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Research Article

High-Resolution Melting Analysis for Mutation Screening of *RGSL1*, *RGS16*, and *RGS8* in Breast Cancer

Emilia Wiechec[1,2], Carsten Wiuf[3], Jens Overgaard[2], and Lise Lotte Hansen[1]

Abstract

Background: Identification of specific mutation targets in cancer may lead to discovery of the genes modulating cancer susceptibility and/or prognosis. The *RGSL1*, *RGS16*, and *RGS8* genes within the 1q25.3 region belong to the novel family of regulators of G protein signaling (RGS) genes, which increase the GTPase activity of the Gα subunit to attenuate signaling from the G protein–coupled receptor. We evaluated the use of high-resolution melting (HRM) to screen for mutations in the genes of interest and assess their clinical significance.

Methods: The HRM analysis was used to screen 32 coding exons of *RGSL1*, *RGS16*, and *RGS8* in tumors from 200 breast cancer patients. All sequence variants detected by HRM resulted in abnormal shape of the melting curves. The identified mutations and known single nucleotide polymorphisms (SNP) were subsequently confirmed by sequencing, and distribution of the SNP genotypes was determined by SNaPshot analysis. A case–control analysis of genotype frequencies was carried out.

Results: We identified three tumor specific missense mutations in *RGSL1* (*ex6* c.664 G>A (Val222Ile), *ex13* c.2262 C>G (Asp754Glu), and *ex13* c.2316 C>T (Ser772Leu) in three different breast cancer patients. In addition, a total of seven known SNPs were identified in this study. Genotype distributions were not significantly different between breast cancer patients and controls.

Conclusions and Impact: Identification of novel mutations within *RGSL1* provides a new insight into the pathophysiology of breast cancer. Moreover, the HRM analysis represents a reliable and highly sensitive method for mutation scanning of multiple exons. *Cancer Epidemiol Biomarkers Prev; 20(2); 397–407. ©2010 AACR.*

Introduction

Breast cancer is the most frequent female cancer and second cause of cancer death worldwide (1). The disease is genetically heterogeneous; therefore, susceptibility to breast cancer may be due to inherited or acquired genetic variations as mutations and single nucleotide polymorphisms (SNP), alone or in combination, as a disease causing haplotype (2). Single point mutations in various cell growth and apoptotic pathways such as ERK (extracellular signal regulated kinase)/MAPK (mitogen-activated protein kinase) pathway, phosphoinositide-3-kinase (PI3-K) pathway, G protein–coupled (GPCR) signaling, and p53-driven cell death are responsible for growth advantage of tumor cells, tissue invasion, and metastasis formation (3–9). Identification of specific SNP genotypes is also essential to predict drug response and toxicity of cancer treatment, as they may affect the transcriptional activity of the genes which metabolize the chemotherapeutic drugs (10–12). Screening genes for unknown mutations comprises a prerequisite for discovering the genes of importance to a disease or genes in which somatic mutations are responsible for tumor progression.

In this study, we screened 3 members of the regulators of G protein signaling (RGS) family, *RGSL1* (*RGSL2*), *RGS16*, and *RGS8*, for unknown mutations in breast tumors from 200 patients. The 3 RGS genes are clustered at 1q25.3, a region encompassing a strong candidate for hereditary prostate cancer, *RNASEL* (13–15). Because of steroid hormone regulation of prostate and breast cancers (16), we also considered implication of the genes within 1q25.3 in breast cancer development. Because mutation screening of *RNASEL* has not pointed at its unambiguous involvement in breast cancer, we have focused on the group of RGS genes from the region of interest (17, 18). The novel family of RGS proteins plays an essential role in regulating GPCR-induced signaling by enhancing the GTPase activity, thereby attenuating the G protein–dependent activation cycle (19–21). GPCRs that are involved in the regulation of numerous cellular processes such as growth, differentiation, motility, gene

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transcription, and signaling through these receptors have been lately described to have an emerging role in cancer (3, 22, 23). Thus, ability of RGS proteins to inhibit the GPCR-mediated signaling seems to impact cancer progression greatly and RGS proteins are considered as new therapeutic targets for drug development (23). AI/LOH studies along 1q have shown to correlate with DNA copy number changes, which, in addition to new mutations, could alter the RGS protein activity in breast cancer (24–27).

High-resolution melting (HRM) approach has been establishing since 2003 and was originally developed for genotyping and mutation scanning, followed by detection of epigenetic changes such as DNA methylation (28, 29). The HRM analysis is a fast and reliable methodology to detect new DNA sequence variants, which are detected as a shift in the melting temperature of the PCR amplicons. Mutations residing in the melting domain are visualized as alteration of the melting curve derived after PCR amplification by increasing the temperature and measuring the decrease of fluorescence emitted from within the double helix while the DNA strands separate (30–32). This method has a relatively high throughput, a low cost, and is less time-consuming in comparison with a well-established method such as high-performance liquid chromatography (HPLC). A total of 32 coding exonic sequences in the *RGSL1*, *RGS16*, and *RGS8* were screened in the search for unknown mutations by using the HRM methodology. The *RGSL1* comprises 22 exons across 110 kb. Until recently, the first 7 exons comprised the former *RGSL2*, which now is part of the *RGSL1* gene (UCSC Genome Browser, March 2006 Assembly). The neighboring *RGS16* and *RGS8* comprise 5 and 6 exons, respectively. The purpose of the HRM analysis was to search for unknown sequence variants and possible SNPs in the *RGSL1*, *RGS16*, and *RGS8* in breast cancer. Our approach was intended to verify the specificity of the HRM assay when compared with direct sequencing and SNaPshot genotyping, which served as a validating method (33, 34). The results show that the HRM technology holds an improvement in the field of unknown mutation detection technologies and is in concordance with genotype tagging methods such as SNaPshot analysis and DNA sequencing. A total of 7 known SNPs in the *RGSL1*, *RGS16*, and *RGS8* were identified with all 3 methodologies. Three tumor-specific unknown missense mutations in *RGSL1* were found in 3 different breast cancer samples.

Materials and Methods

DNA samples

Matched tumor and peripheral blood DNA samples obtained from 200 patients diagnosed with primary sporadic breast cancer, collected between 1992 and 1994, were accessible for the study. The complete information about the cohort of breast tumors is available (35). A control group of 96 peripheral blood DNA samples from Danish medical students was used for association study. The Local Ethical Committee, Aarhus County, Denmark, approved the use of human material for this study. DNA was purified using a modified protocol of the salting-out method (36).

Design of primers for the HRM assay

The set of primers for HRM, specific for all 32 coding exons in the *RGSL1*, *RGS16*, and *RGS8*, was designed, fulfilling the requirements of the Light Cycler 480 System Gene Scanning Assay. All the amplicons were in the range of 83 to 286 bp. The secondary structure formation and the melting model were determined with the freely available POLAND algorithm provided by the Heinrich-Heine-Universität Düsseldorf (37). All primers were HPLC purified and purchased from TAG Copenhagen A/S.

High-resolution melting

The coding exons of the *RGSL1*, *RGS16*, and *RGS8* were amplified using exon-specific primers and PCR conditions presented in Table 1. The HRM analysis was optimized for each exon by using a group of 6 control samples. The PCR and the subsequent HRM analysis were carried out in a single run on the Light Cycler 480 instrument (Roche Applied Science). All amplicons were amplified using Light Cycler 480 High Resolution Melting Master (Roche Applied Science) according to the manufacturer's guidelines. Briefly, PCR reactions were carried out in 10 μl final volume containing 5 μl of Light Cycler® 480 High Resolution Melting Master, 0.2 μL of each primer (10 μmol/L), 1.2 μL of $MgCl_2$ (25 μmol/L), 1.4 μL of ddH_2O, and 2 μL of template (10 ng/μL). The PCR conditions requiring the SYBR Green I detection format were as follows: 10 minutes of denaturation at 95°C, followed by 45 cycles of 95°C for 10 seconds, annealing as indicated in Table 1 for 15 seconds, and extension at 72°C for 10 seconds with the acquisition mode set to single. The premelting program included denaturation at 95°C for 1 minute and cooling to 40°C for 1 minute. The melting program was carried out at the continuous range from 60°C to 95°C with 25 acquisitions per °C.

Mutation detection

The HRM curve analysis was carried out by the Light Cycler 480 Gene Scanning Software. The melting curves were initially normalized between two temperature ranges, the premelt and postmelt equal to 100% of the initial fluorescence and to 0% (baseline) the fluorescence after DNA dissociation. Next, the normalized curves were shifted to the region of low fluorescence (denatured DNA) in order to identify heteroduplexes. Finally, the difference plots were generated, allowing capturing the differences in melting profile between the reference sample curve and curves from the test samples. All samples displaying clear difference in fluorescence when compared with the wild-type (WT) sample were considered

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Table 1. List of primer sequences in the *RGSL1*, *RGS16*, *RGS8* for HRM and sequencing analysis

Exon	Primer sequence (5′→3′)	Amplicon size, bp	Annealing temperature
RGSL1			
1	F: CAATGGATATAGTTCTATTGAC	137	56°C
	R: CTTATTTGAGAGACTCAAGG		
2	**F: GAATGCAATTCTGGTTCATG**	179	54°C
	R: CAGCTGCTGATGAAATATTTG		
3	F: CATGAAGAGAAGCTCTACTC	158	56°C
	R: CTAAACTCTCTACCTCTGAAG		
4a	F: GCAGTTCGGTTTCACTTAG	139	56°C
	R: AGAATGTAATGGAAACACAAG		
4b	F: CTTGTGTTTCCATTACATT	83	56°C
	R: CGCATGCACACCAATGCTT		
5a	F: GTATAGCTCTGTGCCTCTT	146	56°C
	R: GTGGCCCTCAGCATGAG		
5b	F: CCTGGAAGTGAGAGACTAC	120	56°C
	R: GATGCTTTATAATTCTTACTGA		
6a	F:CCAGAGTCCCTCCTGAACC	174	60°C
	R:CAGACCATGGCATGCTTC		
6b	**F: CACACCAAGATGACCATGG**	165	58°C
	R: GGCTTTCCTGCTTGAGTAC		
6c	**F: GTACTCAAGCAGGAAAGCC**	191	58°C
	R: CCAGGGAACTGATTCTTGT		
6d	F: ACAAGAATCAGTTCCCTGG	159	56°C
	R: CATCTTGGAGGAGTGATTG		
6e	F: CAATCACTCCTCCAAGATG	157	56°C
	R: GTCAAGAAGTTGGATCTCC		
6f	**F: GGAGATCCAACTTCTTGAC**	121	56°C
	R: GAGCTCGCAGATTCTGTC		
6g	F: GACAGAATCTGCGAGCTC	180	56°C
	R: GCCAGATATAACTTATTTCTG		
7	F: GATGCAGCAAGCTGACCA	192	56°C
	R: CCTTTTAAAACAGTAATTACTC		
8a	F: CTGGTCTAGTTCCTGTAAC	193	56°C
	R: CCTATAGTCCATTTCCTTCA		
8b	F: CTCTAGAGTTAAGCCAGGC	152	56°C
	R: ATTACAGTGACTGCTCTGAC		
9	F: TGAACCCAGACTTTGGACAA	233	56°C
	R: AAGATTACAGATTTTCTCTTAG		
10	F: CATTGCTTATAATATAGTGTTC	197	55°C
	R: GCATGTACCTGAAGATGTTG		
11a	F: CAGAATCCAGAATCATCATTC	185	56°C
	R: CATTGGTCTCTATACTGATC		
11b	**F: CTTCAGGGAGTTCCTCAAGGA**	215	56°C
	R: CCCAGGCTATTCTCCTAAG		
12	F: CTATCTCAGAAATGGTAGATG	217	58°C
	R: GCAACTCCAGGAAGAATCC		
13	**F: GTTTGCCTGGACTGTCATC**	230	56°C
	R: GTCTCTAATGTCTTGTTTCAC		
14	F: CAGTAGACTCTCGGTGAAC	278	54°C
	R: CAAAGCAATAAGCTTCTTATG		
15	F: GTAAGAGTGACTTGATCAAAC	246	58°C
	R: GAGAAGTGGAAAACTTACTTC		
16	F: GCCTTGTTAGTCACTGTTTTC	243	60°C
	R: CTTCTTGGTGAAAGGCCAG		

(Continued on the following page)

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Table 1. List of primer sequences in the *RGSL1*, *RGS16*, *RGS8* for HRM and sequencing analysis (Cont'd)

Exon	Primer sequence (5′→3′)	Amplicon size, bp	Annealing temperature
17	F: CTGCCTTCCCGTGGAGC	214	60°C
	R: CCTGACCTAACAGTCACTG		
18	F: TGTGCTCGGTGCTCCAG	234	56°C
	R: CTCTTCACAAGTCTTGGTTG		
19	F: CAGTAGGAGTTGTCGCAG	186	56°C
	R: CCTCTGATTGAGTAGCAAAC		
20	F: CTACATTGGAGGTGTCCG	246	56°C
	R: CTATTGCATGCTTGGACATA		
21	F: CTCAATTACTGCATCATAATTG	125	58°C
	R: CTCTAAGAGATGATTTATCTC		
RGS16			
1a	F: TCCGATTGGTCAGCAGTG	173	56°C
	R: GGATGGTGGCAGGCTC		
1b	F: GAGCCTGCCACCATCC	119	57°C
	R: CCAAGCAGTTGGACAACC		
2	F: CACTGGCTGAGCAAATGAG	187	59°C
	R: TAACAGACCAGCAACAAGTC		
3a	F: CCATCAGAGTTCTGGCTAG	199	56°C
	R: CACATCTTCTGAGAAGTTTC		
3b	F: GAAACTTCTCAGAAGATGTG	223	58°C
	R: CTTTATATGTTGAATAGAGGC		
4a	F: AGGCAGGTCATTGTTCAAG	239	57°C
	R: CAGCTTGGTAGCTGATCG		
4b	F: CGATCAGCTACCAAGCTG	228	56°C
	R: CCTAGTCTCTGGTCACAG		
5a	F: **GACACTGGATCAGATGTG**	238	60°C
	R: **GCTGTGGCAGTCTGCAG**		
5b	F: CTGCAGACTGCCACAGC	197	60°C
	R: CTCACTGCCGTGGAGAC		
RGS8			
1a	F: CTAGAGCATAACGAGAAGAC	233	58°C
	R: AGGACTCTCTCTTCCTGCTTG		
1b	F: CAAGCAGGAAGAGAGTCCT	168	56°C
	R: CTACTTGCATTTGTTATGGAG		
2	F: **CGCTTGCTGCCTGGCT**	286	60°C
	R: **GGATAATGCAATCGGTGC**		
3a	F: CTGTTCATGAAGTCTTCAGA	213	56°C
	R: GCTGTGAAATCACTACACG		
3b	F: CGTGTAGTGATTTCACAGC	196	56°C
	R: CTTTCACTCACGTCCAGG		
4a	F: CAGTGACAGAATTGCAGGA	178	56°C
	R: CTTCTTCTGTCGATAATCTC		
4b	F: GAGATTATCGACAGAAGAAG	167	56°C
	R: GAACACCTAGTACATACCTG		
5a	F: CTGCACCCAGATGAGAATG	227	54°C
	R: AACCAGAATTCCAGGTTCTC		
5b	F: CAGAACCTGGAATTCTGGTT	244	56°C
	R: CAGAGTGATGAGGCTCAG		
6a	F: GTAGATGCTGTCATTCATAAT	175	56°C
	R: CTTGGTCAAAGCAAGTCAG		
6b	F: CTGACTTGCTTTGACCAAG	214	56°C
	R: GAACACCTATGACATTTCAC		

NOTE: Primer sequences used for initial PCR in SNaPshot assay are in bold.
Abbreviations: F, forward; R, reverse.

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to possess a novel genetic variation and underwent sequencing.

Sequencing

Both DNA strands from PCR products showing abnormal HRM profiles were sequenced to confirm the specificity of the HRM analysis. The PCR products (10 μL) generated after HRM were purified with 0.07 μL of Exonuclease (Fermentas) and 3.3 μL of FastAP (Fermentas) for 15 minutes at 37°C, followed by enzyme inactivation at 80°C for 15 minutes. The sequence reaction was carried out in a final volume of 10 μL containing 1 μL of the purified PCR product, 0.5 μL of each primer (10 μmol/L), and 3 μL of the 2.5× buffer, using the BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing program was as follows: 1 cycle of 96°C for 5 minutes, 25 cycles of 96°C for 30 seconds, 59°C for 15 seconds, 60°C for 4 minutes. The sequencing products were precipitated with isopropanol, separated by capillary electrophoresis in the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems), and evaluated using the Molecular Evolutionary Genetics Analysis, v.3.1.

Design of extension primers for SNaPshot assays

A total of 7 SNP loci located within the coding exons of the genes of interest, and previously identified by the HRM assay, were selected for analysis. The primers for PCR amplification and SNaPshot extension reactions (Table 2) were designed with the 3′ end of each primer positioned directly at the site of target SNP, on either the sense or anti-sense DNA strand. Each SNaPshot primer was 5′-tailed with poly(dGACT). The BLAT Search Genome (38) was used to search for an appropriate alignment of each primer. The potential hairpin and self-complementarity formation was checked with Oligonucleotide Properties Calculator (39). All primers were HPLC purified and purchased from TAG Copenhagen A/S.

SNaPshot assay

The PCR amplifications were carried out in a final volume of 15 μL containing 20 ng/μL DNA, 0.16 μL of 25 μmol/L dNTP mix (Ampliqon), 2 μL of each primer (10 μmol/L), 0.15 μL of Taq polymerase 5 U/μL (Ampliqon), 2 μL of 1× buffer supplied with 1.6 mmol/L of $MgCl_2$. The PCR conditions were as follows: 1 cycle of 96°C for 15 minutes, 35 cycles of 94°C for 30 seconds, primer annealing as indicated in Table 1 for 30 seconds, 72°C extension for 30 seconds, followed by 15 minutes of final extension at 72°C, and a 4°C holding step. After amplification, 15 μL of PCR products was purified by incubating at 37°C for 30 minutes with 0.07 μL of Exonuclease (Fermentas) and 3.3 μL of FastAP (Fermentas), followed by enzyme deactivation at 80°C for 15 minutes. Next, 3 μL of the purified PCR product was mixed with 3 μL of SNaPshot reaction mix (Applied Biosystems), 3.8 μL of ddH_2O, and 0.2 μL of the SNaPshot primer (10 μmol/L) in a 10 μL total volume. Primer extension was carried out on the GeneAmp PCR system (Applied Biosystems) for 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds, followed by a 4°C hold. After the SNaPshot PCR, the unincorporated ddNTPs were removed enzymatically by incubating samples with 1 U of FastAP for 60 minutes at 37°C, followed by 15 minutes at 80°C for enzyme inactivation. All products were separated by capillary electrophoresis in the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems) and evaluated using the GeneScan software, version 3.7 (Applied Biosystems).

Statistical tests

To test for differences in SNP genotype frequencies between breast cancer cases and controls a chi-square (χ^2) test was used (5 tests in total). We also used a χ^2 test to assess whether pairs of SNPs (10 pairs in total) had different genotype frequencies. In both situations *P* values were adjusted for multiple testing by using the Bonferroni correction.

The prognostic value of different SNP genotypes in breast cancer was analyzed using a Cox multivariate proportional hazards regression model by backward selection. Adjustment was made for tumor size, nodal status, menopausal status, histopathologic classification, and estrogen receptor status.

Table 2. List of extension primer sequences for the SNaPshot assay

SNP	Primer sequence (5′→3′)	Size, bp	Change
rs1144566	F: **gactgactgactgactgac**AACATTGACCATGAGACCC	42	A/G
rs7535533	R: **gactgactgactgactga**CAAATCTTTTCATTGGTCTCTA	44	T/C
RGSL1 ex6 c.664 G>A	R: **gactgactgactgactgactgact**CTATGTGGGTGTAGCAAA	47	G/A
rs569956	F: **gactgactgactgactgactgactgac**CTGGATGGTCAGAG AGGCT	50	G/T
rs41299607	F: **gactgactgactgactgactgactgactgac**AGAGGATGAAGTCTTTGCC	54	A/G
rs649437	F: **gactgactgactgactgactgactgactgactgact**CTTGACCTCTGGCAGGAC	62	C/T
RGSL1 ex13 c.2316 C>T	F: **gactgactgactgactgactgactgactgactga**GTGCAAAGTGAAGTACAAATTT	64	C/T

NOTE: Poly(dGACT) tail is shown in bold.
Abbreviations: F, forward; R, reverse.

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Table 3. List of mutations and SNPs identified in the *RGSL1*, *RGS16*, and *RGS8*

Exon	Nucleotide	Type	SNP ID	HRM		Sequencing		SNaPshot	
				C	BC	C	BC	C	BC
RGSL1 ex2	A/G	Missense	rs41299607	8/96	22/190	8/8	22/22	8/96	22/190
RGSL1 ex6 c.664 G>A	G/A (Observed C/T)	Missense	Novel	0/96	1/190		1/1		1/190
RGSL1 ex6	C/G	Missense	rs647224	5/96	21/190	5/5	21/21		
RGSL1 ex6	C/T	Synonymous	rs649437	44/96	80/190	25/25	80/80	44/96	80/190
RGSL1 ex11	A/G (Observed T/C)	Missense	rs7535533	44/96	71/181	44/44	71/71	44/96	71/181
RGSL1 ex13 c.2316 C>T	C/T	Missense	Novel	0/96	1/192		1/1		1/192
RGSL1 ex13 c.2262 C>G	C/G	Missense	Novel	0/96	1/192		1/1		
RGS16 ex5	A/G	Missense	rs1144566	2/96	12/175	2/2	12/12	2/96	12/175
RGS8 ex2	C/T	Missense	rs680277	9/96	18/185	9/9	18/18		
RGS8 ex2	G/T	Synonymous	rs569956	9/96	18/183	9/9	18/18	9/96	18/183

Abbreviations: C, controls; BC, breast cancer samples.

The 2-sided power analysis was carried out with the use of power calculator provided by Russel Lenth (40).

Results

HRM analysis

A total of 21 coding exons in *RGSL1*, 4 exons in *RGS16*, and 6 exons in *RGS8* were screened for unknown mutations. To optimize the HRM analysis for each exon, control DNA derived from Danish medical students was used. Each exon was analyzed using 96 DNA templates from control DNA and 200 DNA templates from breast carcinomas.

Using PCR and HRM conditions as listed in Table 1, the 7 known variants and 3 novel mutations in the genes of interest were identified (Table 3) on the basis of a clear separation of differentially shifted melting curves. Four known SNPs (rs41299607, rs647224, rs649437, and rs7535533) within *RGSL1*, 1 known SNP in *RGS16* (rs1144566), and 2 known SNPs in *RGS8* (rs680277 and rs56956) were identified by HRM both in control DNA and in tumor DNA. The 3 unknown point mutations were identified in exons 6 and 13 of *RGSL1*. Examples of atypical melting curves of the identified sequence variations are shown in Figure 1A.

Sequencing

All cases showing melting curves divergent from the wild type underwent direct sequencing of both strands to confirm the HRM results. A total of 95 control amplicons and 227 breast tumor amplicons of different exons in *RGSL1*, *RGS16*, and *RGS8* were sequenced (Table 3). The HRM results were confirmed in 100% of the sequenced amplicons. Three novel missense mutations in *RGSL1* were identified only in tumor and not in blood DNA obtained from the same patient. These mutations were detected in 3 different tumors and comprised a G664A resulting in Val222Ile in exon 6, a C2262G in exon 13 changing Asp754Glu, and a C2316T changing Ser772Leu also in exon 13. Examples of sequence variants confirmed by sequencing with regard to previous HRM findings are shown in Figure 1B.

SNaPshot assay

Of a total of 7 known SNPs previously identified by HRM, 5 (rs41299607, rs649437, rs7535533, rs1144566, and rs569956) were genotyped using the SNaPshot assay. The rs647224 and rs680277 were excluded from genotyping because of the potential linkage with rs649437 and rs569956, respectively. The novel mutations in exons 6 and 13 (*RGSL1 ex6* c.664 G>A and *RGSL1 ex13* c.2316 C>T) were included in the genotyping analysis. Because the frequency of the unknown mutations was relatively low in tumor samples and absent in control samples, the second point mutation (*RGSL1 ex13* c.2262 C>G) was excluded from the genotyping analysis. Besides genotyping purpose of SNaPshot analysis, this methodology was considered as an additional validation method to previous HRM and direct sequencing. All polymorphisms identified by SNaPshot were concordant with the previous results (Table 3). Examples of polymorphisms assessed by SNaPshot assays are presented in Figure 1C.

Comparison of HRM, sequencing, and SNaPshot analyses

A total of 32 amplicons within the *RGSL1*, *RGS16*, and *RGS8* were examined for the presence of unknown mutations and SNPs in breast cancer with the use of the HRM analysis. The HRM-identified sequence variations have

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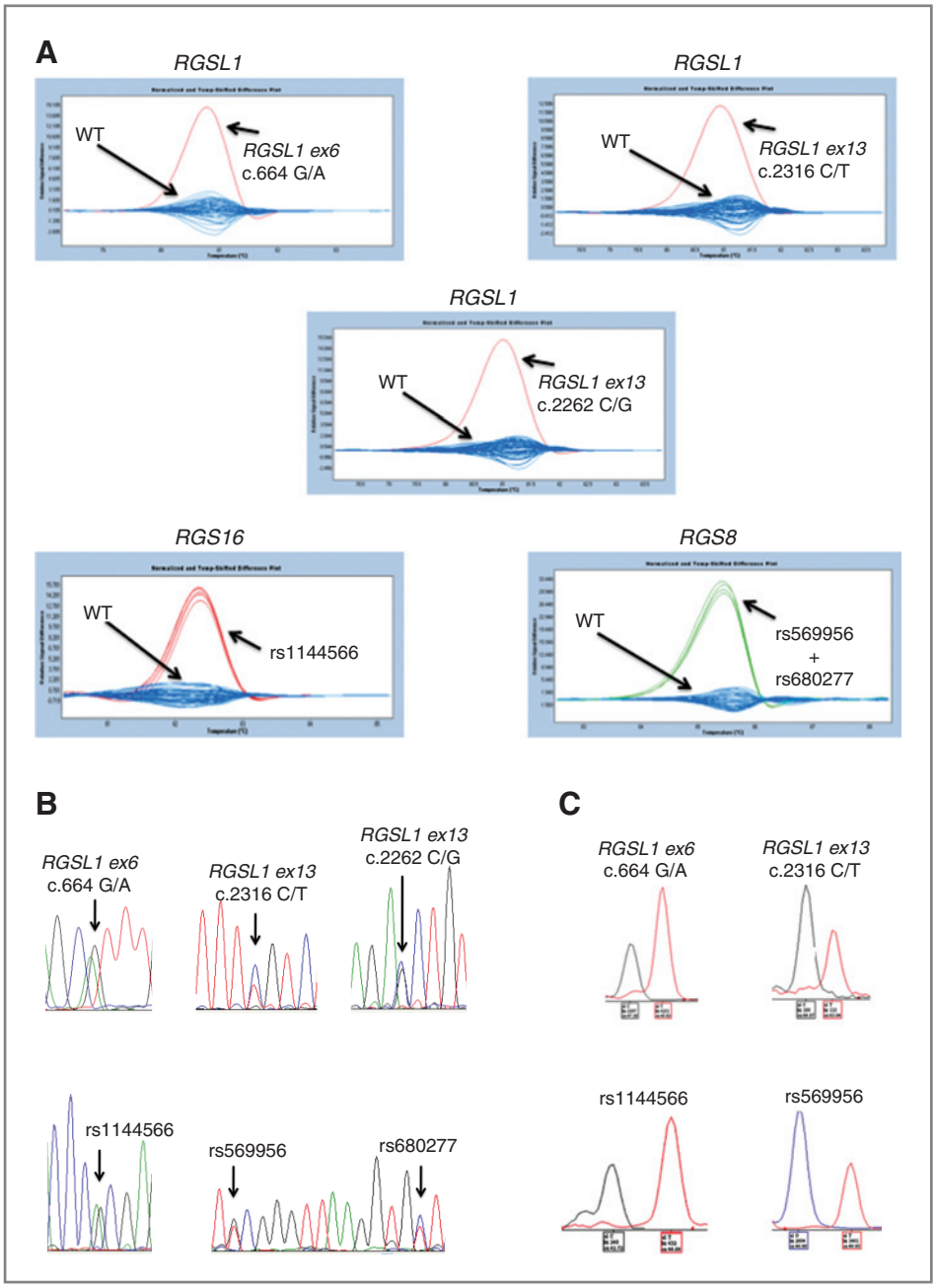

Figure 1. Detection of sequence variants within *RGSL1*, *RGS16*, and *RGS8* by HRM. The HRM-identified variants: *RGSL1 ex6* c.664 A>G, *RGSL1 ex13* c.2316 C>T, *RGSL1 ex13* c.2262 C>G, rs1144566, rs56956, and rs680277 (A), subsequent validation by direct sequencing (B) and SNaPshot genotyping (C) are shown.

been sequenced for cross-validation, followed by SNaPshot genotyping. We did not observe any false-positive results in all sequenced and genotyped cases (Table 3). The specificity of the HRM analysis was 100%. Although the sensitivity of sequencing is known from the literature to be lower than of HRM, our results showed that sensitivity of HRM is highly comparable with sequencing and were also verified with the SNaPshot assay.

Statistics

The frequency of genotypes for the 5 known polymorphisms (rs41299607, rs649437, rs7535533, rs1144566, and rs569956) studied in both control and breast cancer populations is shown in Table 4. The 2 newly identified mutations, *RGSL1 ex6* c.664 G>A and *RGSL1 ex13* c.2316 C>T, which were also confirmed by the SNaPshot analysis, were not covered by statistical analysis because of occurrence only in one breast cancer sample. The genotype distribution of the 5 analyzed SNPs did not differ between control and breast cancer samples. The pairwise comparison of the genotype frequencies in the two groups did not reveal any statistically significant difference. Multivariate analysis has found no association between different genotypes and clinicopathologic

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Table 4. Case–control comparison of SNP genotypes identified by SNaPshot analysis

	rs41299607			rs649437			rs7535533	
Controls								
	AA	88/96 (92%)		CC	12/96 (12.5%)		CC	16/96 (16.5%)
	AG	8/96 (8%)		CT	44/96 (45.5%)		CT	44/96 (46%)
	GG	0/96		TT	40/96 (42%)		TT	36/96 (37.5%)
Breast cancer								
	AA	168/190 (88.5%)		CC	41/190 (21.5%)		CC	31/181 (17%)
	AG	22/190 (11.5%)		CT	80/190 (42%)		CT	71/181 (39%)
	GG	0/190		TT	69/190 (36.5%)		TT	79/181 (44%)
P: χ^2			0.39			0.17		0.54

	rs1144566			rs569956	
Controls					
	AA	94/96 (98%)		GG	0/96
	AG	2/96 (2%)		GT	9/96 (9%)
	GG	0/96		TT	87/96 (91%)
Breast cancer					
	AA	163/175 (93%)		GG	0/183
	AG	12/175 (7%)		GT	18/183 (10%)
	GG	0/175		TT	165/183 (90%)
P: χ^2			0.09		0.9

parameters and overall survival of breast cancer patients. A Cox multivariate proportional hazard analysis was carried out. The analysis included in addition to the different genotypes the following clinicopathologic factors: menopausal status, nodal status, tumor size, estrogen receptor status, and histopathologic classification. No significant association between the different genotypes was observed, whereas the analysis confirmed that the risk of developing metastatic breast disease was significantly associated with the primary tumor size greater than 20 mm (HR = 1.71; 95% confidence limits: 1.08–2.71) and positive lymph nodes (HR = 2.46; 95% confidence limits: 1.57–3.86); the other parameters did not show independent prognostic value.

The 2-sided power analysis was carried out on SNP rs1144566 because of the lowest *P* value from case–control comparison of genotypes among 5 analyzed SNPs. The power analysis showed that the statistical power to detect a significant association in the genotype frequencies is less than 27% (using a type I error of 5%). A 3 times increase in the sample size of both cases and controls is necessary to obtain a 90% power. Using a Bonferroni-corrected type I error (5%/5 = 1%) yields a power of about 4% and 38% with samples sizes increased 3 times.

Discussion

RGS proteins are key players in modulating the signaling initiated by essential mediators of cancerous transduction such as GPCRs (22, 41). These are also capable of interactions with non-GPCR signaling components such as epidermal growth factor receptor (EGFR), phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), calmodulin, and 14-3-3, suggesting their role as signaling scaffolds (42). Several RGS proteins including RGS16 have been described as differentially expressed in various types of cancer (23, 27). To date, genetic mutations in RGS genes, which affect the final gene product, have been mostly associated with schizophrenia, celiac disease, hypertension, and recently lung and bladder cancer (23, 43–46). In this study, we carried out mutational scanning of the *RGSL1*, *RGS16*, and *RGS8* located at 1q25.3, which has been shown to be genetically unstable in breast cancer due to high rate of allelic imbalance and chromosomal breakpoints. Downregulation of RGS16 caused partially by promoter methylation and chromosomal breakpoints has been shown (27). Furthermore, loss of RGS16 has been associated with proliferation of breast cancer cells and phosphorylation of Akt, a downstream effector of the prosurvival PI3-K pathway (47). To our knowledge, there is no evidence for genetic variability in *RGSL1*, *RGS16*, and *RGS8*, which can confer the susceptibility to breast cancer. We used a real-time PCR-based system, namely, HRM, to detect new sequence variants in the genes of interest. The advantage of HRM, besides its robustness, simplicity, and less labor-intensiveness, is the possibility of performing the melting analysis of the amplicon in connection with the PCR. However, careful design of the amplicon is essential to avoid false-positive signals and nonreproducible results. In the search for unidentified mutations, an abnormal melting curve shape is an indication of the putative

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existence of the sequence variation; however, its genotype has to be further determined. Despite this condition, HRM comprises an efficient tool for large-scale gene scanning. Direct sequencing-based validation was applied to all mutations and all 7 SNPs (rs41299607, rs647224, rs649437, rs7535533, rs1144566, rs680277, and rs569956) and 3 unknown mutations in *RGSL1* (*RGSL1 ex6* c.664 G>A, *RGSL1 ex13* c.2262 C>G, *RGSL1 ex13* c.2316 C>T) identified by the HRM assay. The SNaPshot genotyping method was utilized to confirm previous findings and assess the genotype frequencies of single SNPs in both controls and the breast cancer group. The SNaPshot assay was highly concordant with HRM, and sequencing proving the existence of the 3 unknown, missense mutations within *RGSL1* only in 3 different breast cancer samples. The amino acid change in the *RGSL1ex6* c.664 G>A (Val222Ile) and *ex13* c.2262 C>G (Asp754Glu) may not influence the protein activity significantly due to the functional similarities of Val and Ile, and Asp and Glu, respectively. However, the Ser to Leu substitution affecting codon 772 may affect the protein structure, stability, and further intracellular interactions, due to changes in posttranslational modification. This has to be established by further functional studies of *RGSL1*. However, because of a lack of commercially available antibody against RGSL1 and RNA from the patients, we could not determine expression of RGSL1 in breast cancer samples with and without newly identified mutations. Noticeably, the additional study on an independent cohort of breast tumors should be conducted to elucidate the clinical significance of mutations in *RGSL1* and their implication in a mechanism of breast cancer development.

Furthermore, we have not found any mutations in *RGS16* including the Tyr168 residue of RGS16, which is necessary for EGFR-mediated phosphorylation of RGS16 resulting in the attenuation of RGS16 GTPase activity (48). This suggests that the Tyr168 residue of RGS16 is unlikely to undergo mutational regulation in breast cancer. Our previous study has shown reduced expression of RGS16 in breast cancer samples affected by allelic imbalance, intragenic chromosomal breakpoint, and methylation (27). Other alternative mechanisms leading to the inactivation of RGS16 might rely on upstream regulatory genes such as *p53*, which is known to possess binding site on RGS16. However, we could not find any meaningful correlation between the established *p53* mutation status of our cohort of breast cancer samples, genetic alterations affecting *RGS16*, and prognosis for the patients (27, 49). The reduced expression of RGS16 might result in cell proliferation and cancer progression due to failure in mediating inhibition of the prosurvival MAPK pathway (50, 51). The latest study on RGS16 has shown that underexpression of RGS16 correlates with enhanced proliferation of breast cancer cells and activation of the downstream molecule of the prosurvival PI3-K pathway, namely, Akt (47).

Unlike RGS16, RGS8 has never been described as a potential cancer susceptibility gene. To date, its role has mostly been described as an inhibitor of G protein signaling in the neural system due to predominant expression in cerebellum (52). In our earlier study, similarly to RGS16, expression of RGS8 was abrogated by the presence of chromosomal breakpoints within *RGS8* (manuscript in preparation) in breast cancer. We did not observe any tumor-specific mutations in our cohort of breast cancer samples, which could contribute to the elevated level of RGS8. Thus, expression pattern of RGS16 and RGS8 seems to depend on the tumor-specific genomic alterations and epigenetic changes.

Moreover, association study including genetic variations in *RGSL1*, *RGS16*, and *RGS8* did not provide any significant correlation, neither of genotype and allele distribution between 96 controls and 200 breast tumors nor prognostic value of genotypes and clinicopathologic parameters in breast cancer. However, further investigation, including a higher number of breast cancer samples and controls, is required to clarify the implication of the genes of interest in modulating the susceptibility to breast cancer.

In summary, identification of the 3 novel mutations in *RGSL1* improves our knowledge of the etiology of breast cancer but should be followed by further investigations in larger cohort of breast cancer samples. The potential mechanism of RGS protein regulation within 1q25.3 is most likely associated with epigenetic regulation and chromosomal rearrangements with DNA copy number changes at the forefront. Although the genotypes of *RGSL1*, *RGS16*, and *RGS8* do not seem to be directly linked to risk, they may interact with other markers in the development of breast cancer. Furthermore, we support the HRM of being currently one of the most efficient methods of screening genes for unknown mutations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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