Aberrant Methylation of the *Ras-related Associated with Diabetes* Gene in Human Primary Esophageal Cancer

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Abstract. *Background/Aim: Ras-related associated with diabetes (RRAD), a member of the Ras-related GTPase superfamily, is frequently methylated in several human cancers, though its methylation profile remains unclear in esophageal cancer. Materials and Methods: We examined RRAD promoter hypermethylation using real-time quantitative methylation-specific PCR in 229 primary human esophageal tissues of contrasting histological types. Results: RRAD hypermethylation showed highly discriminative receiver-operator characteristic curve profiles, clearly distinguishing esophageal squamous cell carcinoma (ESCC) from esophageal adenocarcinoma (EAC) or normal esophagus (NE) ($p<0.01$ and $p<0.01$, respectively). RRAD normalized methylation values were significantly higher in ESCC (0.0242) than in NE (0.0057, $p<0.05$) or EAC (0.0139, $p<0.01$). RRAD hypermethylation frequency was also significantly higher in ESCC (23.1%) than in NE (0%, $p<0.05$) or EAC (5.4%, $p<0.05$). Conclusion: Promoter hypermethylation of RRAD is a frequent, tissue-specific event in ESCC, and is uncommon in EAC. The aberrant methylation of RRAD may be involved in the pathogenesis of a subset of ESCC, but not in EAC.*

Esophageal cancer ranks eighth as most common cancer and the sixth as most frequent cause of cancer-related death worldwide (1). There are two principal forms of this malignancy, each possessing distinct pathological characteristics: esophageal squamous cell carcinoma (ESCC), which occurs in high frequencies in many developing countries, especially in Asia, including China (2); and esophageal adenocarcinoma (EAC), which is more prevalent in Western countries. In 2008, there were an estimated 482,000 new cases and 407,000 deaths worldwide related to esophageal cancer (1). Despite therapy, five-year survival rates remain dismal (*i.e.*, 17% 5-year survival) (3). Clearly, novel early detection biomarkers and therapeutic targets are needed.

Emerging evidence has suggested that epigenetic changes play a crucial role in esophageal carcinogenesis (4-8). It is now well-established that DNA methylation correlates with inactivation of tumor suppressor genes in human malignancies including esophageal cancer (4, 5, 8, 9). Ras-related associated with diabetes (*RRAD*), a member of the Ras-related GTPase superfamily, is originally identified as an up-regulated mRNA in skeletal muscle from individuals with type II diabetes by subtraction cloning (10), and is most highly expressed in lung, skeletal and cardiac muscle in humans (10, 11). *RRAD* displays a wide spectrum of cellular functions, including glucose uptake in cultured muscle and fat cells (11), cytoskeletal remodeling (12, 13), osteoblast differentiation (14), voltage-gated calcium channel activity (15), cell migration (16-18) and cardiomyocyte apoptosis (19). It has

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Table I. *Clinicopathologic characteristics and methylation status of RRAD in human esophageal tissues.*

Histological type	Number of samples	Age (year) mean	NMV		Methylation Status (cut-off 0.02)[3]	
			Mean	$p^{\$}$	Frequency	$p^{\dagger}$
Normal esophagus	56	63.6	0.0057		0	
Barrett's metaplasia	52	64.8	0.0093	>0.05*	7.7% (4/52)	>0.05*
Dysplasia in Barrett's esophagus	39	64.8	0.0100	>0.05*	10.5% (4/39)	<0.05*
Esophageal adenocarcinoma	56	64.5	0.0139	>0.05*	5.4% (3/56)	>0.05*
Esophageal squamous cell carcinoma	26	62.5	0.0242	<0.05*/**	23.1% (6/26)	<0.05*/**

NMV: Normalized methylation value; $^{\$}$Mann-Whitney *U*-test; †Fisher's exact test; *comparisons made to normal esophagus; **comparisons made to esophageal adenocarcinoma.

been shown that *RRAD* is frequently methylated in multiple human malignancies, including malignant mesothelioma, prostate, cervical, lung, breast and nasopharyngeal cancer (20-24); however, the methylation profiles of *RRAD* remain uncharacterized in human esophageal cancer.

Our goal was to determine the methylation profiles of *RRAD* in human esophageal cancer (EAC and ESCC), pre-malignant lesions (henceforth referred to as Barrett's esophagus (BE)) or Barrett's esophagus with dysplasia (D) and normal esophageal epithelium (NE) by real-time quantitative methylation-specific PCR (qMSP).

Materials and Methods

Tissue samples. In the current study, 56 NEs, 52 BEs, 39 Ds, 56 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 obtained using a standardized biopsy protocol, as described previously (25). Briefly, at each endoscopy, four-quadrant biopsies were obtained at 2-cm intervals throughout the grossly apparent BE segment (or at 1-cm intervals on follow-up after an endoscopy with LGD). 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. 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 up until DNA extraction. Clinicopathologic characteristics of patients are summarized in Table I.

DNA and RNA extraction. Genomic DNA was extracted from biopsies and cultured cells using a DNeasy Tissue Kit (Qiagen, Valencia, CA) and stored at –80˚C before analysis.

Bisulfite treatment and real-time methylation-specific PCR. DNA was treated with bisulfite to convert unmethylated cytosines to uracils prior to qMSP, as described previously (26). Briefly, 1.0 μg genomic DNA was denatured by treatment with 2 mol/L NaOH and modified by 3 mol/L sodium bisulfite. DNA samples were purified using Wizard DNA cleanup resin (Promega, Madison, WI), treated with 3 mol/L NaOH, precipitated with 100% ethanol, and resuspended in 50 μL water. Promoter methylation levels of *RRAD* were determined by qMSP with the ABI 7900 Sequence Detection (Taqman) System, using primers and probes as described previously (26). The PCR mixture consisted of 12.5 μL Taqman Universal Master Mix without UNG (Applied Biosystems, Foster City, CA), 2.0 μL of probe for both *Reprimo* and *β-actin* (2.5 μmol/L), 0.25 μL forward and reverse primer for both *Reprimo* and *β-actin* (10 μmol/L), 50 ng bisulfite-treated DNA, and water (up to a total volume of 25 μL). Normalized methylation value (NMV) was defined as follows: NMV=(*RRAD-S*/*RRAD-FM*)/(*ACTB-S*/*ACTB-FM*), where RRAD-S and *RRAD-FM* represent *RRAD* methylation levels in sample and fully methylated DNAs, respectively, while *ACTB-S* and *ACTB-FM* correspond to *β-Actin* in sample and fully methylated DNAs, respectively.

Data analysis and statistics. Receiver-operator characteristic (ROC) curve analysis was performed using NMVs for the 56 EAC, 26 ESCC and 566 NE by Analyse-it© software (v.1.71, Analyse-it Software, Leeds, UK). Using this approach, the areas under the ROC curve (AUROC) identitifed optimal sensitivity and specificity levels (*i.e.*, cut-offs) at which to distinguish normal from malignant esophageal tissues, and corresponding NMV thresholds were calculated for *RRAD*. The cut-off value determined from this ROC curve was applied to determine the frequency of *RRAD* methylation in each tissue type included in the present study. For all other tests, Statistica (version 6.1; StatSoft, Inc., Tulsa, OK) was used. Differences with $p<0.05$ were deemed significant.

Results

All qMSP assays were performed in duplicate and results were reproducible and concordant. *RRAD* promoter hypermethylation showed highly discriminative ROC curve profiles, clearly distinguishing both ESCC ($p<0.01$) and EAC ($p<0.05$) from NE, as well as EAC from ESCC ($p<0.0001$). ROC curves with AUROCs for *RRAD* of ESCC *vs*. NE, EAC *vs*. NE, and EAC *vs*. ESCC are shown in Figure 1.

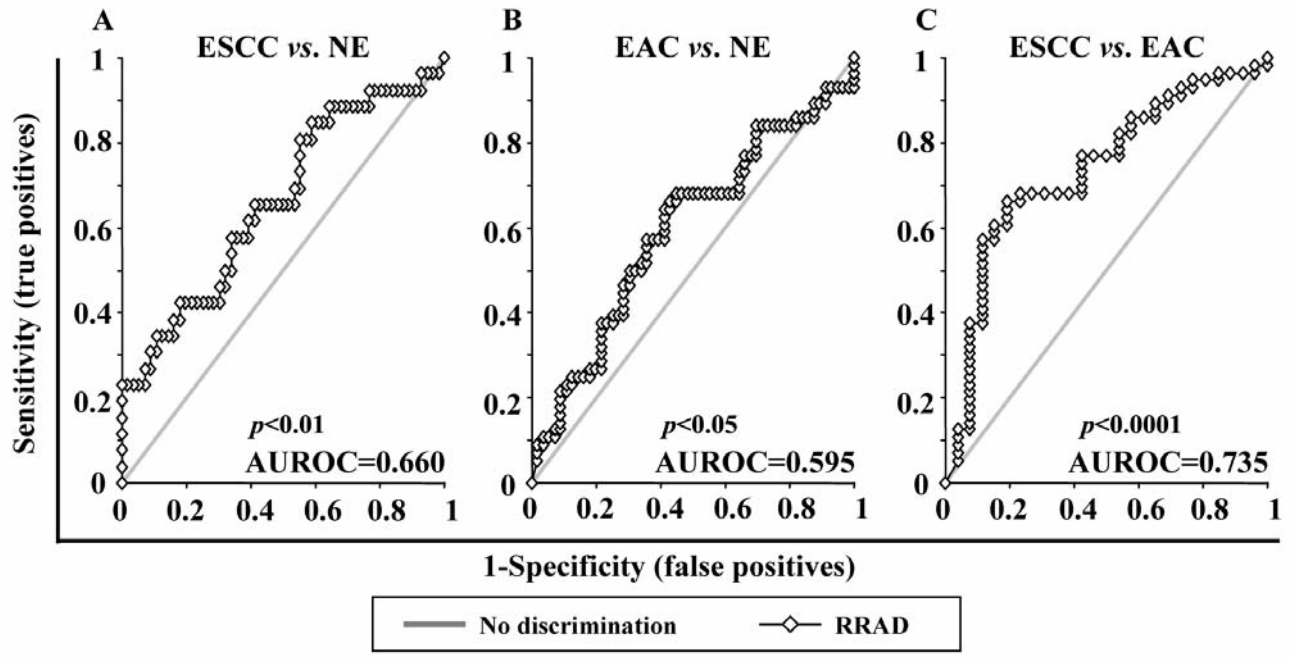

Figure 1. *Receiver-operator characteristic (ROC) curve analysis of normalized methylation value (NMV). ROC curve analysis of RRAD NMVs of normal esophagus (NE) vs. esophageal squamous cell carcinoma (ESCC) (A), NE vs. esophageal adenocarcinoma (EAC) (B) and EAC vs. ESCC (C).*

The cut-off NMV for *RRAD* (0.02) was chosen from the ROC curve in order to maximize for sensitivity and specificity. Mean NMVs and *RRAD* hypermethylation frequencies for each tissue type are shown in Table I. NMVs of *RRAD* were significantly higher in ESCC than in NE or in EAC ($p<0.05$ and $p<0.05$, respectively; Mann-Whitney *U*-test, Figure 2), but not in EAC, in D and in BE than in NE. Mean NMV is stepwise increased from NE (0.0057), to BE (0.0093), D (0.01) and EAC (0.0139). Frequency of *RRAD* hypermethylation was increased relative to NE (0%) in BE (7.7%; $p>0.05$), D (10.5%; $p<0.05$), EAC (5.4%; $p>0.05$) and ESCC (23.1%; $p<0.05$). In addition, frequency of *RRAD* hypermethylation was significantly higher in ESCC (23.1%) than in EAC (5.4%; $p<0.05$).

No significant associations were observed between *RRAD* promoter hypermethylation and patient age, survival, BE segment lengths, tumor stage or lymph node metastasis and smoking or alcohol consumption (data not shown).

Discussion

In the current study, we systematically investigated hypermethylation of the *RRAD* gene promoter in primary human esophageal lesions of differing histological types and neoplastic stages. Our results demonstrate that *RRAD* promoter hypermethylation occurs frequently in human ESCC

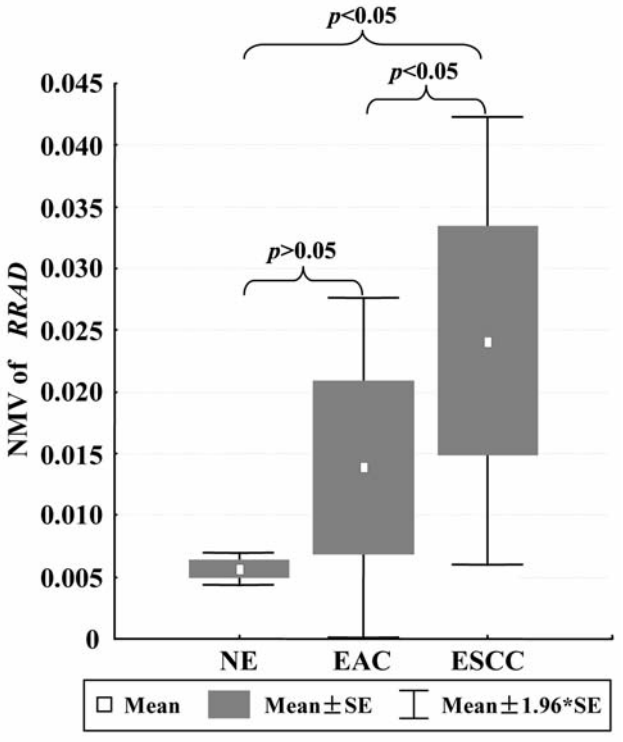

Figure 2. *Methylation levels of RRAD in normal esophagus (NE), esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC).*

(23.1%), but only rarely in EAC (5.4%). In addition, both *RRAD* hypermethylation frequency and mean NMV were significantly higher in ESCC compared to EAC (23.1% *vs.* 5.4%; $p<0.05$ and 0.0242 *vs.* 0.0139; $p<0.05$, respectively). Furthermore, ROC curve analysis clearly distinguished ESCC from EAC (AUROC=0.735, $p<0.0001$). Taken together, these results imply that hypermethylation of *RRAD* is common in human ESCCs but uncommon in EACs, and that it is a cell type-specific event (*i.e.*, common in ESCC but rare in EAC). Thus, *RRAD* hypermethylation appears to be a critical event unique to human ESCC, rather than EAC.

The precise roles of *RRAD* in physiology and pathophysiology remain incompletely elucidated. Few reports show oncogenic effects of RRAD. Over-expression of RRAD promoted cell growth by accelerating cell-cycle transitions by interacting with the GTPase-activating protein, nm23, and was associated with poor prognosis of breast cancer patients (27). *RRAD* may promote carcinogenesis, at least in part, by interacting with GCIP and inhibiting GCIP-mediated reductions of Rb phosphorylation and cyclin-D1 expression (28). *RRAD* was up-regulated in primary cultured invasive phenotype hepatocellular carcinoma cells (29).

Interestingly, it has been suggested that *RRAD* functions as a tumor suppressor gene in some human malignancies. *RRAD* methylation was found and correlated with SV40 infection in human malignant mesothelioma (20). Frequency of *RRAD* methylation was significantly higher in prostate cancer tissues (37%) than in non-malignant prostatic tissues (9%) (21). *RRAD* was frequently methylated in biopsies of invasive cervical cancer (22). *RRAD* hypermethylation was a frequent event in lung and breast cancers (42% and 62%, respectively) and correlated with smoking history and poorer prognosis in lung adenocarcinomas (23). Expression of RRAD was significantly lower in invasive non-small cell lung carcinomas than in non-neoplastic bronchiolar epithelium by immunohistochemistry (30). In lung cancer cells, *p53* up-regulated both mRNA and protein expression of *RRAD*, and inhibited cell migration by disrupting actin dynamics *via* RRAD (as a direct p53 transcriptional target) (30). In nasopharyngeal carcinoma, *RRAD* was epigenetically inactivated, and ectopic expression of RRAD suppresses tumor cell proliferation and migration *in vitro* (24). In addition, RRAD was up-regulated by Platelet-Derived Growth Factor and overexpression of *RRAD* attenuated vascular lesion formation by inhibition of vascular smooth muscle cell migration (18, 31). Taken together, these findings suggest that *RRAD* functions as a tumor suppressor gene in certain human cancers.

As mentioned above, *RRAD* was frequently methylated in human malignant mesothelioma, prostate, cervical, lung, breast and nasopharyngeal cancer (20-24), also in ESCC as shown in the current study. These results reveal that *RRAD* may act as a tumor suppressor, though the mechanistic details by which *RRAD* suppresses tumorigenesis are presently unclear.

The current study indicates, for the first time, that aberrant methylation of *RRAD* may be involved in pathogenesis of a subset of ESCC, but not in EAC. In addition, *RRAD* hypermethylation is uncommon in EAC, appearing to represent a cell type-specific biomarker for ESCC as opposed to EAC. The exact role of the *RRAD* hypermethylation regarding the carcinogenesis of human esophageal cancers should be addressed in future work.

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Conflicts of Interest

All Authors do not have any conflicts of interest relevant to the manuscript.

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