

Germline Mutations in *MSR1*, *ASCC1*, and *CTHRC1* in Patients With Barrett Esophagus and Esophageal Adenocarcinoma

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THE INCIDENCE OF ESOPHAGEAL adenocarcinoma (EAC) in the United States and Europe has increased 350% since 1970, with uncertain etiology.¹ Although early-stage EAC is curable, most cases are detected at an advanced stage with poor survival. Esophageal adenocarcinoma is believed to be preceded by Barrett esophagus (BE), a premalignant metaplasia caused by chronic gastroesophageal reflux disease (GERD).²⁻⁶ GERD-related inflammation and the transforming growth factor β (TGFB) pathway have been implicated in sporadic BE and EAC, just as the role of inflammation has become prominent in a range of human cancers.⁷ Although acknowledged, the role of inflammation in BE and EAC has not been thoroughly

Context Barrett esophagus (BE) occurs in 1% to 10% of the general population and is believed to be the precursor of esophageal adenocarcinoma (EAC). The incidence of EAC has increased 350% in the last 3 decades without clear etiology. Finding predisposition genes may improve premorbid risk assessment, genetic counseling, and management. Genome-wide multiplatform approaches may lead to the identification of genes important in BE/EAC development.

Objective To identify risk alleles or mutated genes associated with BE/EAC.

Design, Setting, and Patients Model-free linkage analyses of 21 concordant-affected sibling pairs with BE/EAC and 11 discordant sibling pairs (2005-2006). Significant germline genomic regions in independent prospectively accrued series of 176 white patients with BE/EAC and 200 ancestry-matched controls (2007-2010) were validated and fine mapped. Integrating data from these significant genomic regions with somatic gene expression data from 19 BE/EAC tissues yielded 12 "priority" candidate genes for mutation analysis (2010). Genes that showed mutations in cases but not in controls were further screened in an independent prospectively accrued validation series of 58 cases (2010).

Main Outcome Measures Identification of germline mutations in genes associated with BE/EAC cases. Functional interrogation of the most commonly mutated gene.

Results Three major genes, *MSR1*, *ASCC1*, and *CTHRC1* were associated with BE/EAC (all $P < .001$). In addition, 13 patients (11.2%) with BE/EAC carried germline mutations in *MSR1*, *ASCC1*, or *CTHRC1*. *MSR1* was the most frequently mutated, with 8 of 116 (proportion, 0.069; 95% confidence interval [CI], 0.030-0.130; $P < .001$) cases with c.877C>T (p.R293X). An independent validation series confirmed germline *MSR1* mutations in 2 of 58 cases (proportion, 0.035; 95% CI, 0.004-0.120; $P = .09$). *MSR1* mutation resulted in CCND1 up-regulation in peripheral-protein lysate. Immunohistochemistry of BE tissues in *MSR1*-mutation carriers showed increased nuclear expression of CCND1.

Conclusion *MSR1* was significantly associated with the presence of BE/EAC in derivation and validation samples, although it was only present in a small percentage of the cases.

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studied.⁸ Barrett esophagus is common in the general population, estimated to occur in 1% to 10%⁶; it develops in 12% to 15% of patients with GERD.⁵ The risk of EAC in patients with BE is approximately 0.4% per year.⁹

Although most BE and EAC are believed to be sporadic, genetic (heritable) etiologies have been supported by observation of familial clustering of cases noted over several decades, although few large families co-segregating BE/EAC have been reported.^{10,11} In 1 referral series, clinical epidemiologic analyses suggest 7% of individuals with BE, EAC, or both have at least 1 affected blood relative.¹² Although shared environmental factors may contribute to such familial aggregation, an autosomal dominant mode of inheritance with incomplete penetrance is consistent with most published studies, and rare reported cases are consistent with autosomal recessive inheritance.¹⁰

The discovery of germline mutations in a gene or genes that predispose to BE/EAC may have ramifications regarding cancer risk assessment, genetic counseling, premorbid diagnosis, and targeted surveillance and management, and also add to the fundamental understanding of the pathophysiology of sporadic BE and EAC. We therefore sought to identify a gene or genes associated with BE/EAC predisposition.

METHODS

Our study (2005-2010), approved by respective institutions' review board for research participants, involved prospective recruitment of all 298 consenting adults with histologically proven BE, EAC, or both, as well as families with 2 or more cases with BE, EAC, or both from 16 academic and community hospitals and clinics nationally (two-thirds originated from Cleveland Clinic, Cleveland, Ohio, and Johns Hopkins Medical Institutions, Baltimore, Maryland; <1% of research participants declined participation). All BE cases were long segment (eMethods; available at <http://www.jama.com>). For discordant sibling pair

studies, the nonaffected sibling had endoscopy documented unaffected status. Only white participants of northern or western European descent were selected and sex-matched in cases and controls.

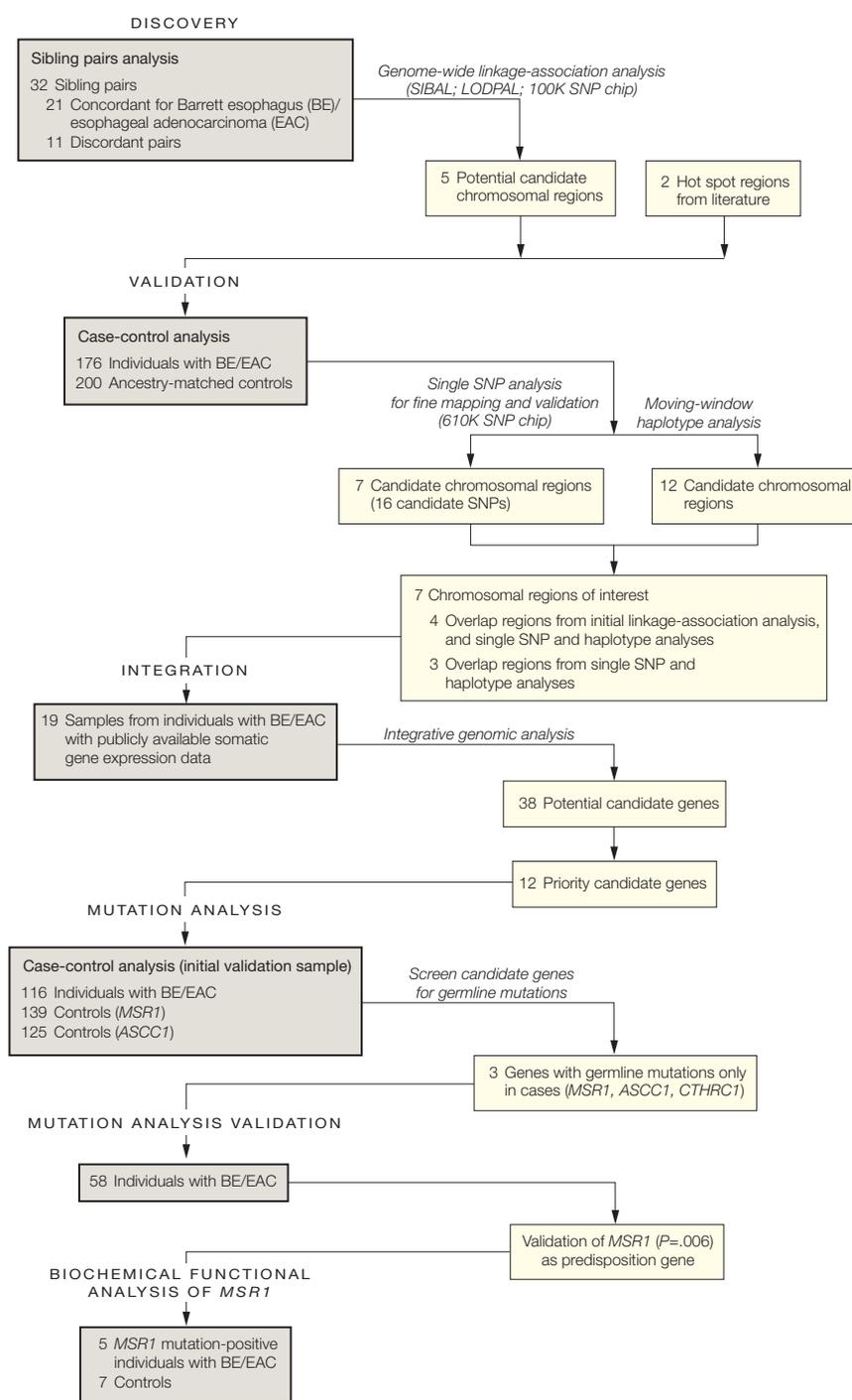
Identification of Loci Using Genome-Wide Mapping Methods

Model-Free Linkage Analysis. Twenty-one concordant-affected sibling pairs (42 individuals with BE/EAC) and 11 discordant sibling pairs (11 with BE/EAC and 11 without BE/EAC) (2005-2006) were genotyped using Affymetrix GeneChip Human Mapping 100K SNP set (Affymetrix, Santa Clara, California) (FIGURE 1). Significant linkage to chromosomal regions found by 1 model-free linkage analysis method was self-replicated by a second model-free linkage analysis approach, which is used for small sample-sized data sets.¹³⁻¹⁵ Genomic regions were considered potentially interesting when $-\log_{10} P \text{ value}(pP) \geq 2.2$ by SIBPAL analysis¹³ had logarithm of odds > 3.2 by LODPAL analysis.¹³ These regions from this pilot linkage-association analysis were considered "potentially interesting" and served as regions to be validated (eMethods and Figure 1).

Independent Validation and Fine Mapping Significant Regions. It is standard in this field to single out significant genomic regions from pilot analysis to follow up with increased sample sizes from independent cases (validation), more genetic markers (fine mapping),¹⁶⁻¹⁹ or both in a second validation stage (Figure 1). We followed this strategy of independent validation and fine mapping of the "potentially interesting" regions identified by the pilot linkage-association analysis. We also paid particular attention to 2 additional regions (1q23 and 8p22), because these regions were found previously to be frequently somatically lost (by array comparative genomic hybridization) in EAC or gastroesophageal junction cancers.²⁰

Population substructure of cases and controls was determined by PLINK and EIGENSTRAT (eMethods).^{21,22} Analysis using EIGENSTRAT software^{21,22} and principal component analysis identified the top eigenvalues from the 376 available eigenvalues. Regression analyses were used to allow for potential population substructure by 2 separate analyses (PLINK-derived and EIGENSTRAT-derived analyses). After population substructure was assessed to be similar ($>85\%$),^{21,22} single single-nucleotide polymorphism (SNP) association analyses of the above targeted genomic regions were performed with an independent validation series totaling 176 patients with BE/EAC and 200 ancestry-matched population controls (2007-2010) whose SNP data were derived from the denser Illumina Human610-Quad BeadChips (Illumina Inc, Hayward, California). Although we only were validating specific regions, we reasoned that it would be more cost-efficient to genotype all markers in a commercially available Chip instead of creating a new automation process for a reduced marker set. If the underlying genetic effect had been negligible, we would not have expected to see any savings on the average sample size, but fortunately the underlying genetic effect was large enough to warrant savings on sample size.

Statistical simulations have indicated that haplotype analysis with multiple SNPs may be more powerful than single SNP analysis,²³⁻²⁵ because multiple alleles at different loci on the same chromosome that are in linkage disequilibrium (LD) are likely to interact with each other to result in a phenotype. Thus, haplotype analysis was performed using PLINK²⁶ to predict the most likely haplotypes and those that were significantly associated with the BE/EAC phenotype. To account for type I error, an empirical *P* value corrected for testing multiple markers was obtained by permuting (10 000 permutations) the affection status across the individual genotypes, as described in PLINK.²⁶

Figure 1. Schema of Strategy for Mapping BE/EAC Loci and Candidate Gene Selection

BE/EAC indicates Barrett esophagus/esophageal adenocarcinoma. The multistage strategy used to identify BE/EAC susceptibility genes via a genome-wide combined linkage-association analysis, followed up by an independent genome-wide single-nucleotide polymorphism (SNP)-based case-control validation. A series of multiple, including functional, platform integration resulted in a prioritized candidate gene list, with the final 12 top priority candidates brought forward to candidate gene mutation analysis in a case-control series followed up by validation in an independent series of patients and by functional interrogation.

Integrating Information From Significant Regions With Publicly Available Somatic Gene Expression Data Sets

To narrow in on one or a subset of genes within and in proximity to the significant SNPs/haplotypes germane to BE/EAC (by tissue-specific expression in oncologic pathways and for functional-genomic validation), we integrated our significant regions with publicly available somatic gene expression data derived from 19 patients with BE/EAC (GDS3472 or GSE13083),²⁷ followed up by unsupervised hierarchical clustering²⁸ of genes within 250 kb flanking the significant SNPs and haplotypes across BE/EAC and unaffected individuals (eFigure 1, TABLE 1, and TABLE 2).

Prioritized Candidate Gene Analysis

A final list of biologically plausible candidate genes ("priority" candidate genes) was then scanned for germline mutations in BE/EAC cases and compared with ancestry-matched population controls (Figure 1). Genes with mutations in cases but not in controls were screened in an independent validation series of 58 cases prospectively accrued from outpatient endoscopy units (2010) (Figure 1 and eTable 1).

MSR1 and *CCND1* Protein Levels and Cell Lines

Proteins were extracted from immortalized lymphoblastoid cells obtained from patients with BE/EAC and normal controls. After processing, protein lysates were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Antibodies specific to *CCND1* (Cell Signaling Technology Inc, Danvers, Massachusetts), *MSR1* (Abcam, Cambridge, Massachusetts), and α -tubulin (Sigma-Aldrich, St Louis, Missouri) were used for Western blotting.

Wild-type *MSR1* or pCMV-FLAG empty vector were transiently transfected into *MSR1*-null HEK293 cells using Lipofectamine LTX and Plus Reagent (Invitrogen, Carlsbad, California). Cells were harvested after 24 hours

and lysates (30 µg of protein) were analyzed by Western blotting using antibodies against FLAG (Sigma-Aldrich, 1:1000), CCND1 (Thermo-Fisher Scientific Inc, Waltham, Massachusetts, 1:200), and α-tubulin (Sigma-Aldrich, 1:5000).

CCND1 Immunohistochemical Analysis

CCND1 immunohistochemical analysis was performed using an avidin-biotin complex immunoperoxidase technique.

RESULTS

Linkage and Association Analyses

A pilot combined linkage-association analysis based on modification of established criteria²⁹ revealed 5 candidate regions (1q24.1-25.3, 1q41,

8q21.11-22, 10q21-22, and 11q21) (Table 1 and Figure 1).

Subsequently, we performed a validation study in an independent series of 176 cases and 200 controls (Figure 1), using a denser SNP-marker set but focusing only on the germline regions of interest and the 2 somatically lost hot spot regions (1q23 and 8p22). We were able to validate 4 (1q24.1-25.3, 1q41, 8q21.11-22, and 10q21-22) of these 5 pilot-derived germline candidate regions, while excluding one (11q21). Three additional loci at locations remote from the pilot linkage peaks (1q21.2, 8p22, and 11q25) were also found (Table 1). The most significant SNPs from this validating association analysis were located within or in the vicinity of the most promising pilot-derived linkage peaks (Table 1).

Moving-Window Haplotype Analysis

Haplotype and LD analysis conducted on the 176 cases and 200 controls confirmed our findings—any single SNP that was significant in the above single SNP analysis always revealed a haplotype block containing significant SNPs within the haplotypes (at least in LD) ($P < .005$) (Table 2). In the haplotype analysis, we considered regions of highest priority as those haplotypes exhibiting significance across multiple SNPs. The combined P values from the significant single SNPs and the significant haplotypes facilitated prioritization of regions of interest for further follow-up. There were 4 significant regions that overlapped from the linkage, single SNP

Table 1. Significant Single SNP Association Results From Pilot-Combined Linkage-Association and Independent Validation Case-Control Analyses in Patients With BE/EAC

Region	db SNP	Location, Build 36.1	P Value	Association Analysis, FDR-Corrected	Linkage Analysis, LOD Scores (pP ^{SP}) ^a	Single SNP Association, pP ^{Assoc} ^b	Gene at the Significant SNP
1q21.2	rs2809811	100,805,788	<.001	0.0185		4.80	
1q24.1-25.3	rs10494465	164,810,325	.007		4.31 (2.17)		
	rs950302	165,350,678	.004		4.38 (2.40)		
	rs10489191	165,772,769	.004		3.68 (2.36)		
	rs10489211	166,579,946	.004		3.32 (2.35)		
	rs6659944	167,046,343	<.001	0.0175		5.03	
	rs10494476	167,267,368	.009		3.10 (2.06)		
	rs3853181	169,241,785	<.001	0.0198		4.47	<i>C1orf129</i>
1q41	rs6661125	178,493,273	<.001	0.0175		5.06	<i>LHX4</i>
	rs10209401	217,912,676	<.001	0.0214		4.07	<i>DIRC3</i>
	rs12070516	218,894,580	<.001	0.0199		4.75	<i>MARK1</i>
	rs12062054	229,020,356	<.001	0.0199		4.93	
	rs2355230	237,851,538	.007		4.12 (2.18)		
	rs4498839	243,586,955	<.001	0.0173		5.22	<i>KIF26B</i>
	rs381111	16,090,070	<.001	0.0253		3.29	<i>MSR1</i>
8p22	rs4469448	75,457,415	.01		3.19 (2.01)		
	rs4739755	81,665,497	.01		3.39 (2.00)		
	rs3097418	94,851,657	<.001	0.0253		3.33	<i>TMEM67</i>
8q22.1-24.22	rs3098224	104,515,622	<.001	0.0253		3.24	<i>WDSOF1</i>
	rs3098233	104,463,670	<.001	0.0253		3.24	<i>CTHRC1</i>
	rs4388439	133,277,554	<.001	0.0253		3.10	<i>KCNQ3</i>
	rs11001056	53,599,547	<.001	0.0463		3.04	<i>PRKG1</i>
10q21-22	rs2050381	55,029,991	<.001		3.604 (3.52)		
	rs10509021	56,518,547	<.001		2.566 (4.00)		
	rs11000190	73,577,964	<.001	0.0262		3.46	<i>ASCC1</i>
	rs7107185	94,342,447	<.001		4.588 (3.40)		
11q21	rs1255537	94,958,558	<.001		4.54 (3.52)		
	rs11223500	132,917,451	<.001	0.0500		3.23	<i>OPCML</i>

Abbreviations: BE, Barrett esophagus; EAC, esophageal adenocarcinoma; FDR, false discovery rate; LOD, logarithm of odds; SNP, single-nucleotide polymorphism.

^a LOD score was derived from LODPAL. ¹⁵⁻¹⁸ pP^{SP} indicates $-\log_{10}(P$ value) derived from SIBPAL (considered $-\log_{10}(P$ value) ≥ 2.00), from analysis of the 32 sibling pairs (21 concordant-affected sibling pairs and 11 discordant sibling pairs). ¹⁵⁻¹⁸

^b pP^{Assoc} indicates $-\log_{10}(P$ value) derived from the validation case-control association analysis using independent $n=376$ (comprising 176 cases and 200 controls).

Table 2. Haplotypes Significantly Associated With BE/EAC Cases vs Controls^a

Chromosome Region	Haplotype	SNPs	P Value	Significant Genes
1q21.2	12212	rs3806237, rs12060945, rs2270694, rs12722868, rs2809811 ^b	<.001	
	22121	rs12060945, rs2270694, rs12722868, rs2809811 ^b , rs34552536	<.001	
	221	rs2270694, rs12722868, rs2809811 ^b	<.001	
	212	rs12722868, rs2809811 ^b , rs34552536	<.001	
1q24.2	122	rs6659944 ^b , rs12067866, rs12069349	<.001	
	222	rs6659944 ^b , rs12067866, rs12069349	<.001	
1q24.3	222	rs16828284, rs3853181 ^b , rs1800822	<.001	<i>C1orf129</i>
	21212	rs16828284, rs3853181 ^b , rs1800822, rs2066530, rs2066536	<.001	<i>C1orf129</i>
	222	rs3853181 ^b , rs1800822, rs2066530	<.001	<i>C1orf129</i>
1q25.2-25.3	22112	rs6661125 ^b , rs17300107, rs6670868, rs16856123, rs17302632	<.001	<i>LHX4</i>
	122	rs6661125 ^b , rs17300107, rs6670868	<.001	<i>LHX4</i>
1q41	11112	rs1338775, rs6694126, rs17007991, rs12070516 ^b , rs17008285	<.001	<i>MARK1</i>
	11122	rs6694126, rs17007991, rs12070516 ^b , rs17008285, rs17008643	<.001	<i>MARK1</i>
	11222	rs17007991, rs12070516 ^b , rs17008285, rs17008643, rs17008806	<.001	<i>MARK1</i>
	12222	rs12070516 ^b , rs17008285, rs17008643, rs17008806, rs3806325	<.001	<i>MARK1</i>
	22222	rs6694126, rs17007991, rs12070516 ^b , rs17008285, rs17008643	<.001	<i>MARK1</i>
	1122	rs17007991, rs12070516 ^b , rs17008285, rs17008643	<.001	<i>MARK1</i>
	2222	rs17007991, rs12070516 ^b , rs17008285, rs17008643	<.001	<i>MARK1</i>
	1112	rs6694126, rs17007991, rs12070516 ^b , rs17008285	<.001	<i>MARK1</i>
	1222	rs12070516 ^b , rs17008285, rs17008643, rs17008806	<.001	<i>MARK1</i>
	2222	rs6694126, rs17007991, rs12070516 ^b , rs17008285	<.001	<i>MARK1</i>
	2222	rs1338775, rs6694126, rs17007991, rs12070516 ^b	<.001	<i>MARK1</i>
	1111	rs1338775, rs6694126, rs17007991, rs12070516 ^b	<.001	<i>MARK1</i>
	222	rs6694126, rs17007991, rs12070516 ^b	<.001	<i>MARK1</i>
	112	rs17007991, rs12070516 ^b , rs17008285	<.001	<i>MARK1</i>
	222	rs17007991, rs12070516 ^b , rs17008285	<.001	<i>MARK1</i>
	122	rs12070516 ^b , rs17008285, rs17008643	<.001	<i>MARK1</i>
222	rs12070516 ^b , rs17008285, rs17008643	<.001	<i>MARK1</i>	
111	rs12070516 ^b , rs17008285, rs17008643, rs17008806, rs3806325	<.001	<i>MARK1</i>	
8p22	22221	rs4265186, rs268387, rs354521, rs354517, rs3811111 ^b	.002	<i>MSR1</i>
	22112	rs354521, rs354517, rs3811111 ^b , rs2959634, rs2959631	.004	<i>MSR1</i>
8q22.1	21222	rs3097422, rs3097418 ^b , rs6989157, rs6987276, rs4392869	.002	<i>TMEM67</i>
	12222	rs3097418 ^b , rs6989157, rs6987276, rs4392869, rs987036	.002	<i>TMEM67</i>
	212	rs3097422, rs3097418 ^b , rs6989157	<.001	<i>TMEM67</i>
	221	rs3097418 ^b , rs6989157, rs6987276	<.001	<i>TMEM67</i>
8q22.1-23.1	22122	rs3098233 ^b , rs3098224 ^b , rs3098218, rs3098212, rs2959025	<.001	<i>CTHRC1</i> , <i>WDSOF1</i>
	11211	rs3098233 ^b , rs3098224 ^b , rs3098218, rs3098212, rs2959025	<.001	<i>CTHRC1</i> , <i>WDSOF1</i>
	22112	rs6988793, rs6987078, rs3098233 ^b , rs3098224 ^b , rs3098218	.001	<i>CTHRC1</i> , <i>WDSOF1</i>
	12212	rs6987078, rs3098233 ^b , rs3098224 ^b , rs3098218, rs3098212	.002	<i>CTHRC1</i> , <i>WDSOF1</i>
	21121	rs6987078, rs3098233 ^b , rs3098224 ^b , rs3098218, rs3098212	.002	<i>CTHRC1</i> , <i>WDSOF1</i>
	22211	rs2959644, rs6988793, rs6987078, rs3098233 ^b , rs3098224 ^b	.002	<i>CTHRC1</i> , <i>WDSOF1</i>
8q24.2-24.22	12112	rs3098224 ^b , rs3098218, rs3098212, rs2959025, rs2957452	.002	<i>WDSOF1</i>
	22222	rs6989059, rs6986982, rs6988942, rs6989209, rs4388439 ^b	<.001	<i>KCNQ3</i>
10q21.1	12212	rs6986982, rs6988942, rs6989209, rs4388439 ^b , rs3843561	.002	<i>KCNQ3</i>
	22222	rs11000400, rs11000436, rs11000798, rs11001056 ^b , rs11001210	.003	<i>PRKG1</i>
	22222	rs11001056 ^b , rs11001210, rs11001213, rs11001447, rs11001702	.002	<i>PRKG1</i>
	2212	rs11000436, rs11000798, rs11001056 ^b , rs11001210	.002	<i>PRKG1</i>
	2122	rs11000798, rs11001056 ^b , rs11001210, rs11001213	.003	<i>PRKG1</i>
	221	rs11000436, rs11000798, rs11001056 ^b	<.001	<i>PRKG1</i>
	212	rs11000798, rs11001056 ^b , rs11001210	<.001	<i>PRKG1</i>
222	rs11001056 ^b , rs11001210, rs11001213	<.001	<i>PRKG1</i>	

(continued)

association, and haplotype-LD analyses (1q24.1-25.3 [encompassing 1q24.2, 1q24.3, and 1q25.2-25.3 fine-mapped regions], 1q41, 8q21.11-22 [encompassing 8q21.11-22 and 8q22.1-24.22 fine-mapped regions], and 10q21-22). Additionally, there were 3 significant regions that overlapped in the single SNP association

and haplotype analyses (1q21.2, 8p22, and 10q22.1). Thus, we selected these regions, shown in Table 2, as “regions of interest” (Figure 1, eTable 2, and eTable 3). Each of these regions contained SNPs that were statistically significant at $P < .005$ and also had multiple haplotype windows showing significance ($P < .01$).

Functional-Genomic Validation

Integration of our significant SNP and haplotypes with publicly available somatic BE/EAC transcriptome data (Figure 1) yielded 38 genes located within 250 kb flanking each significant SNP, within significant haplotypes, or both that accurately clustered BE/EAC cases from controls

Table 2. Haplotypes Significantly Associated With BE/EAC Cases vs Controls^a (continued)

Chromosome Region	Haplotype	SNPs	P Value	Significant Genes
10q22.1	2221	rs11000101, rs11000108, rs11000122, rs11000152, rs11000190 ^b	.003	<i>ASCC1</i>
	22211	rs11000108, rs11000122, rs11000152, rs11000190 ^b , rs11000202	.002	<i>ASCC1</i>
	12222	rs11000122, rs11000152, rs11000190 ^b , rs11000202, rs11000348	.009	<i>ASCC1</i>
	21122	rs11000152, rs11000190 ^b , rs11000202, rs11000348, rs11000828	<.001	<i>ASCC1</i>
	1122	rs11000190 ^b , rs11000202, rs11000348, rs11000828	<.001	<i>ASCC1</i>
	2211	rs11000122, rs11000152, rs11000190 ^b , rs11000202	.002	<i>ASCC1</i>
	2221	rs11000108, rs11000122, rs11000152, rs11000190 ^b	.002	<i>ASCC1</i>
	221	rs11000122, rs11000152, rs11000190 ^b	.003	<i>ASCC1</i>
	211	rs11000152, rs11000190 ^b , rs11000202	.001	<i>ASCC1</i>
	112	rs11000190 ^b , rs11000202, rs11000348	.003	<i>ASCC1</i>
	11q14	22112	rs1381720, rs12146457, rs3924745 ^b , rs665153, rs2926467	<.001
11221		rs1381722, rs1381720, rs12146457, rs3924745 ^b , rs665153	<.001	
12212		rs1381720, rs12146457, rs3924745 ^b , rs665153, rs2926467	.001	
21122		rs12146457, rs3924745 ^b , rs665153, rs2926467, rs1871684	.001	
12211		rs1381722, rs1381720, rs12146457, rs3924745 ^b , rs665153	.003	
2112		rs12146457, rs3924745 ^b , rs665153, rs2926467	<.001	
1122		rs3924745 ^b , rs665153, rs2926467, rs1871684	.001	
1122		rs1381722, rs1381720, rs12146457, rs3924745 ^b	.002	
221		rs1381720, rs12146457, rs3924745 ^b	<.001	
211		rs12146457, rs3924745 ^b , rs665153	<.001	
112		rs3924745 ^b , rs665153, rs2926467	<.001	
122		rs1381720, rs12146457, rs3924745 ^b	.002	

Abbreviations: BE, Barrett esophagus; EAC, esophageal adenocarcinoma; SNPs, single-nucleotide polymorphisms.

^aThese results were obtained from the haplotype analysis of the independent validation data set comprising 176 cases and 200 controls. In the “Haplotype” column, 1 represents the major allele and 2 represents the minor allele at each respective marker.

^bRepresent SNP associations significant in both the single SNP analysis and the haplotype analysis.

Table 3. Germline Mutations in 3 Candidate Genes in BE/EAC Cases

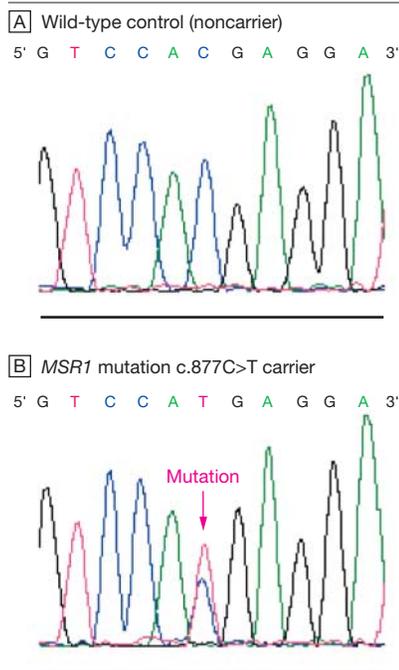
Gene	Variant	Total No.	No./Total (%) With Mutations		Proportion of Cases With Variant (95% CI)	P Value
			Cases	Controls		
<i>MSR1</i> (mutation analysis) ^a	c.877C>T,p.R293X	255	8/116 (6.9)	0/139	0.069 (0.030-0.130)	<.001
<i>MSR1</i> (validation) ^b	c.877C>T,p.R293X	197	2/58 (3.4)	0/139	0.034 (0.004-0.120)	.09
<i>MSR1</i> (pooled) ^c	c.877C>T,p.R293X	323	10/184 (5.4)	0/139	0.054 (0.026-0.098)	.006
<i>MSR1</i> (mutation analysis) ^a	c.760C>G,p.L254V	255	2/116 (1.7)	0/139	0.017 (0.021-0.061)	.19
<i>ASCC1</i> (mutation analysis) ^a	c.869A>G,p.N290S	220	2/95 (2.1)	0/125	0.021 (0.003-0.074)	.18
<i>CTHRC1</i> (mutation analysis) ^a	c.131A>C,p.Q44P	214	1/89 (1.1)	0/125	0.011 (0.0003-0.061)	.42
<i>CTHRC1</i> (validation) ^b	c.131A>C,p.Q44P	183	1/58 (1.7)	0/125	0.017 (0.0004-0.092)	.32
<i>CTHRC1</i> (pooled) ^c	c.131A>C,p.Q44P	272	2/147 (1.4)	0/125	0.014 (0.0009-0.026)	.50

Abbreviations: *ASCC1*, activating signal cointegrator 1 complex subunit 1; BE, Barrett esophagus; CI, confidence interval; *CTHRC1*, collagen triple-helix repeat-containing 1; EAC, esophageal adenocarcinoma; *MSR1*, macrophage scavenger receptor 1.

^aCandidate gene mutation analysis in BE/EAC cases and controls.

^b*MSR1* and *CTHRC1* mutations validated in small independent series of BE/EAC cases.

^cPooled series comprising series of cases and controls used for candidate gene mutation analysis and independent validation series.

Figure 2. Chromatogram of Germline *MSR1* Mutation

A, Wild-type sequence. B, Representative example of chromatogram showing *MSR1* exon 6 c.877C>T (p.R293X) mutation that was observed in approximately 5% (10 of 184) of Barrett esophagus and esophageal adenocarcinoma cases, but not in any of 139 controls (wild-type sequence, control). The heterozygous single-nucleotide variant is indicated by the arrow.

(eFigure 2). An additional filtering step based on known organ-specific functions resulted in a final short list of 12 priority candidate genes (*LHX4*, *DIRC3*, *MARK1*, *KIF26B*, *MSR1*, *TMEM67*, *WD-SOF1*, *CTHRC1*, *KCNQ3*, *PRKG1*, *ASCC1*, and *OPCML*), which were also functionally plausible, within our regions of interest (Table 1 and Table 2).

Mutational Analyses of Priority Candidate Genes

Mutational analyses of these 12 priority candidate genes in BE/EAC cases and controls revealed germline mutations in 3 genes (*MSR1* [macrophage scavenger receptor 1] [MIM153622], *ASCC1* [activating signal co-integrator 1 complex subunit 1] [NC_000010.10], or *CTHRC1* [collagen triple-helix repeat-

containing 1] [MIM610635]) in 13 of 116 patients (11.2%) with BE/EAC (TABLE 3 and eTable 1). No sequence variants were found in the remaining 9 genes that were not also present to the same degree in controls.

Among the 116 patients with BE/EAC, 8 patients (proportion, 0.069; 95% confidence interval [CI], 0.030-0.130; $P < .001$) had a germline truncating mutation in *MSR1* c.877C>T, resulting in p.R293X (FIGURE 2 and Table 3), and 2 additional patients (proportion, 0.017; 95% CI, 0.021-0.061; $P = .19$) with BE/EAC carried germline *MSR1* p.L254V (c.760C>G) in exon 5 (Table 3 and eFigure 3A). These mutations were not found in 139 ancestry-matched population controls. Additionally, we identified 2 germline missense mutations—c.869A>G in exon 8 of *ASCC1*, resulting in p.N290S in 2 patients (proportion, 0.021; 95% CI, 0.003-0.074; $P = .18$); and c.131A>C in exon 1 of *CTHRC1*, resulting in p.Q44P in 1 patient (proportion, 0.011; 95% CI, 0.0003-0.061; $P = .42$), neither of which were found among 125 controls (Table 3, eFigure 3B, and eFigure 3C).

Independent Validation of Germline *MSR1*, *ASCC1*, and *CTHRC1* Mutations

To confirm the mutations found in the 3 candidate genes (Table 3), mutational analyses were then performed in an independent series of 58 cases obtained from outpatient endoscopy units. These samples confirmed the presence of germline *MSR1* c.877C>T, p.R293X mutation in 2 of 58 cases (proportion, 0.034; 95% CI, 0.004-0.120; $P = .09$) and *CTHRC1* c.131A>C, p.Q44P mutation in 1 of 58 cases (1.7%). After pooling the original 116 cases with the validation series of 58, a total of 10 cases with BE/EAC carried p.R293X (proportion, 0.054; 95% CI, 0.026-0.098; $P = .006$) (Table 3).

MSR1 and *CCND1* Protein Levels

Western blotting of germline protein lysates from 5 *MSR1* mutation-

positive patients with BE/EAC and 7 controls revealed variable decreases in *MSR1* protein levels in 3 cases (FIGURE 3). All 5 *MSR1*-mutation positive patients had increased *CCND1* levels compared with controls (Figure 3). Barrett esophagus tissues from patients who were mutation-positive showed increased nuclear expression of *CCND1* by immunohistochemistry compared with control esophageal specimens (FIGURE 4). We then proceeded with the converse experiment by overexpressing wild-type *MSR1* in HEK293 cells, resulting in decreased *CCND1* protein (Figure 3).

COMMENT

Barrett esophagus is prevalent in the general population and has the potential to progress to EAC. Because late-stage EAC carries a poor outcome, it is desirable to identify predisposition or risk alleles that will eventually allow premorbid risk assessment and affect subsequent management. Herein, we have identified germline mutations in 3 candidate genes in approximately 11% of our series of patients with BE/EAC, with the most commonly affected being *MSR1* (approximately 7%), followed by *ASCC1* and *CTHRC1*. Findings of germline *MSR1* and *CTHRC1* mutations were replicated in an independent validation series.

MSR1 on 8p22 encodes the class A macrophage scavenger receptor, which are macrophage-specific trimeric integral membrane glycoproteins implicated in many macrophage-associated, hormonal, and pathological processes, including inflammation, innate and adaptive immunity, oxidative stress, and apoptosis.³⁰⁻³² The *MSR1* c.877C>T sequence variant, resulting in p.R293X, located within a highly conserved collagen-like domain of the *MSR1* protein,³³ would be expected to disrupt function. The *MSR1* p.R293X was previously shown to associate with prostate cancer in specific ancestries, although this association is controversial.^{30,33-36} Given our observations and

taking the p.R293X-prostate cancer association at face value, one possible explanation is that this particular mutation is associated with both BE/EAC and prostate cancer predisposition, with the latter at lower penetrance. In many inherited neoplasia syndromes, single gene mutations predispose to cancers in more than 1 organ.³⁷ We found both p.R293X and p.L254V (also within the conserved coiled-coil domain and near the glycosylation site at the 249th amino acid) in the germline of our BE/EAC cases, but not in the ancestry-matched population controls, which strongly suggest that these mutations contribute to BE/EAC risk, or at least are necessary for BE/EAC predisposition. Whether they are also sufficient is currently unknown.

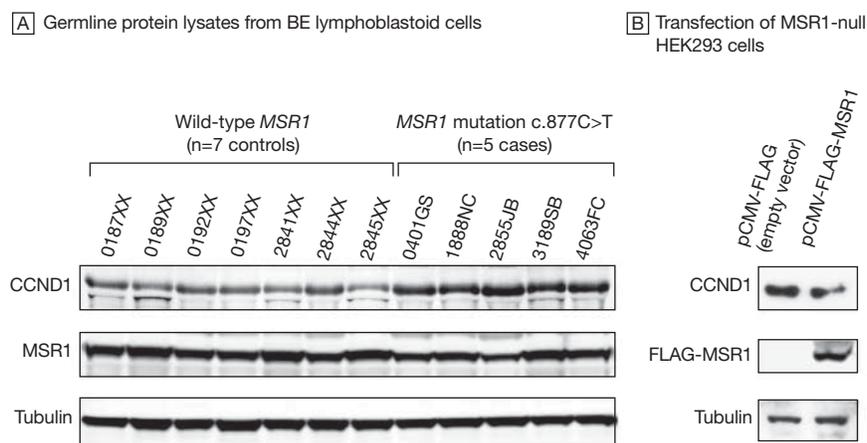
Accumulating evidence links *MSR1* to inflammatory events.³⁸ Barrett esophagus/EAC may also be associated with inflammatory events,⁷ thus supporting our observations that *MSR1* is a plausible candidate susceptibility gene for BE/EAC. The molecular mechanisms underlying the pathogenesis of inflammation-associated cancer are complex and involve both the innate and adaptive immune systems.³⁹⁻⁴²

More and more examples linking inflammatory and carcinogenic path-

ways, such as the cell cycle, are surfacing. For example, signal transducer and activator of transcription 3 and nuclear factor kappa-B link phosphatase and tensin homologue, deleted on chromosome 10 to inflammatory pathways.⁴³ Beyond genetic evidence, we have additionally shown up-regulation of key cell cycle molecule *CCND1* by both

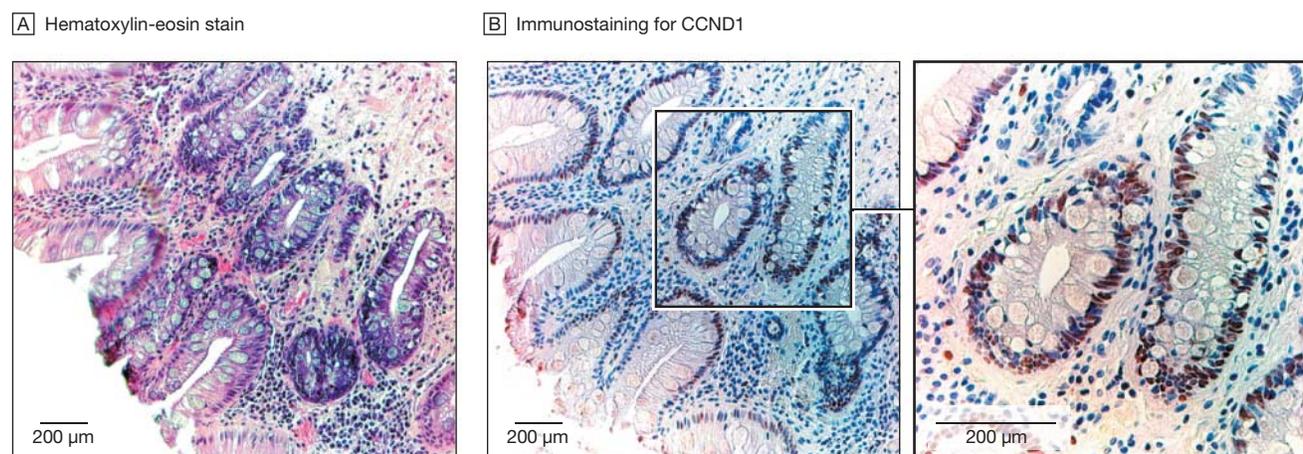
Western blotting of germline proteins and immunohistochemistry of *MSR1* mutation-related BE tissue, in which *CCND1* is overexpressed in the nucleus. *MSR1* p.R293X results in a truncated protein (affecting cytoplasmic topology, the transmembrane and parts of the collagen-like motifs), which still expresses, but variably. We ob-

Figure 3. Western Blot Detection of *MSR1* and *CCND1* Protein Levels



A, Representative Western blot showing *CCND1* protein levels from lymphoblastoid cells derived from patients with Barrett esophagus (BE) (n=5) and from population controls (n=7). The Western blot shown is representative of 2 independent experiments. Note variably decreased *MSR1* accompanied by increased *CCND1* protein expression in patients with BE compared with controls. B, Representative Western blot of *MSR1* and *CCND1* protein levels after HEK293 cells were transiently transfected with empty vector or wild-type *MSR1* constructs. Tubulin was used as a loading control for both A and B.

Figure 4. Immunohistochemistry Detection of *CCND1* in Esophageal Specimen From a Patient With BE



A, Hematoxylin-eosin staining of an esophageal lesion from biopsy specimen displaying characteristic goblet cells from a representative patient with Barrett esophagus (BE). B, *CCND1*-positive staining (brown by immunoperoxidase) in the nuclei of BE lesion cells from a patient who was germline *MSR1*-mutation positive. Hematoxylin counterstain. Detail at higher magnification.

served germline *MSR1* mutation, with variably decreased *MSR1* protein levels, was associated with overexpression of nuclear *CCND1* in BE tissues in *MSR1*-mutation carriers (but not in control normal epithelium). This observation suggests a linkage of inflammation to the cell cycle and a potential etiology for BE, via loss of control of the G1-S transition consistent with checkpoint-mediated cell cycle delays.^{44,45} *CCND1* elevation in both sporadic and heritable BE/EAC as an important final common pathway has precedent,⁴⁶⁻⁴⁹ linking the WNT and adenomatous polyposis coli protein (APC) cascades, and lending credence to our observations. It remains to be determined whether increased expression of *CCND1* in the setting of germline *MSR1* mutation can by itself, or in combination with other oncogenic events, lead to neoplastic transformation in BE.

We also found that *ASCC1* germline p.N290S, affecting a region conserved across species, occurred in 2.1% of BE/EAC cases, but not in ancestral-matched population controls. The candidacy of *ASCC1* as a risk allele for BE/EAC is supported by somatic expression array data comparing normal esophageal epithelium, premalignant BE, and EAC samples.⁵⁰ *ASCC1* enhances nuclear factor kappa-B and activator protein 1 transactivation by directly binding to JUN kinase.⁵¹ Although little else is known about *ASCC1*/TRIP4 function, its putative cross-talk with JUN and nuclear factor kappa-B again links inflammatory to tumor suppressive/oncogenic pathways. Its role in potentially co-regulating the androgen receptor⁵² may also begin to explain the known epidemiologic increased BE risk in men. In addition, although we found only 1 germline missense mutation in *CTHRC1*, this gene is intriguing in its cross-talk with 2 established pathways in sporadic BE/EAC pathogenesis, TGFB, and WNT⁵³ via APC.

Although this sequence alteration is a rare variant, and this study may not be powered to differentiate between 1 of 89 and 0 of 125, a change from glu-

tamine to proline results in an alteration from a single-branch polar (negative) amino acid to a small hydrophobic cyclic amino acid. This change is predicted to disrupt the protein's ability to form secondary structure and the helix-loop-helix structure necessary for collagen deposition, fibrosis, and involvement in the WNT and APC pathways. *CTHRC1* is expressed in tissue repair processes and may be important in the host's response to GERD. Given its role in collagen matrix deposition and its expression in myofibroblasts,⁵³ an alteration of *CTHRC1* might predispose to decreased lower esophageal sphincter tone and consequent tendency toward GERD and BE.

Because we sought to identify risk alleles that contribute to a reasonable subset of heritable BE/EAC that also yield clinically useful attributable risks (or protection), we consciously used a relatively small series. We did this because the larger the series, the more likely the genetic effect sizes shrink, such that case-control series in the thousands result in odds ratios in the 1.1 to 1.3 range. We added rigor by using a second-stage independent validation series of cases and controls that were sufficiently powered based on our pilot data. In addition, rather than performing brute-force sequencing of at least 38 genes in just our 9 top priority regions (which would have increased the likelihood of finding "noise"), we used a systems-biology approach of statistically prioritizing regions of interest by integrating multiple platforms to finally come up with 12 top priority genes, which eventually yielded germline mutations in *MSR1*, *ASCC1*, and *CTHRC1* in patients with BE/EAC but not in population controls. These 3 genes together accounted for 11% of our cases, reflecting what is normally considered a moderate- to high-penetrance genetic load for a disease. The functional interrogation and immunohistochemistry results support the pathogenicity of these germline *MSR1* mutations. Nonetheless, future independent studies are needed to replicate our data in other pa-

tient populations to confirm the conclusions.

In summary, germline mutations in *MSR1*, *ASCC1*, and *CTHRC1* in patients with BE/EAC appear physiologically relevant to BE, encoding proteins involved in apoptosis, innate immunity, polarity, and mobility that affect inflammatory and TGFB/WNT signaling pathways. Larger cohort studies may be necessary to determine the usefulness of these genes and their variants in risk assessment and premorbid diagnosis.

Author Contribution: Dr Eng had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs Meltzer and Eng contributed equally to the manuscript.

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Analysis and interpretation of data: Orloff, He, Ganapathi, Bebek, Romigh, Meltzer, Eng.

Drafting of the manuscript: Orloff, He, Bebek, David, Meltzer, Eng.

Critical revision of the manuscript for important intellectual content: Orloff, Peterson, He, Ganapathi, Heald, Yang, Bebek, Romigh, Song, Wu, David, Cheng, Meltzer, Eng.

Statistical analysis: Orloff, He, Bebek, Eng.

Obtained funding: Meltzer, Eng.

Administrative, technical, or material support: Peterson, He, Ganapathi, Heald, Yang, Romigh, Song, Wu, David, Cheng, Meltzer, Eng.

Study supervision: Meltzer, Eng.

Accruing research subjects documented and verified phenotypes and/or participated in material acquisition: Heald, Song, Wu, David, Cheng, Meltzer, Eng. **Genetic counselor coordinator:** Heald.

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