($n=94$) or invasive ($n=38$). Normal skin specimens ($n=20$) were collected from the penises of 20 of the extramammary Paget's disease patients (age-, and ethnicity-matched with the patients described above) by peritomy, and were used as normal noncancerous tissue controls.

Methylation-Sensitive, High-Resolution Melting Analysis

Genomic DNA was extracted from each formalin-fixed, paraffin-embedded dermatic biopsy section by using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The purified DNA was chemically modified by using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions, to detect methylation sites. Briefly, DNA exposure to sodium bisulfite converted all unmethylated cytosines to uracils, whereas leaving methylated cytosines unaltered.

A methylated reference sample, the CpGenome Universal methylated human male genomic DNA (Chemicon, Billerica, MA, USA), and an unmethylated reference sample, genomic DNA isolated from the peripheral blood mononuclear cells of a healthy male individual, were subjected to the bisulfite modification procedure and used as control standards. To create a standard dilution range of methylated and unmethylated alleles, the above two controls were mixed in 0, 1, 10, 30, 50, 80, and 100% ratios of methylated to unmethylated references. Each of the methylation-sensitive, high-resolution melting analysis experimental runs included these seven methylated to unmethylated standard dilutions. The PCR amplification and high-resolution melting analysis were carried out sequentially. The PCR amplification reaction mixture (20 μl) was composed of 50 ng bisulfate-treated template, 10 μl Premix *Taq* Hot Start polymerase (TaKaRa, Dalian, China); 0.25 mM primers (forward: 5′-TCGTTACGGTTTTAGAAAGAAA-3′ and reverse: 5′-TTCGCTCCCAACCAAAACATAA-3′), and 5 mM SYTO-9 intercalating dye (Invitrogen, Carlsbad, CA,

USA). The thermal-cycling reaction was performed on a 9700 GeneAmp PCR system (Applied Biosystems, Carlsbad, CA, USA) using the following conditions: 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C, and a final extension at 72 °C for 5 min. Immediately after the PCR amplification, high-resolution melting analysis was performed on a Roter-Gene Q real-time PCR cycler (Qiagen) using a +0.1 °C/s temperature-ramping gradient from 72 to 90 °C. Fluorescence acquisition was set as recommended by the manufacturer. The *DLC1* methylation level was quantitated by analyzing the PCR-product melting curves with the accompanying Rotor-Gene Q software.

Bisulfite DNA Sequencing

PCR primers were designed as previously described by Guan *et al*,[11] to amplify a 292 bp region of the *DLC1* promoter that encompasses 35 CpGs (Figure 1a). The PCR product was then subcloned into the pMD19-T expression vector by using a TA Cloning Kit (TaKaRa). Recombinant plasmids were sent to MAP Biotech (Shanghai, China) to complete the bisulfite DNA-sequencing procedure.

Immunohistochemistry

Immunohistochemical (IHC) studies were performed on 5-μm-thick sections of formalin-fixed, paraffin-embedded extramammary Paget's disease and normal tissue samples using standard techniques. The primary antibodies used targeted cytokeratin 7 (CK7, 1:100 dilution; Dakocytomation, Carpinteria, CA, USA), carcinoembryonic antigen (CEA, 1:100; Dakocytomation), and DLC1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Biotinylated secondary antibodies of rabbit anti-mouse IgG (1:50) and HRP enzyme conjugate (all from MultiScience Biotech, Shanghai, China) were used to amplify and detect immunoreactivity. The 3,3′-diaminobenzidine chromogen substrate was used to visualize the results. All IHC analyses were carried out by a technician who was blinded to the clinical information. The CK7 and CEA expression (in cytoplasm and on the cell membrane) was defined as 'positive' if >10% of the cells showed immunostaining. Cytoplasmic expression of DLC1 was defined as 'reduced' if <25% of the cells were immunostained; otherwise, the sample was defined as having 'normal' DLC1 expression.

PIK3CA Mutation Analysis

Purified genomic DNA from all samples were subjected to PCR to amplify the *PIK3CA* exon 9 and exon 20 regions, according to the method previously described by Moroni *et al*.[31] The PCR products were sent to MAP Biotech for direct DNA sequencing to detect the mutation status in each exon region for each sample.

Statistical Analysis

The χ^2-test and Fisher's exact test were used to evaluate the association of *DLC1* methylation status and *PIK3CA* mutations with the patients' clinical data. All statistical procedures were carried out by using the STATA 10.0 software package (StataCorp, College Station, TX, USA). A two-sided *P*-value of <0.05 was set as the threshold for statistical significance.

Results

Methylation Levels of DLC1 CpG Islands in Extramammary Paget's Disease Tumors

To evaluate the *DLC1* methylation levels in extramammary Paget's disease tumors, we examined promoter hypermethylation by using the methylation-sensitive, high-resolution melting procedure. Methylation-sensitive, high-resolution melting analysis was implemented by initially constructing a series of normalized melting profiles for samples with different ratios of methylated to unmethylated template. These profiles were then used to generate a standard curve by which the methylation status of the experimental samples could be quantified (Figure 2). Using this method, 51 (39%) of the total 132 extramammary Paget's disease tumor samples that were methylated on the *DLC1* promoter were identified. Moreover, the samples with methylation had >1% of the promoter sequence methylated. Methylation-sensitive, high-resolution melting analysis of the 20 normal skin DNA samples detected no methylation in the *DLC1* promoter. Therefore, the cancer samples were sub-classified into two groups: methylation-negative (<1% methylation) and methylation-positive (1–100% methylation). Correlation analysis of the two *DLC1* methylation level subgroups indicated that a higher methylation level was significantly associated with advanced patient age ($P=0.002$; Table 1). In contrast, methylation level was not significantly associated with sex or extramammary Paget's disease invasion ($P=0.729$ and $P=0.360$, respectively; Table 1).

Bisulfite DNA Sequencing

Bisulfite DNA sequencing was employed to determine the comprehensive extramammary Paget's-disease-related methylation pattern of the 5′-CpG islands in the *DLC1* promoter. A 292-bp fragment in the *DLC1* promoter, covering 35 CpG dinucleotides, was amplified from a set of 6 extramammary Paget's disease samples that were identified by methylation-sensitive, high-resolution melting analysis as methylated and from a normal skin tissue sample (N1). Bisulfite DNA sequencing showed that the

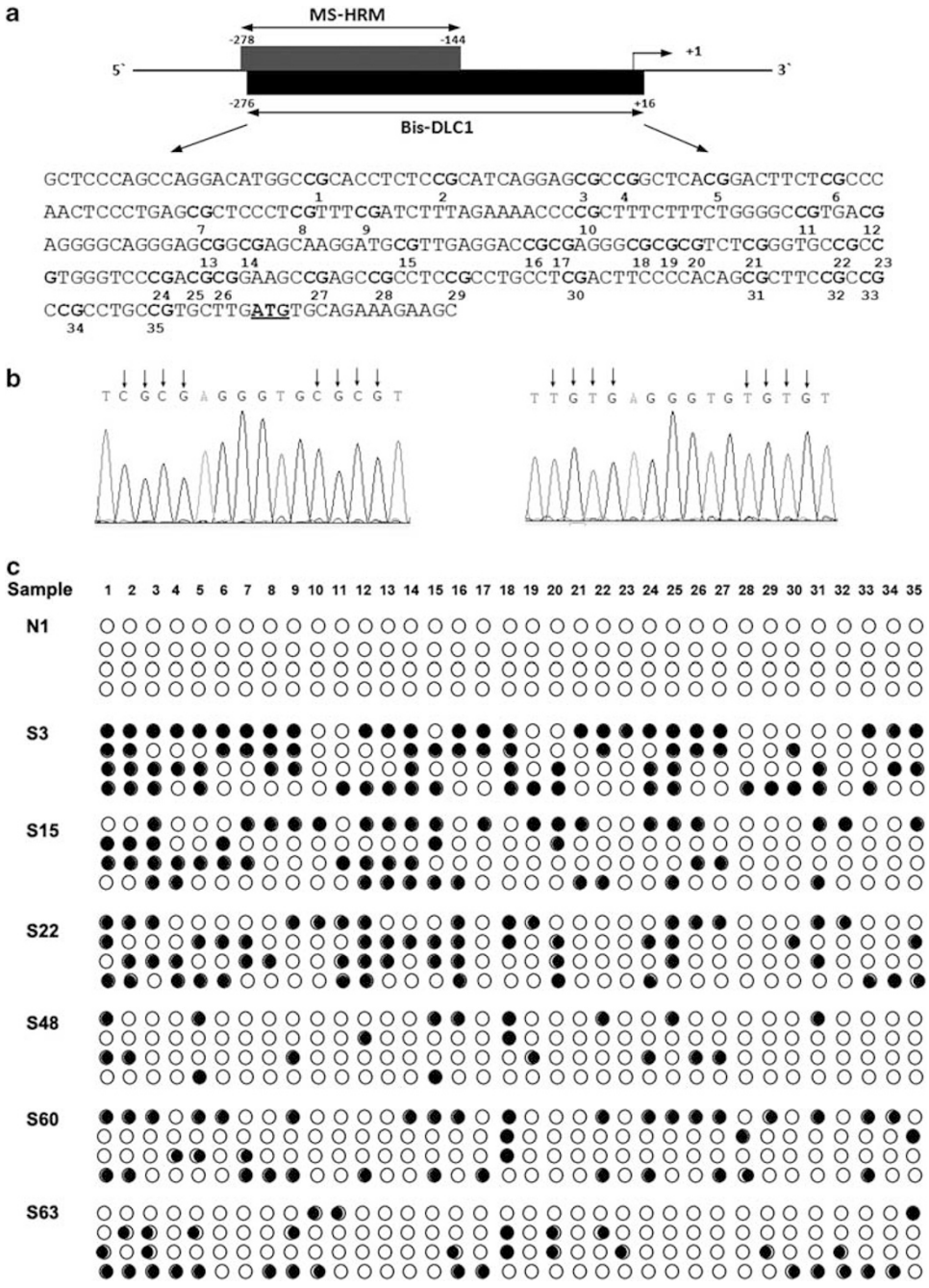

Figure 1 Methylation status of the CpG island region in the deleted in liver cancer 1 (*DLC1*) promoter of extramammary Paget's disease patients. (**a**) Schematic depiction of the *DLC1* promoter-associated CpG island, which spans the region from −278 to +16, with respect to the ATG start site (+1). Regions analyzed by MS-HRM and bisulfite genomic sequencing (Bis-*DLC1*) are shown. The Bis-*DLC1* region encompassed 292 bp and contained 35 CpG dinucleotides. (**b**) Examples of a highly methylated *DLC1* CpG island (sample S3, left) and an unmethylated CpG island (sample N1, right), as determined by bisulfite sequencing analysis. Arrows indicate the positions of CpG dinucleotides. (**c**) Methylation patterns of the Bis-*DLC1* region of the *DLC1* CpG island in a normal tissue sample, and six extramammary Paget's disease tumors and hyperplasia samples that were identified by MS-HRM as methylated. Methylated and unmethylated CpG sites are shown as solid circles and open circles, respectively.

CpG islands in normal skin samples were unmethylated, and that there was frequent methylation in the six extramammary Paget's disease samples (Figure 1c). Although each of the six tumor samples shared some common methylation sites, the overall methylation patterns were distinct.

IHC Expression

The CK7 and CEA expression are key pathological features of Paget cells, and IHC detection of these two proteins is generally considered an accurate diagnostic method for extramammary Paget's

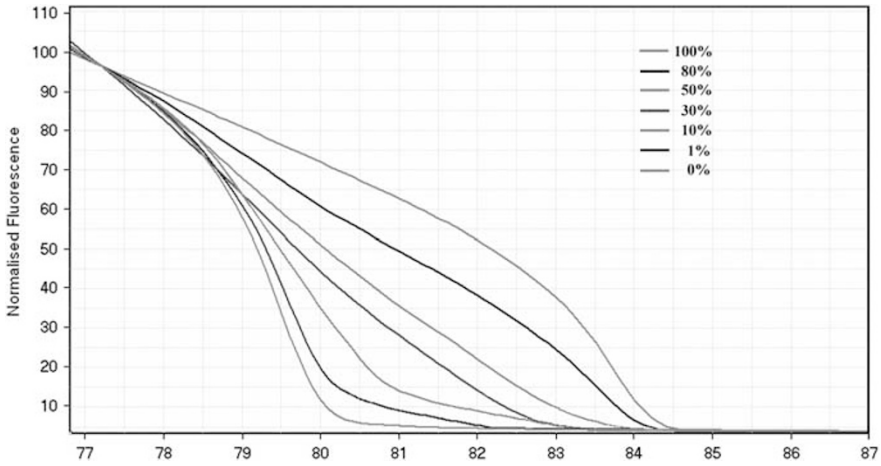

Figure 2 The normalized methylation-sensitive, high-resolution melting analysis standard curve used to evaluate methylation levels of extramammary Paget's disease cases.

Table 1 *DLC1* methylation and protein levels in extramammary Paget's disease

	Total	*Methylation level*[a]		P-*value*[b]	*Protein level*[c]		P-*value*[b]
		0–1%	*1–100%*		*Normal*	*Reduced*	
Case, *n*	132	81	51	—	89	43	—
Age, years							
>70	60	26	31	0.002	34	26	0.016
≤70	72	52	20	—	55	17	—
Sex							
Male	110	67	43	0.729	78	38	0.352
Female	22	13	7	—	17	5	—
Invasion status							
Invasive	38	21	17	0.360	23	15	0.282
In situ	94	60	34	—	66	28	—

Abbreviations: DLC1, deleted in liver cancer 1; IHC, immunohistochemistry.
[a]Detected by methylation-sensitive, high-resolution melting analysis.
[b]Determined by χ^2-test.
[c]Detected by IHC.

disease. In our study, all extramammary Paget's disease samples showed positive CK7 and CEA staining by IHC (Figure 3). In addition, the IHC findings were used to evaluate the potential correlation between DLC1 protein expression and the methylation status of the *DLC1* promoter. The IHC staining indicated that DLC1 was reduced in 43 (33%) of the 132 tumor samples. Moreover, a significant correlation was found between DNA methylation of CpG islands in the *DLC1* promoter and reduced DLC1 protein expression in the extramammary Paget's disease tumor samples ($P=0.011$; Table 2).

Strong immunostaining for DLC1 was observed in all normal skin tissue samples. These samples were considered to represent the baseline level of DLC1 expression, and all of these samples exhibited an absolute lack of methylation in the *DLC1* promoter region. In contrast, the tumor tissues were heterogeneous, having methylated and unmethylated *DLC1* alleles (Figure 1a).

PIK3CA Mutations in Extramammary Paget's Disease

To determine the prevalence of *PIK3CA* mutations in extramammary Paget's disease patients, we analyzed the *PIK3CA* exon 9 and exon 20 coding sequences, both of which are known as mutation hotspots and include the frequently mutated codons 542, 545, and 1047. Of the 35 mutations that were detected in *PIK3CA* of the 132 patients in our study, 25 cases were detected in exon 9, which encodes the helical domain of PI3K (5 cases with c.1624G>A, 1 c.1633G>A, and 20 c.1634A>C), and 10 cases were detected in exon 20, which encodes the catalytic domain of PI3K (2 c.3296C>T and 8 c.3297A>G; Table 3). Although mutations were found in only 32 (24%) of the extramammary Paget's disease cases, the correlation between *PIK3CA* mutations and *DLC1* methylation was significant ($P=0.001$). In addition, *PIK3CA* mutations were found to be significantly associated with invasive extramammary Paget's disease ($P=0.006$).

Discussion

The rarity of extramammary Paget's disease has precluded its comprehensive characterization. As the majority of studies to date have focused on IHC detection of one or several proteins,[32,33] our knowledge of the genetic and epigenetic features of extramammary Paget's disease remain largely unknown. In this study, we collected extramammary Paget's disease tumor samples from 132 unrelated

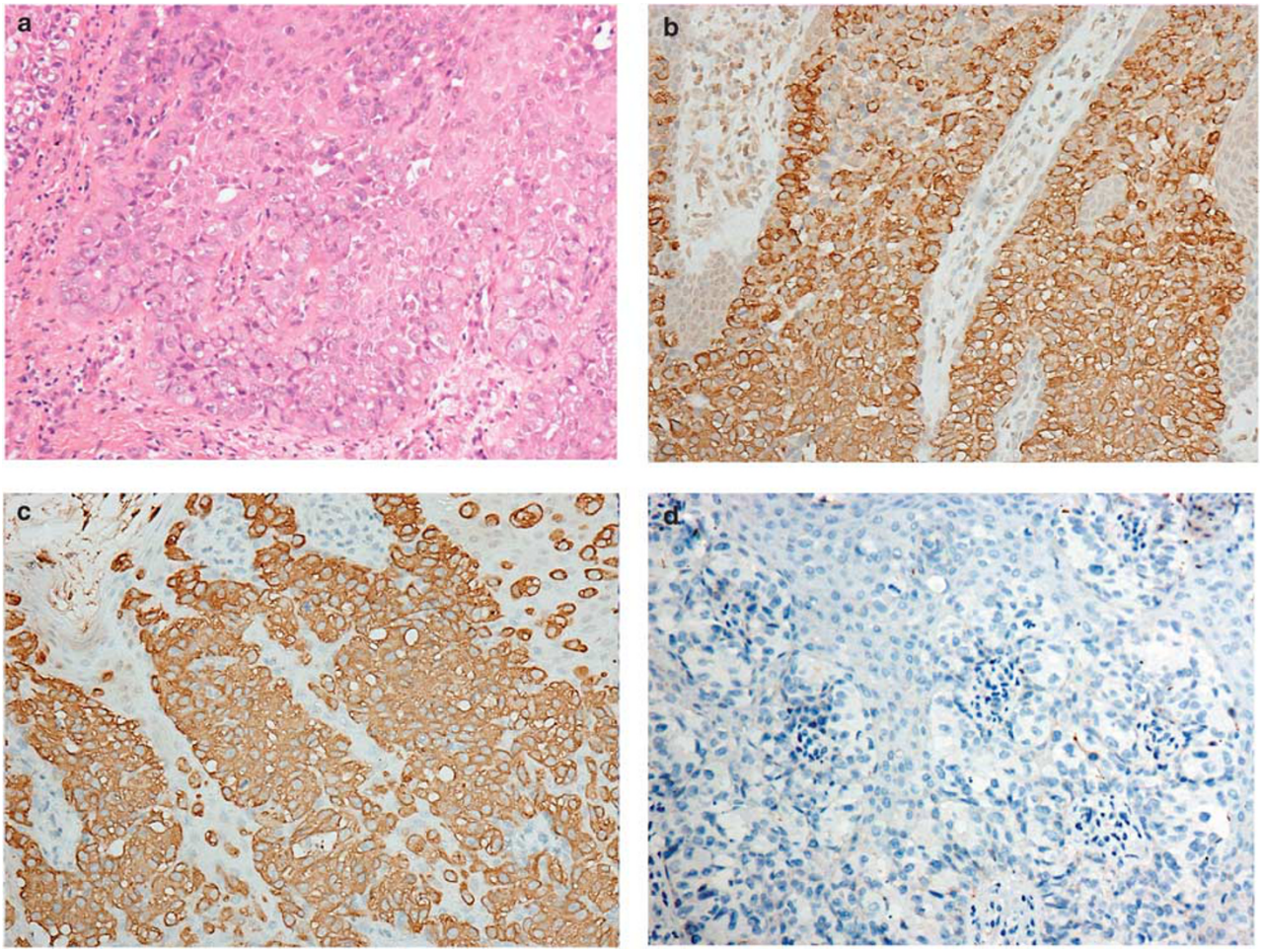

Figure 3 Immunohistochemical analysis of the deleted in liver cancer 1 (DLC1) protein expression in extramammary Paget's disease. (**a**) Extramammary Paget's disease skin pathological section stained with hematoxylin and eosin staining (× 200). (**b**) DLC1 protein expression in a normal skin tissue was localized to the cytoplasm (× 200). (**c**) DLC1 protein expression in an extramammary Paget's disease tissue with a 0% methylation level was localized to the cytoplasm (× 200). (**d**) DLC1 protein expression was completely undetectable in an extramammary Paget's disease tissue with 50–80% methylation (× 200).

Table 2 *DLC1* methylation and protein expression levels in extramammary Paget's disease patients with *PIK3CA* mutations

Methylation level	DLC1 *protein*		P-*value*[a]	PIK3CA *mutations*		P-*value*[a]
	Reduced	*Normal*		*Positive*	*Negative*	
>1%	23	28	0.011	20	31	0.001
<1%	20	63		12	69	

Abbreviation: DLC1, deleted in liver cancer 1.
[a]χ^2-test.

patients over a 5-year period. Our China-based study population accurately reflected the previously reported male bias for extramammary Paget's disease in Asians; specifically, our patients represented a male to female ratio of 1:5. To the best of our knowledge, the set of extramammary Paget's disease patients collected for evaluation from our institute represents the largest set ever reported on in a single study. Twenty normal skin tissues were collected from the same patients with extramammary Paget's disease; it is important to note that this small number of controls, as compared with the experimental sample number, represents a limitation of the present study.

In this study, we found a high prevalence of downregulation and hypermethylation of the *DLC1* gene in cases of this rare disease (33 and 39%, respectively). In addition, activating *PIK3CA* mutations were detected in 24% of the extramammary Paget's disease cases. Taken together, these findings provide solid evidence that epigenetic and genetic alterations are common in extramammary Paget's disease and may be involved in the disease pathogenesis.

Rho GTPases are proteins with pleiotropic cellular functions, including the regulation of actin

Table 3 *PIK3CA* mutations detected in extramammary Paget's disease cases

Case	*Age/sex*	*Tumor*	PIK3CA *mutations*	
			Nucleotide mutation	*Amino acid substitution*
1	74/F	*In situ*	c.3297A>G	p.His1047Arg
2	72/M	*In situ*	c.1634A>C	p.Glu545Ala
3	84/F	*In situ*	c.1624G>A	p.Glu542Lys
4	79/M	*In situ*	c.1634A>C	p.Glu545Ala
5	63/M	*In situ*	c.1624G>A, c.1634A>C	p.Glu542Lys, p.Glu545Ala
6	59/F	*In situ*	c.1634A>C	p.Glu545Ala
7	78/M	*In situ*	c.1634A>C	p.Glu545Ala
8	54/M	*In situ*	c.3297A>G	p.His1047Arg
9	70/M	*In situ*	c.1634A>C	p.Glu545Ala
10	63/M	*In situ*	c.1634A>C, c.3297A>G	p.Glu545Ala, p.His1047Arg
11	76/M	*In situ*	c.1634A>C	p.Glu545Ala
12	79/M	*In situ*	c.1634A>C	p.Glu545Ala
13	63/M	*In situ*	c.1634A>C	p.Glu545Ala
14	81/M	*In situ*	c.1634A>C	p.Glu545Ala
15	73/M	*In situ*	c.1634A>C	p.Glu545Ala
16	79/M	*In situ*	c.1634A>C	p.Glu545Ala
17	72/M	*In situ*	c.1624G>A	p.Glu542Lys
18	54/M	Invasive	c.1634A>C	p.Glu545Ala
19	59/M	Invasive	c.1634A>C	p.Glu545Ala
20	75/M	Invasive	c.1634A>C, c. 3296C>T	p.Glu545Ala, p.His1047Tyr
21	58/M	Invasive	c.1633G>A	p.Glu545Lys
22	70/M	Invasive	c.3297A>G	p.His1047Arg
23	58/M	Invasive	c.1634A>C, c.3297A>G	p.Glu545Ala, p. His1047Arg
24	89/M	Invasive	c.3297A>G	p.His1047Arg
25	75/M	Invasive	c.3296C>T	p.His1047Tyr
26	81/M	Invasive	c.1624G>A	p.Glu542Lys
27	70/M	Invasive	c.3297A>G	p.His1047Arg
28	62/M	Invasive	c.1634A>C	p.Glu545Ala
29	80/M	Invasive	c.1634A>C	p.Glu545Ala
30	62/F	Invasive	c.1634A>C	p.Glu545Ala
31	66/M	Invasive	c.3297A>G	p.His1047Arg
32	79/M	Invasive	c.1624G>A	p.Glu542Lys

Abbreviations: F, female; M, male.

cytoskeletal dynamics, proliferation, migration, and invasion. Thus, deregulation of Rho activity is involved in oncogenic transformation of cells.[34] Accumulating evidence has recently indicated that DLC1 may act as a tumor suppressor by inhibiting Rho signaling. Moreover, loss of DLC1 expression has been clinically detected in many types of human tumors. Herein, we reported that reduced DLC1 expression significantly correlated with *DLC1* promoter methylation ($P=0.011$). Surprisingly, our data also showed that hypermethylation of *DLC1* occurred preferentially in older subjects (>70 years of age). Given the known association between aging and extramammary Paget's disease,[3,35] we speculate that *DLC1* methylation has an active role in neoplastic transformation. It is possible that age-dependent methylation events involving the *DLC1* promoter may represent an important underlying mechanism that links aging and extramammary Paget's disease. Nonetheless, our findings demonstrated that epigenetic abnormality of the *DLC1* promoter (hypermethylation) is a common and major mechanism for the silencing of this gene in extramammary Paget's disease, and may represent an important oncogenic inducer.

Previous studies have suggested that persistent activation of AKT is a frequent finding in extramammary Paget's disease.[36,37] Presently, we detected *PIK3CA* mutations in a large series of extramammary Paget's disease tissue samples to investigate whether the mutation is involved in the pathogenesis of this disease. Our data indicated that 24% of patients had *PIK3CA* mutations, suggesting that the PI3K/AKT pathway is constitutively activated in these individuals and might contribute to the development of extramammary Paget's disease. This finding led us to focus our attention on the role of *PIK3CA* mutations in the etiology and progression of extramammary Paget's disease. By performing a correlative analysis of the clinical data of patients with the *PIK3CA* mutation status, we found that individuals harboring *PIK3CA* mutations are more likely to develop invasive extramammary Paget's disease. It has been reported that dermal invasion in extramammary Paget's disease may be the most significant risk factor for death.[38] Unfortunately, we do not have follow-up data on each of our extramammary Paget's disease patients and are unable to determine the survival rates of those with invasive extramammary Paget's disease and *PIK3CA* mutations.

Nonetheless, a key biological implication of our study is that targeted molecular therapies to inhibit the PI3K/ATK signaling pathway may provide a new strategy for treating invasive extramammary Paget's disease.

As noted earlier in this report, nearly 80% of cancer-related *PIK3CA* mutations occur at three hotspots in the gene: Glu542Lys and Glu545Lys located within the helical domain, and His1047Arg located within the kinase domain.[27] These mutations are known to result in elevated catalytic activity, which leads to oncogenic transformation.[39] However, in our study, the Glu545Ala (c. 1634A > C) mutation in exon 9 of *PIK3CA* showed a high frequency in *PIK3CA* mutation-positive extramammary Paget's disease cases (20 of 32), as compared with only 5 cases with the Glu542Lys, 1 case with Glu545Lys, 2 cases with His1047Tyr, and 8 cases with His1047Arg. To date, very few reports of human cancers have identified the Glu545Ala mutation.[40–42] Yet, in our study, its frequency was even higher than the three mutation hotspots, suggesting that the Glu545Ala mutation may contribute to some distinct biological characteristics of Paget cells, possibly even supporting the distinctive properties of extramammary Paget's disease. However, it remains unknown precisely how the Glu545Ala mutation influences the pathogenesis and progression of Paget cells.

Kudo *et al*[29] reported that *DLC1* was downregulated in tumors derived from hepatocyte-specific *PIK3CA* transgenic mice, indicating that *DLC1* is negatively regulated by activation of the PI3K/AKT pathway. Consistent with those findings, our results revealed that a strong correlation exists between *PIK3CA* mutation and *DLC1* methylation ($P = 0.001$), highlighting the interaction between genetic and epigenetic alterations in extramammary Paget's disease, although, the mechanism underlying this phenomenon requires further study. On the other hand, overactivation of certain oncogenic pathways is known to affect the activity of methytransferases and regulation of gene transcription, possibly affecting components of the MAPK pathway, such as RAS, RAF, MEK, and ERK.[43,44] For example, activating gene mutations in *PIK3CA* have been shown to lead to aberrant methylation and expression of the *PTEN* gene.[45] Collectively, the results from our study and previous work by others suggest that epigenetic alterations of *DLC1* might occur as a consequence of overactivation of the PI3K/AKT pathway in extramammary Paget's disease.

In conclusion, our study demonstrated that *DLC1* promoter methylation occurs at a higher frequency in tumor tissues than in normal tissues from extramammary Paget's disease patients, and that downregulation of *DLC1* protein level strongly correlates with methylation of the *DLC1* promoter. Importantly, we have revealed a significant association of *DLC1* hypermethylation with *PIK3CA* mutations in extramammary Paget's disease. Activation of the PI3K/AKT pathway may have an important role in development of extramammary Paget's disease, and may represent a novel therapeutic target for treating this disease.

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Disclosure/conflict of interest

The authors declare no conflicts of interests.

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