

## ORIGINAL ARTICLE

**Hypermethylation of the *nel-like 1* gene is a common and early event and is associated with poor prognosis in early-stage esophageal adenocarcinoma**Z Jin<sup>1</sup>, Y Mori<sup>1</sup>, J Yang<sup>1</sup>, F Sato<sup>1</sup>, T Ito<sup>1</sup>, Y Cheng<sup>1</sup>, B Paun<sup>1</sup>, JP Hamilton<sup>1</sup>, T Kan<sup>1</sup>, A Oлару<sup>1</sup>, S David<sup>1</sup>, R Agarwal<sup>1</sup>, JM Abraham<sup>1</sup>, D Beer<sup>2</sup>, E Montgomery<sup>3</sup> and SJ Meltzer<sup>1,4</sup><sup>1</sup>Division of Gastroenterology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; <sup>2</sup>Division of General Thoracic Surgery, Department of Surgery, University of Michigan School of Medicine, Ann Arbor, Michigan, USA; <sup>3</sup>Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA and <sup>4</sup>Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

The *nel-like1* (*NELLI*) gene maps to chromosome 11p15, which frequently undergoes loss of heterozygosity in esophageal adenocarcinoma (EAC). *NELLI* promoter hypermethylation was examined by real-time methylation-specific polymerase chain reaction in 259 human esophageal tissues. Hypermethylation of this promoter showed highly discriminative receiver–operator characteristic curve profiles, clearly distinguishing esophageal squamous cell carcinoma (ESCC) and EAC from normal esophagus (NE) ( $P < 0.001$ ). *NELLI* normalized methylation values were significantly higher in Barrett's metaplasia (BE), dysplastic Barrett's (D) and EAC than in NE ( $P < 0.0000001$ ). *NELLI* hypermethylation frequency was zero in NE but increased early during neoplastic progression, to 41.7% in BE from patients with Barrett's alone, 52.5% in D and 47.8% in EAC. There was a significant correlation between *NELLI* hypermethylation and BE segment length. Three (11.5%) of 26 ESCCs exhibited *NELLI* hypermethylation. Survival correlated inversely with *NELLI* hypermethylation in patients with stages I–II ( $P = 0.0264$ ) but not in stages III–IV ( $P = 0.68$ ) EAC. Treatment of KYSE220 ESCC and BIC EAC cells with 5-aza-2'-deoxycytidine reduced *NELLI* methylation and increased *NELLI* mRNA expression. *NELLI* mRNA levels in EACs with an unmethylated *NELLI* promoter were significantly higher than those in EACs with a methylated promoter ( $P = 0.02$ ). Promoter hypermethylation of *NELLI* is a common, tissue-specific event in human EAC, occurs early during Barrett's-associated esophageal neoplastic progression, and is a potential biomarker of poor prognosis in early-stage EAC.

*Oncogene* advance online publication, 23 April 2007; doi:10.1038/sj.onc.1210461

**Keywords:** hypermethylation; *NELLI*; EAC; ESCC; biomarker

**Introduction**

Esophageal cancer ranks sixth among all cancers worldwide, with 400 000 new cases being diagnosed per year (Stewart and Kleihues, 2003). This malignancy exists in two principal forms, each possessing distinct pathological characteristics: esophageal squamous cell carcinoma (ESCC), which occurs at high frequencies in many developing countries, particularly in Asia, and esophageal adenocarcinoma (EAC), which is more prevalent in Western countries, with a rapid rate of increase in recent years (Stewart and Kleihues, 2003). Although significant advances have been made in the treatment of esophageal cancers, these aggressive malignancies commonly present as locally advanced disease with a very poor prognosis (approximately 14% 5-year survival) (Jemal *et al.*, 2005). Therefore, it is important to discover novel early detection biomarkers and targets for future chemoprevention or therapy.

The *Nel* (strongly expressed in neural tissues and containing epidermal growth factor (EGF)-like domains) gene, encoding a protein containing six EGF-like domains, was first cloned from a chick embryo-derived cDNA library in 1995 (Matsuhashi *et al.*, 1995, 1996). Later, Watanabe *et al.* (1996) cloned a human *Nel* gene designated *nel-like type1* (*NELLI*) from a human fetal-brain cDNA library in 1996. The *NELLI* gene has been mapped to chromosome 11p15 (Watanabe *et al.*, 1996), a locus that frequently shows loss of heterozygosity (LOH) in human cancers, including EAC (Dolan *et al.*, 1998). LOH is one mechanism of gene inactivation, but other mechanisms exist, including point mutation, allelic or homozygous deletion, and promoter hypermethylation (Knudson, 2001). It is now well established that promoter hypermethylation correlates with silencing of gene transcription in cancers (Herman and Baylin, 2003), including ESCC and EAC (Fang *et al.*, 2005; Schulmann *et al.*, 2005). Recently, data from our laboratory showed that the *NELLI* promoter was hypermethylated in 15 (44%) of 34 human colon cancers, and that the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-dC) reversed *NELLI* promoter hypermethylation and

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Received 8 January 2007; revised 8 March 2007; accepted 12 March 2007

restored *NELL1* mRNA expression in colon cancer cell lines (Mori *et al.*, 2006). There is a growing body of evidence showing that the abnormal methylation of DNA is an early event in carcinogenesis and can serve as an early detection biomarker in some cancers (Herman and Baylin, 2003). Based on these findings, we hypothesized that *NELL1* might be inactivated via promoter hypermethylation in human esophageal cancers, and that hypermethylation of *NELL1* could occur early in the genesis of EAC, while simultaneously constituting a potentially useful early detection biomarker in this disease.

To test this hypothesis, we studied methylation of the *NELL1* gene promoter by real-time quantitative methylation-specific polymerase chain reaction (PCR) (qMSP) in 259 endoscopic esophageal biopsy specimens of differing histologies. The effect of a DNA methyltransferase inhibitor, 5-Aza-dC, on the re-expression of epigenetically silenced *NELL1* was also studied in esophageal cancer cell lines. Our results show that promoter hypermethylation of *NELL1* is a common event in EAC but uncommon in ESCC, occurs early during Barrett's-associated esophageal neoplastic progression, and is associated with a poor prognosis in early-stage EAC patients.

## Results

### *NELL1* promoter hypermethylation in different esophageal tissues

Promoter hypermethylation of *NELL1* was analysed in 66 normal esophagi (NE), 60 Barrett's metaplasias without dysplasia (BE, including 36 BEs from patients with Barrett's alone (Ba) and 24 BEs obtained from patients with Barrett's accompanied by EAC (Bt)), 19 low-grade (LGD) and 21 high-grade (HGD) dysplasias occurring within BE, 67 EACs, and 26 ESCCs. All assays were performed in duplicate format, and data showed reproducible and concordant results. *NELL1* promoter hypermethylation showed highly discriminative receiver-operator characteristic (ROC) curve profiles, clearly distinguishing both ESCC ( $P < 0.001$ ) and EAC ( $P < 0.0001$ ) from NE, as well as EAC from ESCC ( $P < 0.0001$ ). ROC curves with corresponding areas under the ROC curve (AUROCs) for *NELL1* of EAC vs NE, ESCC vs NE, combined esophageal cancer (T) vs NE and EAC vs ESCC are shown in Figure 1.

The cutoff normalized methylation value (NMV) for *NELL1* (0.1) was chosen from the ROC curve in order to maximize sensitivity and specificity. Mean NMVs and *NELL1* hypermethylation frequencies for each tissue type are shown in Table 1. NMVs of *NELL1* were significantly higher in ESCC, EAC, D, HGD, LGD, BE, Ba and Bt than in NE ( $P < 0.01$ , Student's *t*-test). The frequency of *NELL1* hypermethylation was significantly increased relative to NE (0%) in Ba (41.7%;  $P < 0.0001$ ), D (52.5%;  $P < 0.0001$ ) and EAC (47.8%;  $P < 0.0001$ ). Both *NELL1* hypermethylation frequency and mean

NMV tended to be higher in Bt than in Ba (54.2% vs 41.7%;  $P = 0.34$  and 0.2083 vs 0.1687;  $P = 0.49$ , respectively). Among 15 cases with corresponding NE, BE and EAC, two (Nos. 3 and 13) were hypermethylated only in EAC, four (Nos. 9, 16, 17 and 21) were hypermethylated only in BE, and the remaining nine were either unmethylated (five cases: Nos. 1, 2, 5, 7 and 14) or hypermethylated (four cases: Nos. 4, 6, 8 and 28) in both BE and EAC (Figure 2a). The *NELL1* NMVs of EACs (mean = 0.1905) were significantly higher than those of matched NEs from the same patients (mean = 0.001) in 27 cases having matched NE and EAC available ( $P = 0.0003$ , Student's paired *t*-test; Figure 2b). Three (11.5%) of 26 ESCCs showed hypermethylation of *NELL1*. There was no significant difference between ESCC and NE in *NELL1* NMVs for 13 cases with matched ESCC (mean = 0.0295) and NE (mean = 0.006;  $P = 0.2$ , Student's paired *t*-test; Figure 2c). Both *NELL1* hypermethylation frequency and mean NMV were significantly higher in EAC than in ESCC (47.8% vs 11.5%;  $P = 0.0016$  and 0.1527 vs 0.0233;  $P = 0.0015$ , respectively), as well as in D vs ESCC (52.5% vs 11.5%;  $P = 0.0007$  and 0.183 vs 0.0233;  $P = 0.0007$ , respectively), and in BE vs ESCC (46.7% vs 11.5%;  $P = 0.0016$  and 0.1846 vs 0.0233;  $P = 0.0003$ ; summarized in Table 1).

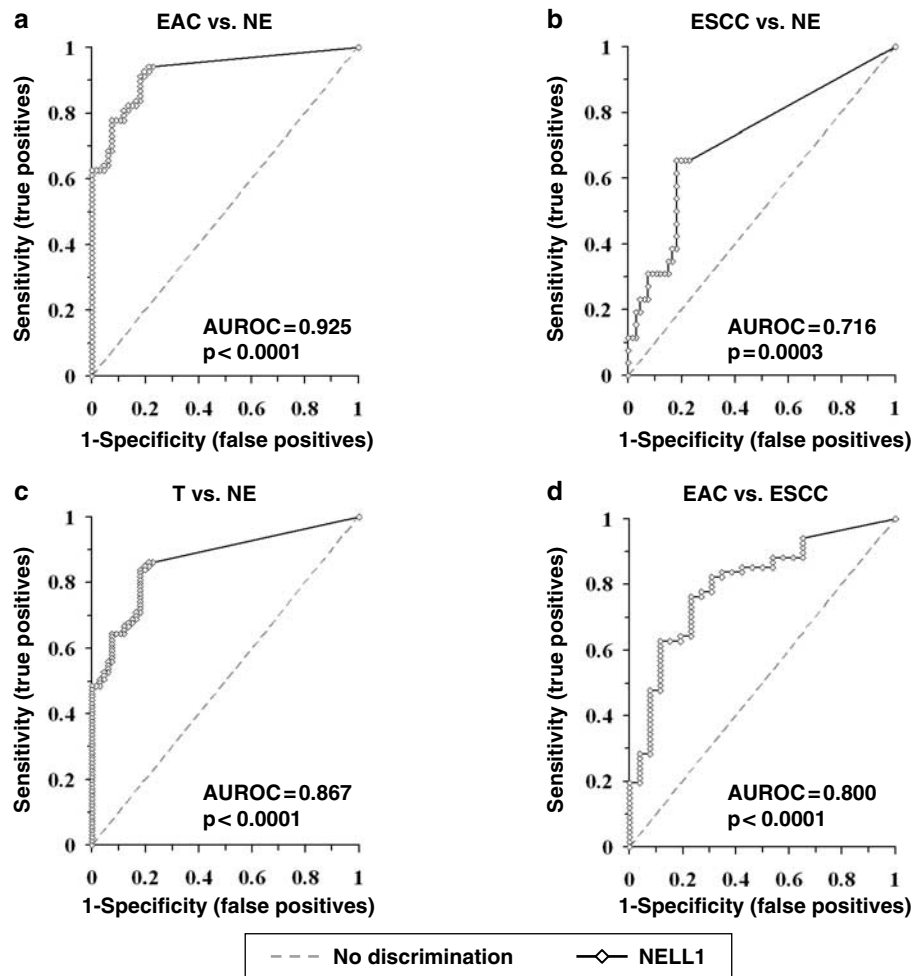
Survival curves were analysed according to UICC classifications (International Union against Cancer, 2002). Overall patient survival correlated with *NELL1* methylation status in stages I–II EAC patients, but not in stages III–IV EAC patients (Figure 3). Stages I–II EAC patients with *NELL1* hypermethylation had significantly shorter survivals than did patients without *NELL1* hypermethylation (mean 25.7 months vs 45.4 months;  $P = 0.0264$ , Log-rank test; Figure 3a). There was no statistically significant difference between *NELL1* hypermethylation and overall survival in stages III–IV EAC patients ( $P = 0.6827$ , Log-rank test; Figure 3b).

BE was defined as long-segment (LSBE) if it was equal to or greater than 3 cm in length, or short-segment (SSBE) if less than 3 cm, according to generally accepted criteria (Rudolph *et al.*, 2000). The mean NMV of *NELL1* was significantly higher in LSBE (mean = 0.2948) than in SSBE (mean = 0.0654;  $P = 0.0061$ , Student's *t*-test, Figure 4a). Similarly, segment lengths of BEs with hypermethylated *NELL1* promoters (mean = 5.86 cm) were significantly greater than segment lengths of BEs with unmethylated *NELL1* promoters (mean = 2.81 cm;  $P = 0.0098$ , Student's *t*-test; Figure 4b), and the frequency of *NELL1* hypermethylation was significantly higher in LSBE than in SSBE ( $P = 0.0061$ , Fisher's exact test; Table 2).

No significant associations were observed between *NELL1* promoter hypermethylation and patient age (data not shown), tumor stage or lymph node metastasis (Table 3), smoking or alcohol drinking status (Table 3), or degree of histologic differentiation in EACs (Table 1).

### *NELL1* methylation and mRNA levels in esophageal cancer cell lines after 5-Aza-dC treatment

Seven (78%) of nine ESCC and two (67%) of three EAC cell lines showed high *NELL1* NMV levels above the

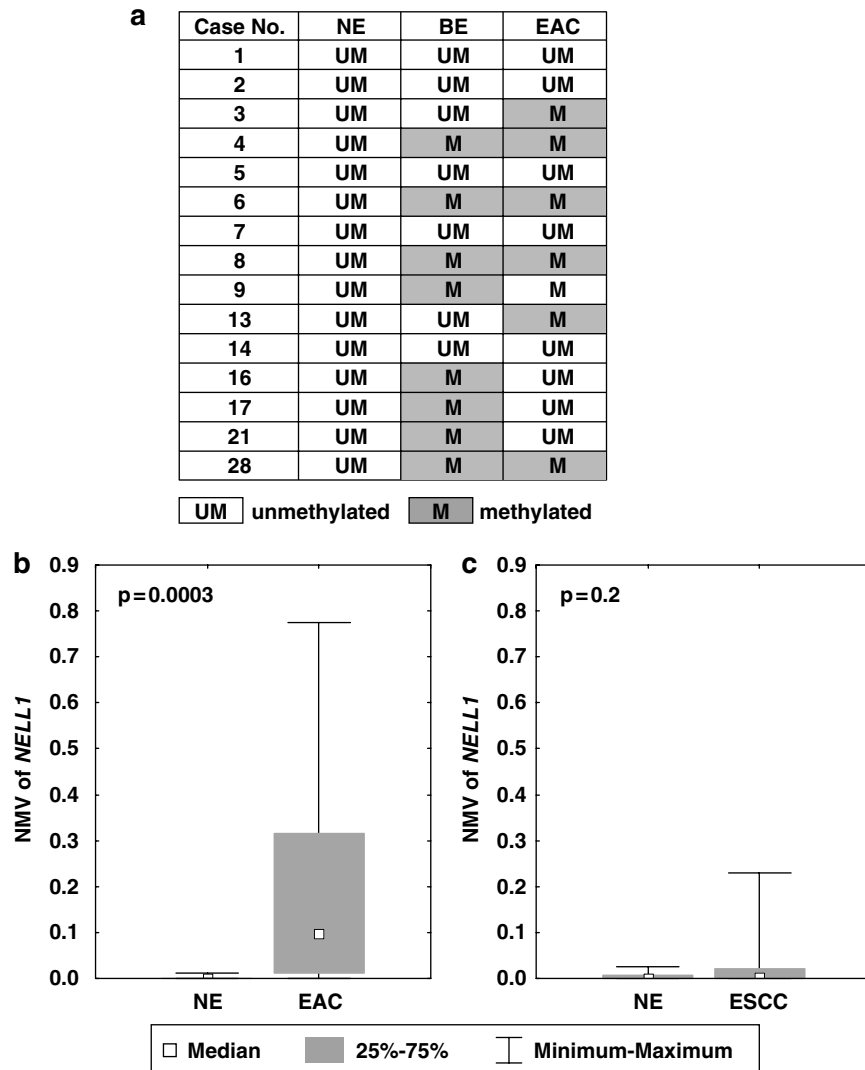


**Figure 1** ROC curve analysis of NMV. ROC curve analysis of *NELL1* NMV for NE vs EAC (a), NE vs ESCC (b), NE vs malignant esophageal tissues (T) (c) and ESCC vs EAC (d). The AUROC for the *NELL1* gene conveys this gene's accuracy in distinguishing NE from EAC, ESCC and T, and ESCC from EAC in terms of its sensitivity and specificity.

**Table 1** Clinicopathologic characteristics and methylation status of *NELL1* in different human esophageal tissues

Histology <sup>a</sup>	Number of samples	Age (years) Mean	NMV <sup>b</sup>		Methylation Status (cutoff 0.1) <sup>c</sup>			
			Mean	P	Frequency	UM	M	P <sup>§</sup>
Normal esophagus	66	64.3	0.0018		0.0%	66	0	
Barrett's metaplasia	60	63.7	0.1846	<0.0000001*/<0.001**	46.7%	32	28	<0.002 <sup>§,†</sup>
Barrett's from non-EAC patients	36	62.5	0.1687	<0.0000001*	41.7%	21	15	>0.05 <sup>‡</sup>
Barrett's from EAC patients	24	65.5	0.2083	<0.0000001*	54.2%	11	13	
Dysplasia in Barrett's esophagus	40	65.3	0.183	<0.0000001*/<0.001**	52.5%	19	21	<0.001 <sup>§,†</sup>
Low-grade dysplasia	19	65.3	0.1975	<0.0000001*	42.1%	11	8	>0.05 <sup>‡</sup>
High-grade dysplasia	21	65.2	0.1698	<0.0000001*	61.9%	8	13	
EAC	67	65.1	0.1527	<0.0000001*/<0.002**	47.8%	35	32	<0.002 <sup>§,†</sup>
Well differentiation	10	66.2	0.263	<0.0000001*	70.0%	3	7	>0.05 <sup>‡</sup>
Moderate differentiation	24	66.1	0.1612	<0.0000001*	50.0%	12	12	
Poor differentiation	22	65.5	0.122	<0.0000001*	36.4%	14	8	
Unknown	11	61	0.0952		45.5%	6	5	
ESCC	26	62.5	0.0233	<0.01*	11.5%	23	3	

<sup>a</sup>EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma. <sup>b</sup>NMV: normalized methylation value; \*Student's *t*-test (comparisons made to normal esophagus); \*\*Student's *t*-test (comparisons made to ESCC). <sup>c</sup>UM, unmethylated; M, methylated; <sup>§</sup>Excludes cases with unknown status; <sup>†</sup>Fisher's exact test; <sup>‡</sup> $\chi^2$  for independence test; <sup>§</sup>comparisons made to ESCC.



**Figure 2** Methylation status of *NELL1* in corresponding esophageal samples. (a) Among 15 cases with corresponding NE, BE and EAC, two (Nos. 3 and 13) were methylated only in EAC, four (Nos. 9, 16, 17 and 21) were methylated only in BE, and the remaining nine were either unmethylated (five cases, Nos. 1, 2, 5, 7 and 14) or methylated (four cases, Nos. 4, 6, 8 and 28) in both BE and EAC. (b) The *NELL1* NMVs of EAC (mean = 0.1905) were significantly higher than those of corresponding NE (mean = 0.001) ( $P = 0.0003$ , Student's paired *t*-test). (c) The *NELL1* NMVs of ESCC (mean = 0.0295) were not statistically different from those of corresponding NE (mean = 0.006) ( $P = 0.2$ , Student's paired *t*-test).

cutoff level of 0.1 (Figure 5a). Cell lines BIC and KYSE 220, which exhibited some of the highest NMVs between the EAC and ESCC cell lines, respectively, were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment, the NMV of *NELL1* was diminished in both BIC EAC cells and KYSE220 ESCC cells, while the mRNA level of *NELL1* showed a dramatic increase in BIC cells, but only a trend toward increasing in KYSE220 cells (Figure 5b and c).

#### Correlation between hypermethylation and mRNA expression of *NELL1* gene in EACs

To further elucidate the relationship between DNA hypermethylation and mRNA expression of *NELL1*, we determined *NELL1* mRNA levels in 22 EAC samples

using quantitative real-time reverse transcription (RT)-PCR (qRT-PCR). *NELL1* mRNA levels in EACs with unmethylated *NELL1* were significantly higher than those in EACs with hypermethylated *NELL1* ( $P = 0.02$ , Mann-Whitney *U*-test; Figure 6).

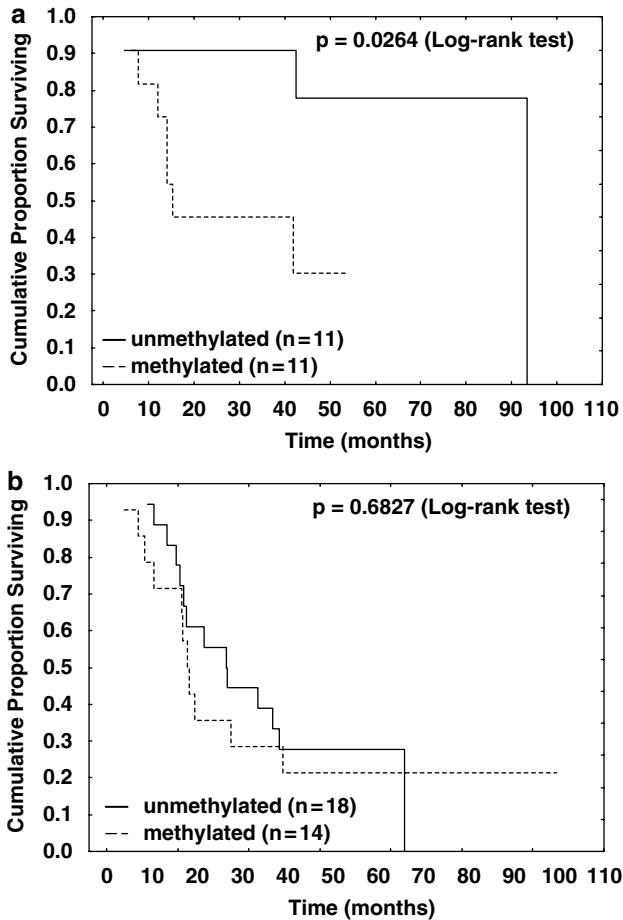
#### Discussion

The *NELL1* gene encodes a protein kinase C-binding protein that contains six EGF-like domains and belongs to a new class of cell-signaling molecules controlling cell growth and differentiation (Matsushashi *et al.*, 1995; Watanabe *et al.*, 1996; Kuroda and Tanizawa, 1999; Desai *et al.*, 2006). The precise roles of *NELL1* in

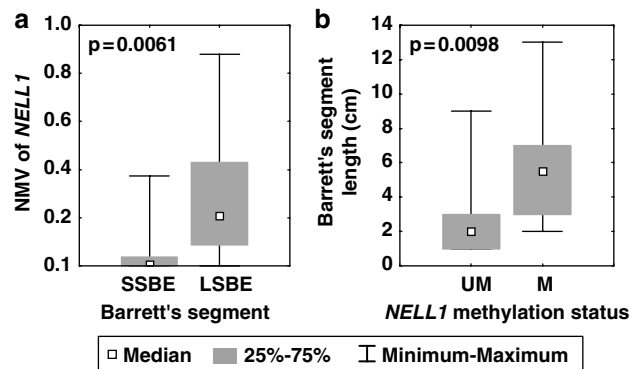
physiology and pathophysiology remain incompletely elucidated. Overexpression of *NELL1* increased osteoblast differentiation and reduced cell proliferation in transgenic mice, while downregulation of *NELL1* using *in vitro* approaches demonstrated inhibited osteoblast differentiation and suggested that decreased levels of *NELL1* protein lead to promoted cell proliferation at the suture line (Zhang et al., 2002). It has also been reported that overexpression of *NELL1* promotes

apoptosis in osteoblasts both *in vitro* and *in vivo* (Zhang et al., 2003), and that this apoptotic activity may be associated with the *Fas* signaling pathway (Zhang et al., 2006). In *NELL1*<sup>6R</sup> mutant mice, loss of *NELL1* expression was associated with reduced expression of genes encoding tumor necrosis factor receptor superfamily member 11b and extracellular matrix proteins (Desai et al., 2006), which have also been implicated in human carcinogenesis (Ingber, 2002; Rowinsky, 2005). Furthermore, the high frequency (44%) of *NELL1* promoter hypermethylation in colon cancer suggests a potential role for *NELL1* inactivation in colon tumorigenesis (Mori et al., 2006). The *NELL1* chromosomal locus reveals a high frequency of LOH or allelic imbalance in human cancers, including EAC (Dolan et al., 1998; Gleeson et al., 1998). Taken together, these findings suggest that *NELL1* functions as a tumor suppressor gene in certain human cancers.

In the current study, we systematically investigated hypermethylation of the *NELL1* gene promoter in primary human esophageal lesions of differing histological types and neoplastic stages, as well as in esophageal carcinoma cell lines. Our results demonstrate that hypermethylation of *NELL1* occurs early in some subjects, that the frequency of this epigenetic event increases early during EAC carcinogenesis, and that this event is common in human EACs, but uncommon in ESCCs. Thus, *NELL1* hypermethylation appears to be a critical and unique event in human EAC, rather than in ESCC. However, four of 15 cases with corresponding NE, BE and EAC were hypermethylated only in BE, but



**Figure 3** Relationship between survival of EAC patients and *NELL1* hypermethylation. Overall patient survival correlated with *NELL1* methylation status in stages I–II EAC patients (a), but not in stages III–IV EAC patients (b). (a) Stages I–II EAC patients manifesting *NELL1* hypermethylation had significantly shorter survivals than did patients without *NELL1* methylation (mean, 25.7 months vs 45.4 months,  $P=0.0264$ , log-rank test). (b) The *NELL1* hypermethylation was not correlated with overall patient survival in stages III–IV EAC patients ( $P=0.6827$ , log-rank test).



**Figure 4** Relationship between Barrett's segment length and *NELL1* hypermethylation. (a) Mean NMV of *NELL1* was significantly higher in LSBE than in SSBE ( $P=0.0061$ , Student's *t*-test). (b) *NELL1* hypermethylation was associated with BE segment length ( $P=0.0098$ , Student's *t*-test).

**Table 2** Relationship of Barrett's segment length and *NELL1* hypermethylation

Barrett's segment	Number of samples	Age (years) Mean	NMV		Methylation Status (cutoff 0.1)			
			Mean	P*	Frequency	UM	M	P <sup>§</sup>
Short-segment (< 3 cm)	14	62.3	0.0654	0.0061	21.4%	11	3	0.0136
Long-segment (≥ 3 cm)	16	62.8	0.2948		68.8%	5	11	

Abbreviations: M, methylated; NMV, normalized methylation value; UM, unmethylated. \*Student's *t* test, <sup>§</sup>Fisher's exact test.

**Table 3** Relationship of clinicopathologic characteristics and *NELL1* hypermethylation in esophageal adenocarcinoma patients

Clinical characteristics	Number of samples	Age (years) Mean	NMV		Methylation Status (cutoff 0.1)			
			Mean	P	Frequency	UM	M	P
UICC stage								
I	7	63	0.2297	> 0.05*	57.1%	3	4	> 0.05 <sup>†</sup>
II	15	65.2	0.1849		46.7%	8	7	
III	25	64.6	0.1176		40.0%	15	10	
IV	7	66.3	0.157		57.1%	3	4	
Lymph node metastasis								
Negative	25	64.9	0.1978	> 0.05**	48.0%	13	12	> 0.05 <sup>‡</sup>
Positive	25	64.6	0.1186		40.0%	15	10	
Smoking status								
Never	6	58.5	0.177	> 0.05*	83.3%	1	5	> 0.05 <sup>†</sup>
Former	24	68.5	0.1023		41.7%	14	10	
Current	13	60.8	0.1702		46.2%	7	6	
Alcohol drinking status								
Never	16	65.3	0.1	> 0.05*	62.5%	9	7	> 0.05 <sup>†</sup>
Former	15	63	0.1751		53.3%	6	9	
Current	10	65.7	0.1311		60.0%	6	4	

Abbreviations: M, methylated; NMV, normalized methylation value; UM, unmethylated. \*Kruskal–Wallis test, \*\*Student's *t* test, <sup>†</sup>Fisher's exact test, <sup>‡</sup> $\chi^2$  for independence test.

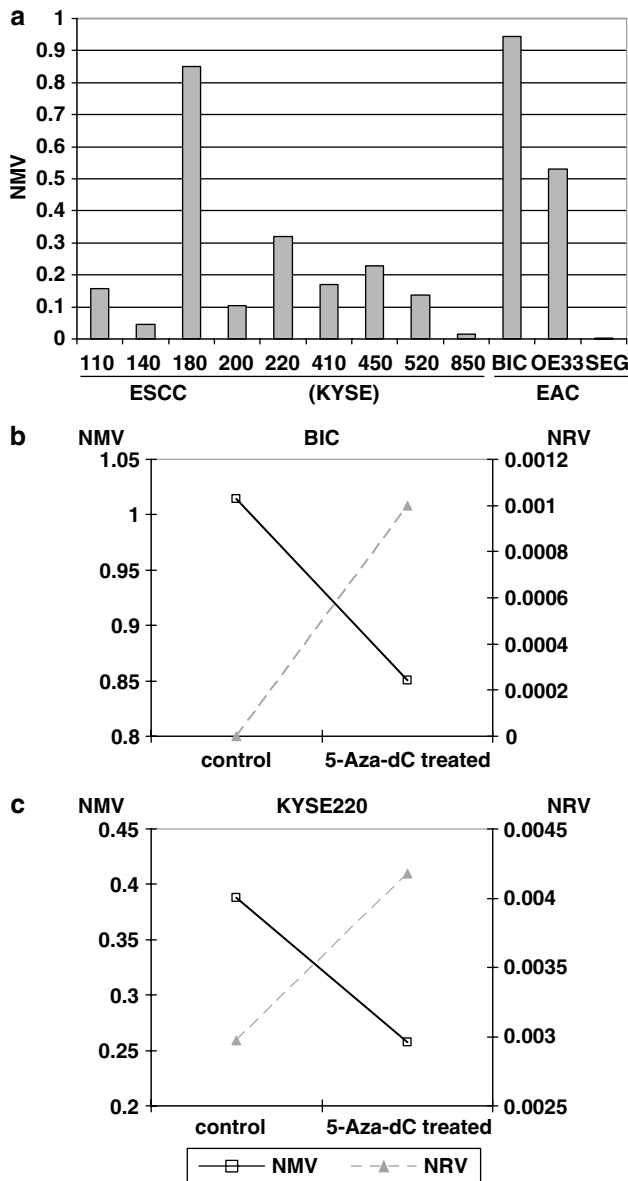
not in matching EAC. We speculate that this unexpected finding may have been due, in these particular cases, to the cancer having originated from a clone of Barrett's cells distinct from the BE clone analysed by us. In other words, a 'field methylation defect' may have involved the Barrett's segment from which the EAC arose, but not the BE segment that we analysed.

There is increasing evidence that promoter hypermethylation has prognostic value in cancer patients, including those with esophageal cancer (Kawakami *et al.*, 2000; Brock *et al.*, 2003; Lee *et al.*, 2006). Brock *et al.* (2003) demonstrated that hypermethylation of multiple genes was a powerful predictor of poor prognosis in EAC patients. Lee *et al.* (2006) reported that the *Fragile Histidine Triad* gene was methylated in 85 (33%) of 257 ESCCs and associated with a poor prognosis for stages 1–2 cases. Furthermore, our previous work showed that methylation of *Adenomatous Polyposis Coli* gene DNA could be detected in plasma and was associated with a poor prognosis in EAC patients (Kawakami *et al.*, 2000). In the current study, hypermethylation of *NELL1* was significantly associated with shortened survival in stages I–II EAC patients. Thus, hypermethylation of *NELL1* may constitute a useful biomarker of biologically aggressive disease in early-stage EAC patients.

Conflicting results have been reported from different studies regarding the length of Barrett's esophagus as a predictive factor for BE progression. While several previous studies stated that patients with SSBE may develop dysplasia (Sharma *et al.*, 1997) and EAC (Rudolph *et al.*, 2000), some prospective studies have shown an increased risk of EAC development only in patients with LSBE (Weston *et al.*, 1997; Hirota *et al.*, 1999; Hage *et al.*, 2004). Rudolph *et al.* (2000) demonstrated that segment length was not related to cancer risk in a prospective cohort study of 309 Barrett's

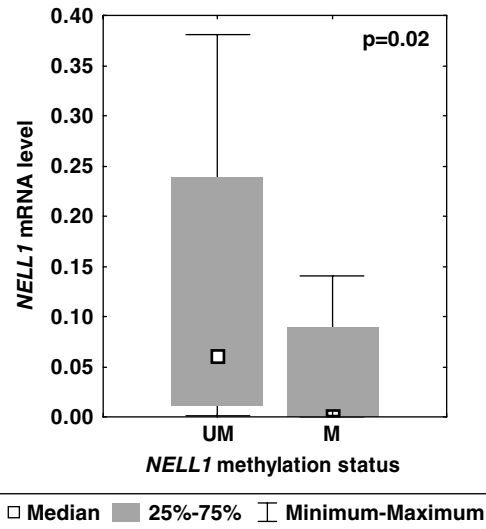
patients followed in the Seattle Barrett's Esophagus Project ( $P > 0.2$ ); however, when patients with HGD at entrance were excluded, a strong trend was observed, with a 5 cm difference in length associated with a 1.7-fold increase in cancer risk (95% confidence interval, 0.8- to 3.8-fold). Weston *et al.* (1997) reported significant differences between SSBE and LSBE in the frequency of both dysplasia and EAC, at 8.1 vs 24.4% for dysplasia ( $P < 0.0001$ ) and 0 vs 15.4% for EAC ( $P < 0.0005$ ). Hirota *et al.* (1999) reported that the prevalence of dysplasia and cancer differed significantly in patients with SSBE vs patients with LSBE in a comprehensive prospective study of 889 consecutive patients. More recently, Hage *et al.* (2004) reported a significantly increased risk of progression to HGD or EAC with LSBE after a mean follow-up of 12.7 years. Thus, it is likely that length of Barrett's epithelium is an important risk factor for both the prevalence of concurrent dysplasia and cancer and the incidence of future malignant progression. Interestingly, *NELL1* hypermethylation showed a strong relationship to BE segment length in the current study. Therefore, *NELL1* methylation may constitute both a molecular correlate of BE segment length and a potential biomarker for the prediction of BE progression.

In this study, hypermethylation of *NELL1* in BIC and KYSE220 cell lines was associated with silenced or reduced expression of *NELL1* mRNA. As expected, treatment of these cells with 5-Aza-dC led to concomitant increases in mRNA expression and reductions in *NELL1* methylation levels. This restoration of *NELL1* mRNA expression by demethylating agent treatment is consistent with the hypothesis that DNA hypermethylation was responsible for silencing of *NELL1*. The involvement of CpG island hypermethylation in silencing of the *NELL1* gene is also supported by the current



**Figure 5** *NELL1* methylation status and levels of *NELL1* methylation and mRNA expression in esophageal cancer cell lines after treatment with 5-Aza-dC. (a) Seven of nine ESCC and two of three EAC esophageal cancer cell lines showed high *NELL1* NMV levels, which exceeded the cutoff value of 0.1. (b and c) BIC and KYSE 220 that had some of the highest NMVs between the EAC and ESCC cell lines, respectively, were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment, the NMV of *NELL1* was diminished, whereas the normalized mRNA value (NRV) of *NELL1* was increased in both BIC and KYSE220 cell lines.

study's finding that *NELL1* mRNA levels in primary EACs with unmethylated *NELL1* promoters were significantly higher than those in EACs with hypermethylated *NELL1* promoters ( $P=0.02$ ). Furthermore, 5-Aza-dC or its derivatives have shown potential as therapeutic anticancer drugs (Lemaire *et al.*, 2005; Momparler, 2005), and thus *NELL1* represents a novel potential target for molecular-based EAC therapies involving demethylation.



**Figure 6** *NELL1* methylation status and mRNA expression level in EAC. *NELL1* mRNA levels were significantly higher in EAC with unmethylated *NELL1* promoter than that of EAC with methylated *NELL1* promoter ( $P=0.02$ , Mann-Whitney *U*-test).

The current study indicates that hypermethylation of the *NELL1* promoter, which leads to gene silencing, is a common event in human EACs, occurs early in Barrett's-associated esophageal adenocarcinogenesis, and is associated with a poor prognosis in early-stage EAC patients. In addition, *NELL1* hypermethylation is uncommon in human ESCC, appearing to represent a cell type-specific biomarker for EAC as opposed to ESCC. Further large-scale prospective longitudinal validation studies of this biomarker as a predictor of outcome in HGD or early EAC are justified by these data. These results also provide a stimulus for further research into potential applications of selected DNA methyltransferase or other indirect methylation inhibitors in the prevention or treatment of esophageal cancer.

## Materials and methods

### Tissue samples

In the current study, 66 NEs, 60 BEs (36 Ba and 24 Bt), 40 Ds (19 LGD and 21 HGD), 67 EACs and 26 ESCCs were examined. All patients provided written informed consent under a protocol approved by the Institutional Review Boards at the University of Maryland School of Medicine, the Baltimore Veterans Affairs Medical Center, and the Johns Hopkins University School of Medicine. Biopsies were taken using a standardized biopsy protocol as described previously (Schulmann *et al.*, 2005). Research tissues were obtained from grossly apparent normal esophageal mucosa, Barrett's epithelium or mass lesions in patients manifesting these changes at endoscopic examination, and histology was confirmed using parallel aliquots obtained at endoscopy. There was no histological evidence of esophagitis for NE. All normal esophageal tissues in this study were unmatched, and only seven (10.4%) of 67 EAC patients received preoperative chemotherapy and/or radiotherapy. Outcome data were derived from a comprehensive database maintained

by the institution's cancer registry and patients' medical records at the University of Maryland and Baltimore Veterans Affairs Medical Centers. All biopsy specimens were stored in liquid nitrogen until DNA extraction. Clinicopathologic characteristics of patients are summarized in Table 1.

#### Cell lines

Three EAC (BIC, OE33 and SEG) and nine ESCC (KYSE 110, 140, 180, 200, 220, 410, 450, 520 and 850) cell lines were obtained from collaborators at the University of Michigan (Dr David Beer) and Kyoto University (Professor Yutaka Shimada). These cells were cultured in 47.5% Roswell Park Memorial Institute 1640 medium, 47.5% F-12 supplemented with 5% fetal bovine serum.

#### DNA and RNA extraction

Genomic DNA was extracted from biopsies and cultured cells using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Total RNA was isolated from biopsies and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). DNAs and RNAs were stored at  $-80^{\circ}\text{C}$  before analysis.

#### Bisulfite treatment and real-time methylation-specific PCR

DNA was treated with bisulfite to convert unmethylated cytosines to uracils before qMSP, as described previously (Mori *et al.*, 2006). Promoter methylation levels of *NELL1* were determined by qMSP with the ABI 7700 Sequence Detection (Taqman, Applied Biosystems, Foster City, CA, USA) System, using primers and probes as described previously (Mori *et al.*, 2006). NMV was defined as follows:  $\text{NMV} = (\text{NELL1-S}/\text{NELL1-FM})/(\text{ACTB-S}/\text{ACTB-FM})$ , where *NELL1-S* and *NELL1-FM* represent *NELL1* methylation levels in sample and fully methylated DNAs, respectively, while *ACTB-S* and *ACTB-FM* correspond to  $\beta$ -actin in sample and fully methylated DNAs, respectively.

#### Real-time quantitative RT-PCR

To determine *NELL1* mRNA levels, one-step qRT-PCR was performed using a Qiagen QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and the ABI 7700 Sequence Detection (Taqman, Applied Biosystems) System. Primers and probes were the same as reported previously (Mori *et al.*,

2006).  $\beta$ -Actin was used for normalization of data. A standard curve was generated using serial dilutions of qPCR Reference Total RNA (Clontech, Mountain View, CA, USA). Normalized mRNA value (NRV) was calculated according to the following formula for relative expression of target mRNA:  $\text{NRV} = (\text{TarS}/\text{TarC})/(\text{ACTB-S}/\text{ACTB-C})$ , where *TarS* and *TarC* represent levels of mRNA expression for the target gene in sample and control mRNAs, respectively, whereas *ACTB-S* and *ACTB-C* correspond to amplified  $\beta$ -actin levels in sample and control mRNAs, respectively.

#### 5-Aza-dC treatment of esophageal cancer cell lines

To determine whether *NELL1* inactivation was due to promoter hypermethylation in esophageal cancer, two esophageal cancer cell lines (KYSE220 and BIC) were subjected to 5-Aza-dC (Sigma, St Louis, MO, USA) treatment as described previously (Bender *et al.*, 1999; Shibata *et al.*, 2002). Briefly,  $1 \times 10^5$  cells/ml were seeded onto a 100 mm dish and grown for 24 h. Then,  $1 \mu\text{l}$  of 5 mM 5-Aza-dC per ml of cells was added every 24 h for 6 days. DNAs and RNAs were harvested on day 6.

#### Data analysis and statistics

ROC curve analysis (Hanley and McNeil, 1982) was performed using NMVs for the 67 EAC, 26 ESCC and 66 NE by Analyse-it software (Version 1.71; Analyse-it Software, Leeds, UK). Using this approach, the AUROC identified optimal sensitivity and specificity levels (i.e., cutoffs) at which to distinguish normal from malignant esophageal tissues, and corresponding NMV thresholds were calculated for *NELL1*. The cutoff value determined from this ROC curve was applied to determine the frequency of *NELL1* methylation in each tissue type included in the present study. For all other tests, Statistica (version 6.1; StatSoft Inc., Tulsa, OK, USA) was used. Differences with  $P < 0.05$  were deemed significant.

#### Acknowledgements

We thank Dr Yutaka Shimada for his generous gift of excellent cell lines. This work was supported by NIH grants CA085069, CA001808 and CA106763 to SJ Meltzer.

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