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Clinical significance of a novel single nucleotide polymorphism in the 5′ untranslated region of the *Rabphillin-3A-Like* gene in colorectal adenocarcinoma

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Abstract

The recently identified human ortholog of the Rabphillin-3A-Like (*RPH3AL*) gene, located at the *17p13.3* locus, has been assessed for its mutational status and clinical significance in colorectal adenocarcinoma (CRC). Prospectively collected 95 frozen CRCs and their matching benign colonic epithelial tissues were evaluated for mutations and mRNA expression. Since, we observed a higher incidence of a single nucleotide polymorphism (SNP) at the −25 position in the 5′ untranslated region (5′UTR-25) of *RPH3AL*, we performed the genotyping analysis of this SNP in a retrospective CRC cohort (n=134) to assess their clinical importance. Univariate and multivariate outcome analyses were performed. The cDNA analysis has detected point mutations in 6 CRCs, coding region SNPs in 14 CRCs, and non-coding region SNPs in 38 CRCs. Combined analyses of both cohorts has demonstrated that the incidence of SNP at 5′UTR-25 was 41% (95 of 229), and its A/A genotype (9%, 20 of 229) was observed exclusively in non-Hispanic Caucasians, and 19 of these cases were diagnosed with nodal metastasis. Patients who exhibited homozygous for A or C alleles had a significantly decreased levels of mRNA expression, increased risk of CRC recurrence and mortality. Therefore, these findings have significant clinical implications in assessing the aggressiveness of CRC.

Keywords

Colorectal Cancer; *RPH3AL*; Single Nucleotide Polymorphism; Recurrence; Prognosis

2. INTRODUCTION

The human chromosome region *17p* shows frequent allelic loss/mutations in colorectal adenocarcinomas (CRCs) (1–3). Such genetic alterations are a hallmark for the presence of tumor suppressor genes and suggest the existence of additional tumor suppressor genes besides *p53*, which is known to occur at the *17p13.1* locus (4–8). Furthermore, a correlation between allelic loss on *17p* and *p53* mutation has not been found. This suggests that unknown tumor suppressor genes near *p53* may be involved in the development of CRC. In a search for such novel tumor suppressor genes in the *17p* chromosome region, we correlated the *Rabphillin-3A-*

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like (*RPH3AL*) gene at the *17p13.3* locus with a reported association between a hemizygous deletion of *17p13.3* in human medulloblastoma and poor patient survival (9). Subsequently, Smith *et al.* (10) identified the *RPH3AL* gene at the *17p13.3* locus (GenBank # AF129812) and suggested it was the human ortholog of the rat *Rabphillin-3A-like* gene (*Rph3al*) (originally termed *Noc2*) with 77% identity at the amino acid level. In that study, they also cloned, sequenced and performed mutational analysis of the *RPH3AL* gene in medulloblastoma, follicular thyroid carcinoma and ovarian carcinoma specimens. As these studies failed to identify any missense mutations in *RPH3AL*, they concluded that *RPH3AL* might not be involved in the oncogenesis of these neoplasms (10). To our knowledge, only one other study has analyzed the coding region of the *RPH3AL* gene for genetic abnormalities in CRCs (n=50). They reported six missense mutations (12%) and suggested a possible tumor suppressor role of *RPH3AL* in CRCs (11).

Although, the precise function of *RPH3AL* is unknown, the *Rph3al* gene is known to be involved in the regulation of endocrine exocytosis through its interactions with the cytoskeleton, in which the cysteine-rich zinc finger domain of Noc2 plays a major role in its binding with the LIM domain of Zyxin (a cytoskeletal protein) (12,13). The Noc2 protein is abundantly expressed in pancreatic beta-cells and in other endocrine cells (13) and alterations in the cellular level of Noc2 profoundly impairs beta-cell exocytosis, indicating that this protein is a key element of the machinery controlling insulin secretion (14,15). It has been suggested that the *RPH3AL* gene product performs functions including vesicle transport and Ca^{2+}-dependent exocytosis, particularly in the secretion of neurotransmitters (16,17) by interacting with Rab3A, a low molecular weight guanosine triphosphate (GTP)-binding protein.

Since, only one small study in CRC has examined the coding region of the *RPH3AL* gene for mutations (11) and because polymorphisms in the regulatory or non-coding regions of several human genes have been implicated in mRNA transcription, increased risk for cancer development, and prognosis (18–21), in the present study we analyzed the mutational status of *RPH3AL* in prospectively collected primary sporadic CRC and matching normal (benign) samples, covering all exonic regions, both coding and non-coding, of this gene. Fourteen other SNPs within its genomic region (obtained from the dbSNP database of the NCBI-http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=9501) were assessed to examine their effect on the status of *RPH3AL*. Since the incidence of a single nucleotide polymorphism (SNP) at the −25 position in the 5′ untranslated region (5′UTR-25) was more common, we also analyzed its incidence and genotypes in a retrospectively collected CRC sample cohort. Finally, the genotypic status of 5′UTR-25 was correlated with mRNA expression, disease recurrence and patient survival.

3. PATIENTS, MATERIALS AND METHODS

3.1. Patients

Clinical information and tissues required for these studies were collected under Institutional Review Board approved protocols of the University of Alabama at Birmingham (UAB). All patients included in this study had undergone surgery for first primary CRC at the UAB hospital.

3.1.1. Prospective CRC samples—Tissue samples from 95 consecutive, unselected patients with histologically confirmed CRCs and corresponding normal (benign colonic epithelial) tissues, 8 cm away from CRC, were collected fresh at surgery, snap-frozen in liquid nitrogen, and stored in liquid nitrogen vapor phase by the Tissue Procurement (TP) Facility of the UAB-Comprehensive Cancer Center until transferred for analysis. All patients had undergone surgical resection for first primary CRC from January 1996 through December

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2004; however, the majority of these cases (71 of 95) were from years 2002 through 2004. Therefore, this patient cohort did not have a long follow-up period.

3.1.2. Retrospective CRC samples—Due to limited resources, we randomly selected 134 patients from an eligible 1,120 CRC patients who had undergone surgical resection for "first primary" CRC between 1981 and 1994 from UAB. The 1,120 eligible retrospective samples were an 'unselected' patient population. The intent of using patients from this time period was to maximize long post term surgery follow-up. Formalin-fixed, paraffin-embedded tissue blocks from these patients were obtained from the Anatomic Pathology Division at UAB. These histologically confirmed CRCs and corresponding normal (benign colonic epithelial) tissues, 8 cm away from CRC, tissues were analyzed to assess the status of 5′UTR-25 the *RPH3AL* gene.

During our initial selection process, those patients who died within a week of their surgery, those patients with surgical margin-involvement, unspecified tumor location, multiple primaries within the colorectum, multiple malignancies, or those patients with family or personal histories of CRC were all excluded from our study population. However, based on the information in the patient charts, we recognized it would be difficult to identify the familial *vs.* sporadic nature of CRCs; therefore, this retrospective cohort can be described as a '*consecutive*' patient population. The median follow-up period of this cohort was 10.8 years (range <1 to >19 years).

3.1.3. Patient demographics, clinical and follow-up information—As shown in Table 1, patient demographics along with clinical and follow-up information were retrieved retrospectively from the medical charts as well as from the UAB-Tumor Registry. Patients were followed either by the patients' physician or by the UAB tumor registry until their death or the date of the last documented contact within the study time frame. The Tumor Registry ascertained outcome (mortality) information directly from patients (or living relatives) and from the physicians of the patients through telephone and mail contacts. This information was further validated against state death certificates. Demographic data including patient age at diagnosis, gender, race/ethnicity, date of surgery, date of the last follow-up (if alive), date of recurrence (if any) and date of death were collected. The Tumor Registry updated follow-up information every six months and follow-up of our retrospective cohort ended in May 2007.

Our study includes both African-American and non-Hispanic Caucasian patients. Information on the race/ethnicity background was obtained from the patient charts and assignment was likely self-described or self-identified. For example, blacks of the metro-Birmingham area are overwhelmingly classified as African-Americans, because migration of African blacks from other continents was a rare phenomenon during this study period. However, we recognize there is always some diversity in identification within any race/ethnic group.

3.1.4. Pathological features—In our study, a pathologist (WEG) reviewed hematoxylin and eosin (H and E) stained slides of all cases for the degree of histologic differentiation and re-graded all lesions as: well, moderate, poor or undifferentiated. Subsequently, we pooled well and moderately differentiated tumors into a low grade group and poor and undifferentiated tumors into a high grade group (22). The pathologic staging was performed according to the criteria of the American Joint Commission on Cancer (23). The International Classification of Diseases for Oncology (ICD-O) coding schema was used to specify anatomic location of the tumor (24). The anatomic sub-sites were grouped into proximal colon and distal colorectum. Three dimensional tumor size (length, breadth and depth) was taken into consideration and the largest of the three dimensions was used for statistical purposes.

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Before, utilizing the tissues for RNA and DNA extraction, and for mutational and genotyping analyses, a frozen section was cut and stained with H and E to assess the proportion of tumor *versus* uninvolved tissue in the sample and to permit macro-dissection (using simple microscope) to separate tumor from uninvolved tissue.

3.2. Mutational analysis of the *RPH3AL* gene

Using the reverse transcription-polymerase chain reaction (RT-PCR) and DNA sequencing methods, we analyzed 95 frozen specimens of CRCs and corresponding benign colonic epithelial tissues for the mutational status of *RPH3AL*. The resulting cDNA transcripts covered the entire coding region including parts of 5′ and 3′ non-coding regions. The initial study on *RPH3AL* (10) had suggested that the putative start site was in exon 2 at position 193 and the stop site in exon 9 at position 1137 of the cDNA; thus our cDNA transcripts covered the non-coding region of the 5′ end as well as the later part of exon 9 at the 3′ end. We designed these specific primers using the LASERGENE (DNAStar Inc, Madison, WI) software.

3.2.1. RNA extraction, PCR analysis, and DNA sequencing of the *RPH3AL* gene —Total cytoplasmic RNA was extracted directly from frozen tissues with a RNAeasy Kit (Qiagen, Valencia, CA). Total RNA was used as template in oligo (dT)-primed first strand cDNA synthesis with SuperScript III (Invitrogen, Carlsbad, CA). One-tenth of the first-strand cDNA synthesis reaction mix was used as the template in the PCR with the *RPH3AL* forward (5′-GTGCACTTTGGAGACAGCAA-3′) and reverse (5′-GTGGGAGGGGAGGGTAATAA-3′) amplification primers. We used a thermostable FastStart *Taq* DNA polymerase (Roche, Indianapolis, IN) for the *RPH3AL* amplification. Mutation analysis of *RPH3AL* was performed by direct sequencing of the *RPH3AL* cDNAs amplified by RT-PCR. The 25 μL reaction mixture consisted of 10 x PCR buffer, 10 mM of each dNTP, 1.5 mM of $MgCl_2$, 10 pmoles of each primer, and 0.5 μL (2.5 units) of FastStart *Taq* DNA polymerase (Roche). Amplification was achieved by 5 min of initial denaturation at 94°C followed by 40 cycles of 30 seconds each at 94°C, 30 seconds each at 60°C, 1 min at 72°C and a 7-min final extension at 72°C. PCR products were fractionated by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

The appropriate reaction products were used as the template in cycle-sequencing reactions (Perkin-Elmer, Boston, MA) with *RPH3AL*-sequencing primers. Sequencing reaction products were resolved on an ABI Prism 307 automated DNA sequencer (UAB Comprehensive Cancer Center DNA Sequencing Core Facility). Compilation and sequence analyses were performed using the LASERGENE (DNAStar) sequence analysis software, which allows for direct analysis of sequencing electrophoretograms for the detection of duplex sequence signals at each position to identify mutations/polymorphisms. Nucleotide changes in each cDNA sequence were confirmed by sequencing both strands.

3.3. Expression of mRNA of *RPH3AL*

The RNA samples isolated from 95 frozen specimens of CRCs and corresponding benign colonic epithelial tissues were analyzed for the mRNA expression of *RPH3AL* by the quantitative real time-PCR method.

3.3.1. Quantitative real-time PCR analysis—One μg of total RNA was reverse transcribed by PCR as performed in SYBR green reagent supermix (Bio-Rad laboratories, Hercules, CA) in a final volume of 25 μL consisting of 0.5 μL of each primer (5 pmoles), 12.5 μL of 2x supermix containing the reaction buffer, Fast-start Tag DNA double strand-specified SYBR green I dye, 6.5 μL of nuclease free water and 5 μL of cDNA template following incubation of PCR reactions at 95°C for an initial denaturation followed by 45 cycles of 15 sec denaturation at 95°C, and annealing and extension at 57°C for 30 sec. The PCR reactions were

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performed using an i-Cycler real-time PCR system (Bio-Rad). The sequences of primers used for PCR analysis are as follows: *RPH3AL*, 5′-CGAGGATCGTCTGCCTTATT-3 (sense) AND 5′-GCACGTACAAGTGTCCACTACA-3′ (antisense) and beta-actin, 5′-TAAGTAGGCGCACAGTAGGTCTGA-3 (sense) and 5′-AAGTGCAAAGAACACGGCTAAG-3 (antisense). PCR products were subjected to melting curve analysis to exclude non-specific amplification. All of the PCR reactions were performed in sets of four. The mean of the *RPH3AL* mRNA and beta-actin mRNA copy numbers were calculated for each patient separately and a ratio of mean *RPH3AL* mRNA to beta-actin mRNA copy numbers was generated.

3.4. Genotyping of the SNP At 5′UTR-25 of *RPH3AL*

Genomic DNA extracted from frozen and archival CRCs and matching benign colonic epithelial specimens of both prospective (n=95) and retrospective (n=134) cases were analyzed for their genotype at 5′UTR-25 of this SNP utilizing the PCR-confronting two pair primer (PCR-CTPP) method as described below.

3.4.1. DNA extraction from paraffin blocks and frozen tissues—Genomic DNA was extracted from paraffin-embedded archival tissues (both from CRCs and matching benign colonic epithelia) following a previously published method (25). In brief, a 10 μM thick archival tissue section was deparaffinized in 1mL of octane (Fisher Scientific, Sewanee, GA). The tissue pellet was resuspended in 180 μL of digestion buffer [(50mM Tris pH 8, 1mM EDTA [pH 8], 1% Tween 20) plus 20 μL of proteinase K (20mg/ml) (Fisher Scientific)], and the mixture was incubated for 24 hrs at 56°C. Samples were heated to 95°C, then 200 μL of phenol/chloroform/isoamylalcohol (Fisher Scientific) (25:24:1, pH 6.7) was added. The aqueous layer was transferred into microcon YM-100 filter tubes (Fisher Scientific, USA). The DNA was eluted from the microcon filter tubes by adding 125 μL of TE buffer (10mM Tris-hydrochloric acid 0.1mM EDTA [pH 8]). DNA quality and concentration was determined by spectrophotometry. However, genomic DNA from snap frozen tissues was extracted using DNeasy Tissue Kit (Qiagen). The quality of DNA, as defined by the ratio of E260nm/280nm =1.8 – 2.0, was maintained for all samples.

3.4.2. PCR-CTPP method and sequence analysis—The PCR-confronting two pair primer **(PCR-CTPP)** method has been applied successfully for genotyping most single nucleotide variations (26). The amplification of allele-specific bands of different lengths was carried out by using four primers for genotyping by electrophoresis. As shown in Figure 1A, the four primers consist of F1 and R1 for the amplification of one allele, and F2 and R2 for the amplification of the other allele. F1 and R2 produce a common PCR product that is independent of the difference in alleles. F2 and R1 confront each other at the 3′ end with the base specific to the allele. The primers designed to detect the 5′UTR-25 C to A variant were: F1 (5′-GAGGGCACAGAGAACCTGTC-3′), R1 (5′-GGAGCACCCGGCTGGGGGTT-3′), F2 (5′-CATCTCAGATGTGACTCCCC-3′), and R2 (5′-GGCCCCAGAGGTACTCACTT-3′). The 25 μL reaction mixture consisted of 10 × PCR buffer, 10 mM of each dNTP, 1.5 mM of $MgCl_2$, 10 pmoles of each primer, 0.5 μL (2.5 units) of Platinum *Taq* Polymerase (Invitrogen), and 100 ng of genomic DNA. Amplification was achieved by 5 min of initial denaturation at 94°C followed by 35 cycles of 30 seconds each at 94°C, 30 seconds each at 60°C, 1 min at 70°C and a 7-min final extension at 70°C. PCR products were fractionated by electrophoresis in a 2% agarose gel and stained with ethidium bromide (Figure 1*B*). The products of PCR-CTPP were analyzed by direct DNA sequencing to reconfirm the genotype of 5′UTR-25.

3.5. Analysis of other SNPs in the genomic region of *RPH3AL*

Due to limited resources, only the prospectively collected CRCs and their matching benign colonic epithelial tissues were analyzed for 14 other SNPs which are closely linked to the

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genomic region of *RPH3AL* to assess for their association with variant forms of SNP at 5′ UTR-25 as well as other genetic alterations within the *RPH3AL* gene. The information on these 14 SNPs were obtained from the dbSNP database of the NCBI (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=9501). Details of their IDs, the PCR primer sets and their position on the chromosome *17* are shown in Table 2. The PCR and sequencing methods utilized for the analysis of these 14 SNPs were similar to the protocols described in the PCR-CTPP and sequencing method section.

3.6. Statistical analyses

The χ^2-test was used to compare baseline characteristics (27). Recurrence of CRC (local recurrence or distant metastases) and deaths due to CRC were the outcomes (events) of interest. The prognostic significance of the SNP at 5′UTR-25 were analyzed in retrospective cohort (n=134) using Kaplan-Meier (28) and Cox proportional hazards regression analysis methods (29). Demographic variables included in the analysis were age (less than 65 and greater than or equals to 65 years), gender, and ethnicity. Pathological variables included were pT (depth of tumor invasion), pN (nodal involvement), M (distant metastasis), tumor differentiation (low or high grade), tumor size (less than or equals to 5 cm and greater than 5 cm) in maximal dimension, and tumor location (proximal colon or distal colorectum). For recurrence analyses, the time at risk was measured by calculating the number of months from date of surgery to time of recurrence. Patients who had a recurrence of their CRC or who died due to their CRC, were identified and classified as an “event”, while the remaining patients without recurrence as, 1) died due to causes other than CRC, or 2) who were alive at the end of the follow-up period and were “right censored”. For survival analyses, the risk of CRC-specific death was measured by calculating the number of months from the date of surgery to death or the date of last contact. Patients who died of a cause other than CRC or who were alive at the end of the follow-up period were “right censored”.

The log-rank test was used to compare Kaplan-Meier curves based on the genotype status of the SNP at 5′UTR-25. The Kaplan-Meier estimates were also used to obtain recurrence rates or survival probability. Separate multivariate Cox regression models were built to assess the value of the type of genotype at 5′UTR-25 of the *RPH3AL* gene in predicting disease recurrence and patient overall survival. We controlled for all demographic and clinicopathological variables described above in these multivariate analyses. All analyses were performed with SAS statistical software version 9.1 (30,31). Quantitative real time-PCR results were plotted and the Independent-Sample T- Test was performed using SPSS 8.0. Two-sided P values were calculated and significance was analyzed at an alpha level of 0.05.

4. RESULTS

4.1. Mutational status of the *RPH3AL* gene in prospective specimens

the mutational analysis of *RPH3AL* in prospectively collected frozen CRCs and their matching benign colonic epithelial specimens (n=95) detected five novel missense mutations at codons 67 (GTG to ATG; Val to Met) in 2 CRCs, at codon 175 (CCC to TCC; Pro to Ser) in 2 CRC, and at codon 290 (AGG to AAG; Arg to Lys) in 1 CRC. A novel silent mutation at codon 98 (TGC to TGT; Cys to Cys) in 1 CRC was also identified. Additionally, 14 CRCs exhibited SNPs; three at codon 49, nine at codon 62, and two at codon 303 in the coding region of the *RPH3AL* gene which were reported earlier (10). In the non-coding region, we also detected novel SNPs at the −21 position (C to G) in 1 CRC, at the + 1044 position (C to G) in 1 CRC, and at the −25 position (C to A) in 36 (38%) of 95 CRCs (data not shown). Genotyping analysis of the 5′UTR-25 position of these 36 cases suggested that 9 (10% of 95) CRCs were homozygous for the “A” allele, 27 (28%) CRCs were heterozygous for “C/A” alleles and the

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remaining (62%) were homozygous for the "C" allele. The SNP at 5′UTR-25 observed in tumors and in their corresponding normal epithelial tissues is shown in Figure 1*C*.

4.2. Genotyping of the SNP at 5′UTR-25 in retrospective specimens

The incidence of the SNP at 5′UTR-25 in the retrospective cohort was 44% (59 of 134) (Table 1). Similar to the prospective cohort, the pattern of the variant genotype frequencies at 5′ UTR-25 in the retrospective CRC samples was C/C 75 (56%), A/A 11 (8%) and C/A 48 (36%) (Table 1). Overall, the incidence of genetic alterations at 5′UTR-25 of *RPH3AL* was 41.5% (95 of 229) (Table 1).

4.3. Correlation between 5′UTR-25 alterations and clinicopathological features

Distribution of SNP at 5′UTR-25 is demonstrated in Table 1. In total, 95% (19 of 20) of all prospective and retrospective cases with the genotype of A/A at 5′UTR-25 were associated with nodal involvement; in contrast, only 31% (23 of 75) cases with the C/A genotype exhibited nodal involvement. CRCs with the genotype C/C however, were evenly distributed into node positive (51%) and node negative (49%) categories (χ^2 $P<0.0001$). Among the cases with the A/A genotype, the majority of patients were males (14 of 20, χ^2 $P = 0.006$) and all of them were non-Hispanic Caucasians (20 of 20, Fisher Exact $P<0.0001$), and their tumors invaded into the deeper layers of the bowel wall (pT component of the TNM staging) (20 of 20, Fisher exact $P = 0.01$). The majority of CRCs with the A/A variant genotype were larger than ≥ 5 cm in size (13 of 19, χ^2 $P = 0.06$); however, there was no association between the genotype at 5′ UTR-25 and either tumor grade or tumor location (Table 1). Because we have not observed statistically significant correlations between the clinicopathological features and the missense mutations or SNPs of *RPH3AL* other than the SNP at 5′UTR-25 (data not shown), they were not considered in further analyses.

4.4. Expression of mRNA based on the SNP status at 5′UTR-25 of *RPH3AL*

The levels of mRNA expression were significantly higher in normal (benign colonic epithelia) tissues 2.36 (0.15 – 10.32) compared to matching invasive tumor tissues 0.42 (0.038 – 1.70) (Figure 2A). As shown in Figure 2B, significantly decreased mRNA expression of the *RPH3AL* gene was observed in CRCs which exhibited A/A (0.38, range 0.06 – 0.59) or C/C (0.27, range 0.038 – 0.246) variant genotypes compared to the C/A variant genotype (1.659, range 0.88 – 1.78). The down regulation of mRNA expression has been observed in CRCs with A/A and C/C genotypes compared to their corresponding normal tissues. However; there was no significant difference between CRC and the corresponding normal tissues of patients which are heterozygous for the C/A variant genotype (Figure 2B).

4.5. Linkage disequilibrium of the SNP at 5′UTR-25 of RPH3AL

There was no significant association between 14 SNPs of the *RPH3AL* genomic region and 5′ UTR-25 genotypes (A/A, C/C and C/A) (Table 2) or other genetic alterations (SNPs as well as mutations) of the coding region of *RPH3AL* (data not shown), indicating that 5′UTR-25 variants were not in linkage disequilibrium with the other SNPs present its genomic region.

4.6. Risk of recurrence

Kaplan-Meier univariate analysis demonstrated that patients homozygous for the A allele or C allele at 5′UTR-25 had a higher risk of recurrence compared to patients who were heterozygous for the C/A alleles (log-rank $P<0.0001$ and $P = 0.008$, respectively) (Figure 3A). However; there was no significant difference in risk of recurrence between patients homozygous for the A and C alleles (log-rank, $P = 0.10$) (Figure 3A).

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Multivariate Cox Proportional Hazards analysis for disease recurrence demonstrated that patients with the A/A variant genotype had a 13.85 times higher risk of CRC recurrence compared to patients with the C/A variant genotype (CI: 3.12–16.43). Patients with the C/C variant genotype had a 4.64 times higher risk of CRC recurrence compared to patients with the C/A variant genotype (CI: 1.65–13.07); whereas, there was no significant difference in risk of recurrence among patients with A/A and C/C variant genotypes, when adjusted for all demographic and clinicopathological features as shown in Table 3. The analyses have also demonstrated that patients with nodal metastasis were 2.41 times more likely to have a recurrence compared to those without nodal metastasis (CI: 1.12–5.18) when adjusted for all other variables (Table 3).

4.7. Survival analyses

Kaplan-Meier univariate survival analysis of the retrospective CRC cohort demonstrated that patients homozygous for the A allele at 5′UTR-25 had a significantly poorer survival as compared to patients heterozygous for the C/A alleles (log-rank $P<0.001$) (Figure 3B). There was also a significant difference in survival of patients homozygous for the C allele compared to patients heterozygous for the C/A alleles (log-rank, $P < 0.001$). However, there was no significant difference in survival between patients homozygous for the A or C alleles (log-rank, $P = 0.21$) (Figure 3B).

Multivariate Cox proportional hazards analyses demonstrated that patients with the A/A variant genotype were 3.88 times more likely to die due to CRC as compared to patients with the C/A variant genotype (CI: 1.39–10.82); whereas, there was no significant difference in the risk of death for patients with genotypes C/C vs. C/A or A/A vs. C/C when adjusted for all demographic and clinicopathological features (Table 3). Patients with nodal involvement were 2.25 times more likely to die due to CRC compared to those who were node negative (CI: 1.30–3.92) while patients with high tumor grade were 1.95 times more likely to die due to CRC as compared to those with low tumor grade when adjusted for all other features (CI: 1.14–3.31) (Table 3).

5. DISCUSSION

The present study has identified five novel missense point mutations and one silent mutation in the coding region of the *RPH3AL* gene together with three additional novel genetic alterations, including a single nucleotide polymorphism (SNP) at 5′UTR-25 in the non-coding region. Also, this study has confirmed the identity of three previously known polymorphisms in the coding region of the *RPH3AL* gene (10). The genotype analysis of SNPs at 5′UTR-25 suggested that homozygous for the A allele was exclusively present in non-Hispanic Caucasians and was significantly associated with nodal metastasis. Consequently, the levels of *RPH3AL* mRNA were lower in CRCs specifically with homozygous C or A alleles at 5′ UTR-25 when compared to their corresponding benign colonic epithelial tissues. In contrast, the levels of mRNA expression were high both in CRCs and matching controls of cases with heterozygous C/A genotype. In multivariate Cox proportional hazards analyses it was observed that patients with the A/A and C/C variant genotypes had 13.85 times and 4.64 times, respectively a higher risk of CRC recurrence compared to patients with the C/A variant genotype. In comparison, patients with positive nodal metastasis were 2.41 times more likely to have a recurrence when compared to those with lymph nodes free of metastatic disease. Multivariate Cox proportional hazards analyses for survival demonstrated that patients with the A/A variant genotype were 3.88 times and patients with nodal metastasis 2.25 times more likely to die due to CRC compared to patients with the C/A variant genotype and patients with no nodal involvement, respectively.

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Single nucleotide polymorphisms among individuals may significantly advance our ability to understand the process of cancer development and provide insights into its treatment (32). Polymorphisms differ from gene mutations in that they have a frequency of at least 1% in the normal healthy population. Approximately 90% of DNA polymorphisms are due to a single base substitution; while, the remaining SNPs result from insertion, or deletion, or microsatellites (or polymorphisms in them) (33). Although most polymorphisms are functionally neutral, some affect regulation of gene expression or on the function of the coded protein. These functional polymorphisms despite being of low occurrence, could contribute to the differences between individuals in susceptibility and severity of disease (34,35). In addition, polymorphisms in association with other genetic or epigenetic events or in strong linkage disequilibrium with other SNPs are involved in pathogenesis of cancer through modulating gene expression (36–38).

Findings of this current study demonstrate that both homozygous genotypes at 5′UTR-25 of *RPH3AL* (A/A and C/C) confer significantly poor prognoses than the heterozygous genotype (C/A). These findings are similar to previous reports showing the correlations between the homozygous, but not the heterozygous, genotypes of a SNP of the matrix metalloproteinase-1 gene and the aggressive phenotypes of CRCs (20). Contrary to the expectation that the A/A homozygote would have the worst prognosis and the C/C homozygote would have the best prognosis, our results demonstrate that both homozygous genotypes (A/A and C/C) confer significantly worse prognoses than the heterozygous (C/A) genotype. Higher mRNA levels of *RPH3AL* in CRCs with C/A heterozygote variants of SNP at 5′UTR-25 may aid in maintaining adequate amounts of mRNA copies of *RPH3AL*; thus, less “malignant behavior” of CRCs with the C/A genotype and consequent better patient survival were observed. The allele loss (39, 40) or methylation (36–38) are well known genetic events in various cancers which have profound effect on level of gene expression of several tumor suppressor genes and involved in tumor progression, poor survival and early recurrence of several human malignancies (41–43). These genetic events might be the possible mechanisms responsible for differential gene expression among variants of *RPH3AL* in CRCs; however, our further studies are aimed at investigating these possibilities.

Although, the results presented in this study may not elucidate the mechanisms regulating the expression of RPH3AL, a recent *in vivo* experimental study on rat pancreas suggested that the promotor region of NOC2 (RPH3AL) revealed the presence of putative cAMP-responsive elements (CRE) potentially capable of binding with a transcriptional inhibitor, inducible cAMP early repressor (ICER) and demonstrated that a four-fold increase in the expression of ICER decreased the expression of NOC2 (15). Also, this study has demonstrated that expression of NOC2 was dynamically regulated by the glucose levels. However, the future studies will have to determine the precise regulatory mechanisms that are leading to the reduction of mRNA expression in CRCs with homozygous 5′UTR-25 genotypes of *RPH3AL* in relation to the responsive elements of its promoter region.

Our analyses of 14 other SNPs linked to the genomic region of *RPH3AL* have suggested no significant association with the SNP at 5′UTR-25 or with other alterations of *RPH3AL*. Therefore, based on these findings it is reasonable to conclude that there is no genetic linkage between the alterations of *RPH3AL* gene, specifically, with different variants of the SNP at 5′ UTR-25 and other SNPs located its genomic region.

The present study demonstrates a significant association between the *RPH3AL* gene polymorphism at the 5′UTR-25 position and advanced tumor stage. Individuals carrying the variant A/A genotype exhibited nodal metastasis, early recurrence, and both short overall survival and decreased disease-free survival. Previous reports suggested that genetic variations or polymorphisms play an important role in tumor growth, invasiveness, and metastatic

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behavior by influencing either the epithelial cell adhesion to collagen or laminin in the extracellular matrix or by influencing cell-cell adhesive interactions (44) or their involvement in carcinogenesis (45). Although it is not clear how primary tumors progress into invasive or metastatic phenotypes, particularly in relation to the *RPH3AL* gene, our results suggest that the biological events that are specifically mediated by the A/A variant genotype of the *RPH3AL* gene may promote tumor cell invasive and metastatic phenotypes. These findings strongly support our hypothesis that polymorphism at the 5′UTR-25 of *RPH3AL* might have substantial impact on individual susceptibility and biological mechanisms which facilitate tumor aggressiveness leading to early recurrence or poor survival in this sub-set of patients.

Although the role of the *RPH3AL* gene is not fully explored in the initiation or progression of neoplasia in our studies, the evidence that patients with the A/A genotype have a poor prognosis and higher risk of early CRC recurrence reinforces our hypothesis which emphasizes the SNP at 5′UTR-25 of *RPH3AL* in tumor aggressiveness. Therefore, studies are currently in progress in our laboratory to determine the functional effects of these genetic alterations, specifically, to understand how the SNP at 5′UTR-25 of *RPH3AL* contribute to CRC progression. Additionally, we are currently evaluating the clinical value of different genetic abnormalities in this gene in assessing the *risk* of developing colorectal neoplasia in a case-control scenario.

One important property of DNA polymorphisms is that their prevalence can vary substantially between different racial groups (46–48) and gender (49). We have, in fact, found a statistically significant difference in the prevalence of the genotypes based on race or gender. One could only anticipate finding such disparities if the mechanism underlying the function of the polymorphic site is altered by a phenomenon that is associated with race or gender. Thus a polymorphism at 5′UTR-25 in the *RPH3AL* gene appears to be a credible genetic factor related to the susceptibility to CRC for Caucasian males and may be associated with an endocrine mechanism specified by sex hormones or other gender specific mechanism. Furthermore, our results suggest that the SNP with the A/A variant genotype of the *RPH3AL* gene at 5′UTR-25 might be considered as a race specific genetic variant, as we observed in both prospectively and retrospectively collected patients with the A/A variant genotype are Caucasians.

Moreover, in this study, the genotype analysis of the SNP at the 5′UTR-25 of *RPH3AL* in normal (benign colonic epithelium) and CRC tissues demonstrated an identical allelic status. One of the advantages of these findings in clinical use of SNP at the 5′UTR-25 in the *RPH3AL* gene would be that the genotype can be determined through a blood test to assess the potential risk of aggressive behavior of CRCs.

In summary, the current study found that the allele or genotype frequencies of SNPs at the 5′ UTR-25 of *RPH3AL* varied significantly by patient ethnicity and specifically, the A/A variant genotype of this SNP was observed exclusively in non-Hispanic Caucasian patients. The overall prevalence of C/C variant genotype was higher at 5′UTR-25 in the *RPH3AL* gene. The homozygous A/A and C/C variant genotypes were significantly associated with down regulation of *RPH3AL* expression in CRCs. Also, these two genotype variants were associated with regional lymph node metastasis, depth of tumor invasion, higher disease recurrence rates and poor patient survival. The down regulation of intratumoral expression of *RPH3AL* gene compared to matching normal tissues suggests that *RPH3AL* gene is a candidate tumor suppressor gene of CRC. Whatever the underlying mechanisms that down regulate the expression of *RPH3AL* in CRC patients who exhibit A/A or C/C variants, it is apparent from these studies that SNPs at 5′UTR-25 within the *RPH3AL* gene might be involved in the progression of CRC. Thus, these findings have significant clinical implications in assessing the aggressiveness of the disease in a sub-set of colorectal adenocarcinoma patients.

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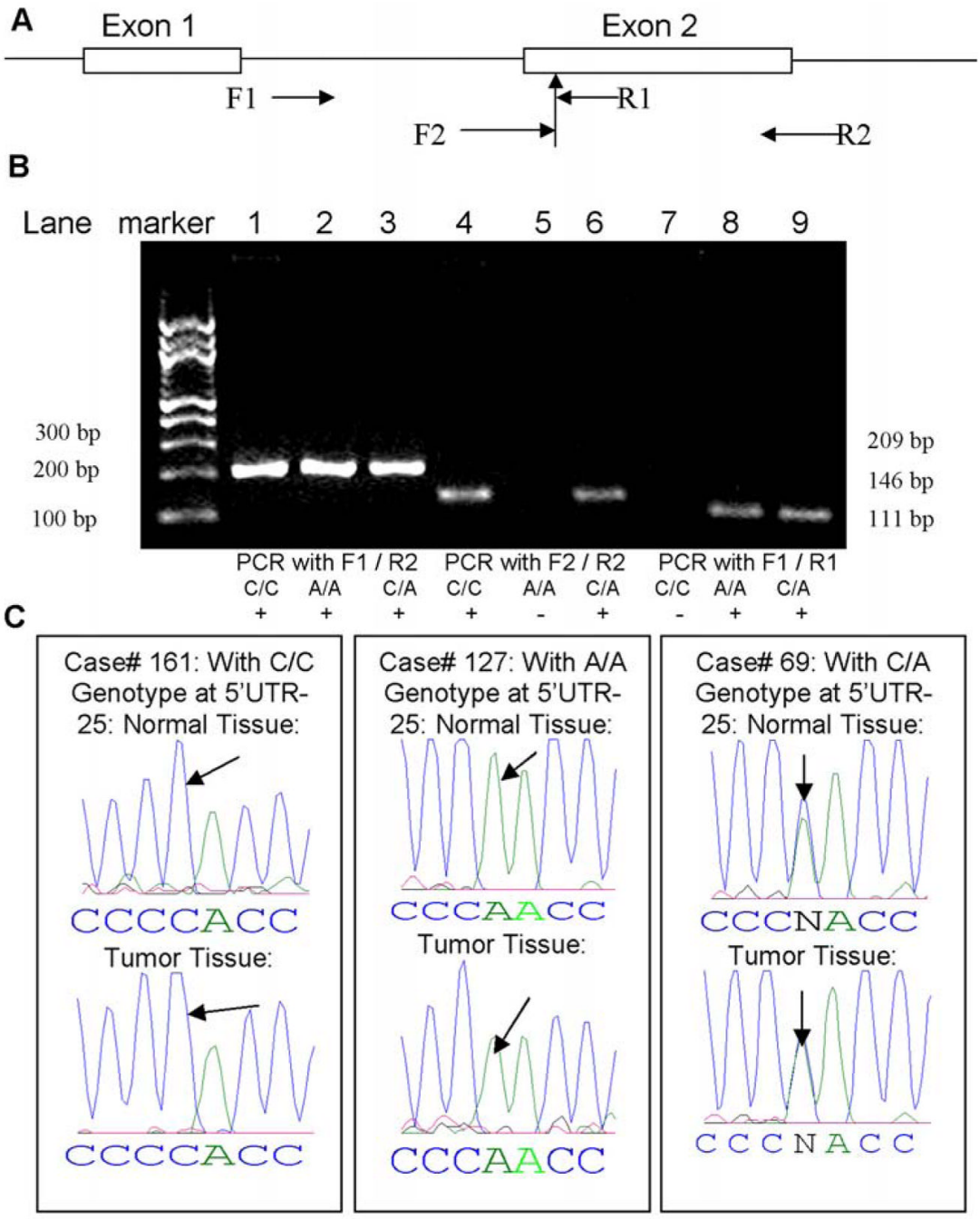

Figure 1.

Genotype analysis of single nucleotide polymorphisms at the 5′UTR of the *RPH3AL* gene using the PCR-CTPP method. (Panel-A), Primers are shown with the horizontal arrows below the scheme representing exon-intron structure. The nucleotide at the 3′ end is the cytosine of the F2 primer and the thymine of the R1 primer. The position of the 5′UTR-25 (C to A) variation is indicated by a vertical arrow. (Panel-B), The agarose gel (2%) electrophoresis panel represents the PCR amplification of the samples with 5′UTR-25 C/C, A/A and C/A genotypes using the primers indicated below. The size of each PCR product (band) is 209 bps for F1/R2 primers set, 146 base pair (bp) for F2/R2 primer set and 111 bps for F1/R1 primer set. Lanes 1, 4 and 7 are DNA from a representative case with the C/C genotype, lanes 2, 5 and 8 are

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DNA from a representative case with the A/A genotype, and lanes 3, 6 and 8 are DNA from a representative case with the C/A genotype. (Panel-C), Sequencing analysis of the PCR product with the F1 and R2 primers. The DNA sequences obtained from a tumor sample and its matching benign epithelium demonstrate a similar nucleotide sequence suggesting that the nucleotide change (from wild-type) is a genetic polymorphism.

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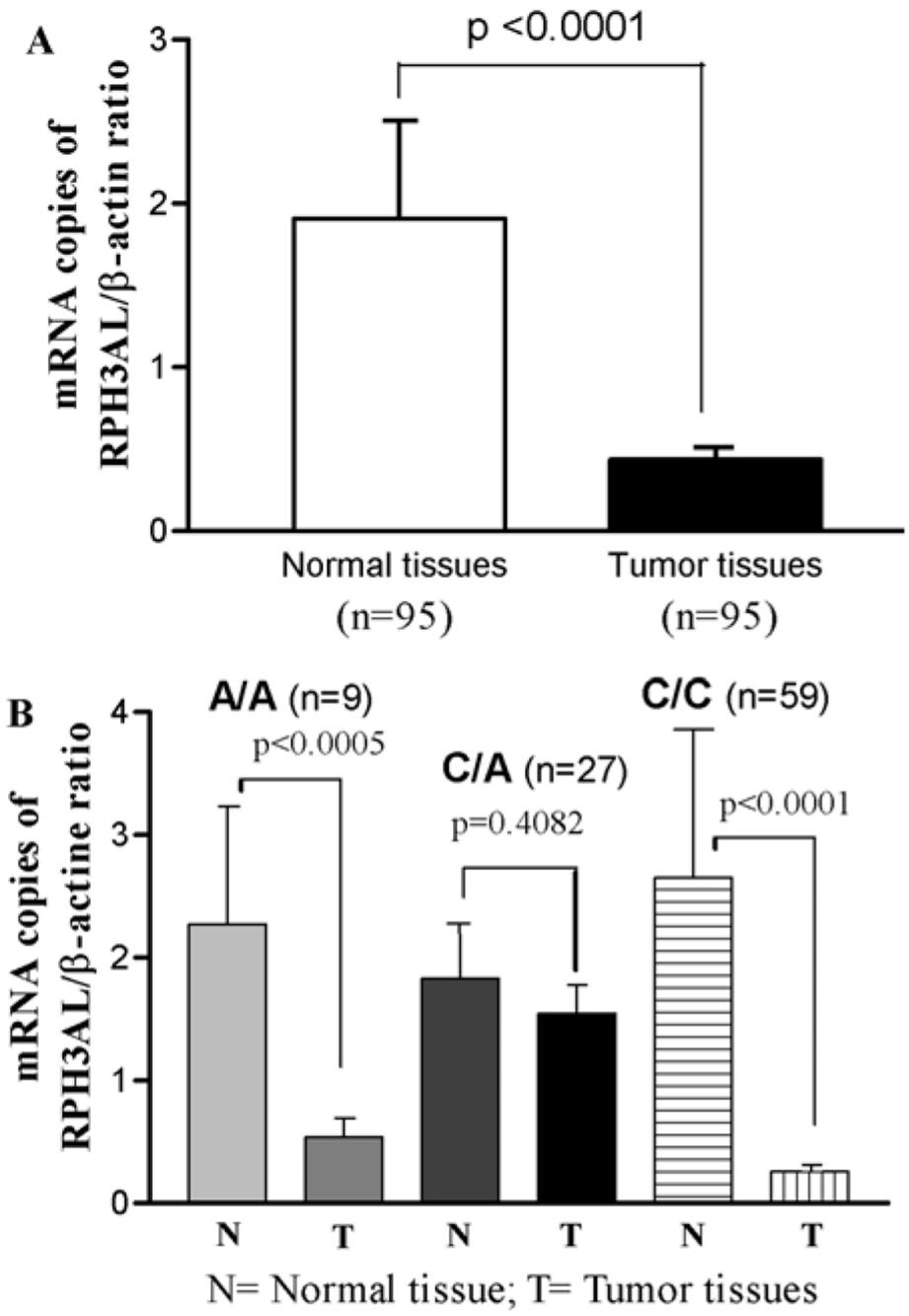

Figure 2.

The mRNA levels in CRCs and matching normal tissues based on different genotypes of the *RPH3AL* gene quantified by real-time PCR. (Panel A), There were significant differences in RNA quantity between CRCs and matching normal tissue samples. The mean number of *RPH3AL* mRNA copies/beta-actin copies for CRC and matching normal tissue samples were 2.36 (0.15–10.32) and 0.42 (0.38–1.78), respectively. (Panel B), mRNA levels quantified in CRCs and matching normal tissues based on the genotype. The mRNA levels were compared between CRCs and matching normal tissues in three genotypes of *RPH3AL*. The mean number of *RPH3AL* mRNA copies/beta-actin copies for C/A, A/A and C/C genotypes in CRCs were 1.659 (0.88 to 1.78), 0.38 (0.06 to 0.59) and 0.246 (0.038 to 0.746), respectively.

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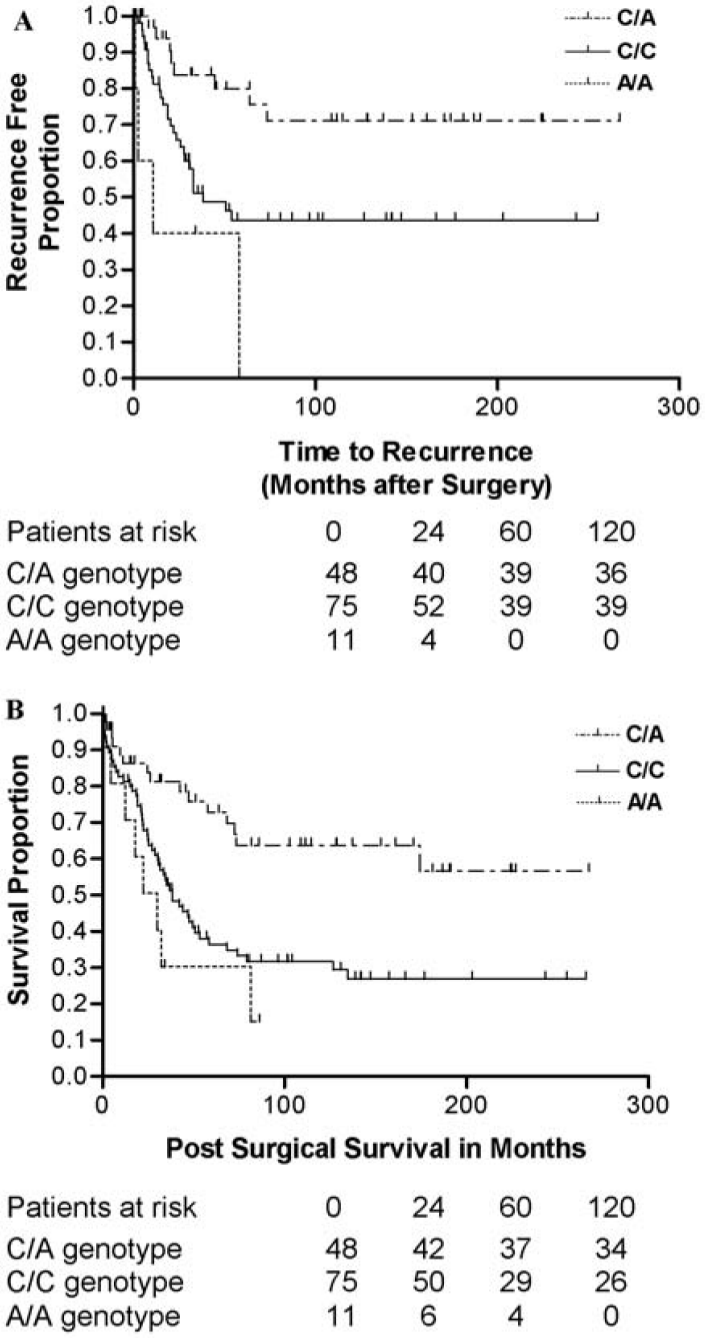

Figure 3.

Kaplan-Meier curves of risk of recurrence and survival based on the genotype status of the *RPH3AL* gene. (Panel-A), The risk of relapse patient with the genotypes A/A and C/C were higher compared to patients with genotype C/A; A/A vs. C/A log-rank P <0.0001; C/A vs. C/C log-rank P = 0.008; However there was no difference in the risk of relapse among patients with genotypes A/A and C/C; A/A vs. C/C log-rank P = 0.10. (Panel-B), Differences in overall survival were observed between genotypes: A/A vs. C/A log-rank P = 0.001; C/C vs. C/A log-rank P < 0.001; However, there was no significant survival difference of genotypes A/A vs. C/C log-rank P = 0.21.

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Table 1

Correlation between the genotypes at 5′UTR-25 of *RPH3AL* and patient characteristics

	Prospective sample (n=95)			Retrospective sample (n=134)			Combined sample (229)			
	Genotype			Genotype			Genotype			
	A/A	C/A	C/C	A/A	C/A	C/C	A/A	C/A	C/C	χ^2
	n=9	n=27	n=59	n=11	n=48	n=75	n=20	n=75	n=134	
Characteristic	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**P-value**
Age Group (years)										
< 65	5 (56)	10 (37)	24 (41)	4 (36)	20 (42)	33 (44)	9 (45)	30 (40)	57 (43)	0.90
≥ 65	4 (44)	17 (63)	35 (59)	7 (64)	28 (58)	42 (56)	11 (55)	45 (60)	77 (57)	
Gender										
Female	3 (33)	9 (33)	31 (53)	3 (27)	19 (40)	45 (60)	6 (30)	28 (37)	76 (57)	0.006
Male	6 (67)	18 (67)	28 (47)	8 (73)	29 (60)	30 (40)	14 (70)	47 (63)	58 (43)	
Ethnicity										
African-American	0 (0)	6 (22)	33 (56)	0 (0)	9 (19)	49 (65)	0 (0)	15 (20)	82 (61)	<0.0001[1]
Caucasian	9 (100)	21 (78)	26 (44)	11 (100)	39 (81)	26 (35)	20 (100)	60 (80)	52 (39)	
Depth of tumor invasion										
T_1	0 (0)	4 (15)	0 (0)	0 (0)	1 (2)	1 (1)	0 (0)	5 (7)	1 (1)	0.01[1]
T_2	0 (0)	6 (22)	2 (3)	2 (18)	13 (27)	14 (19)	2 (10)	19 (25)	16 (12)	
T_3	6 (67)	15 (56)	47 (81)	5 (46)	25 (52)	42 (56)	11 (55)	40 (53)	89 (67)	
T_4	3 (33)	2 (7)	9 (16)	4 (36)	9 (19)	18 (24)	7 (35)	11 (15)	27 (20)	
Nodal Status										
Negative (N_0)	0 (0)	19 (70)	28 (48)	1 (9)	33 (69)	37 (49)	1 (5)	52 (69)	65 (49)	<0.0001
Positive ($N_{1–3}$)	9 (100)	8 (30)	30 (52)	10 (91)	15 (31)	38 (51)	19 (95)	23 (31)	68 (51)	
Distant metastasis										
M_0	8 (89)	23 (85)	48 (83)	6 (55)	43 (90)	62 (83)	14 (70)	66 (88)	110 (83)	0.15
M_1	1 (11)	4 (15)	10 (17)	5 (45)	5 (10)	13 (17)	6 (30)	9 (12)	23 (17)	
Tumor Stage[2]										
I	0 (0)	8 (30)	1 (2)	0 (0)	12 (25)	9 (12)	0 (0)	20 (27)	10 (8)	<0.0001
II	0 (0)	10 (37)	22 (38)	1 (9)	20 (42)	26 (35)	1 (5)	30 (40)	48 (36)	
III	8 (89)	5 (18)	25 (43)	5 (46)	11 (23)	27 (36)	13 (65)	16 (21)	52 (39)	
IV	1 (11)	4 (15)	10 (17)	5 (46)	5 (10)	13 (17)	6 (30)	9 (12)	23 (17)	
Tumor Location[2]										
Proximal Colon	5 (66)	9 (35)	39 (66)	4 (36)	26 (54)	31 (41)	9 (45)	35 (47)	70 (52)	0.71

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	Prospective sample (n=95)			Retrospective sample (n=134)			Combined sample (229)			
	Genotype			Genotype			Genotype			
	A/A	C/A	C/C	A/A	C/A	C/C	A/A	C/A	C/C	χ^2
	n=9	n=27	n=59	n=11	n=48	n=75	n=20	n=75	n=134	
Characteristic	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**P-value**
Distal Colorectum	4 (44)	17 (65)	20 (34)	7 (64)	22 (46)	44 (59)	11 (55)	39 (53)	60 (48)	
Tumor Grade[3]										
Low	6 (75)	19 (86)	36 (67)	8 (73)	30 (63)	44 (59)	14 (74)	49 (69)	80 (62)	0.44
High	2 (25)	3 (14)	18 (33)	3 (27)	18 (37)	31 (41)	5 (26)	22 (31)	49 (38)	
Tumor Size (cms)[3]										
≤ 5	2 (22)	16 (62)	25 (47)	4 (40)	29 (62)	40 (53)	6 (32)	45 (61)	65 (51)	0.06
> 5	7 (78)	10 (38)	28 (53)	6 (60)	18 (38)	35 (47)	13 (68)	29 (39)	63 (49)	

[1] Fisher Exact Test; Data for tumor size and grade were not available in 100% of cases

[2] Data for tumor stage and location for one case was not available

[3] Data for tumor grade and size were not available in 100% of cases.

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Table 2

The SNPs of the genomic region of *RPH3AL* and their correlation with the SNP at 5′UTR-25

SNP Cluster ID	Primer sequence (5′-3′)	Alleles	Position on chromosome 17	No. of tumors with alterations	Correlation with genotype at 5′UTR-25 A/A (n=9) No. of Mut (%)[1]	C/A (n=27) No. of Mut (%)[1]	C/C (n=59) No. of Mut (%)[1]
SNPs in the non-coding region of RPH3AL							
rs4985611	F1: CTTGGAACCCAGTTTGTTGG R1: ACACCATCAGCAACCTTTCC	G/T	203345	1	0(0)	0(0)	1(10)
rs7215343	F1: AGATTTGGGACGCTTCCTCT R1: TTCTCTGTGCGTCAATTTGC	C/T	202893	3	0(0)	1(4)	2(3)
rs7223403	F1: GTCAGCATCTCATCGCTTCC R1: GTGCAGGGGTTCATTTCATT	A/G	203005	2	0(0)	1(4)	1(2)
rs9891032	F1: CCAAGAGGCTCTCAAACTGG R1: TGGGTTGGGCTGTAATAACC	A/G	203362	0	0(0)	0(0)	0(0)
rs9907777	F1: AATTGCCAAGAGGCTCTCAA R1: CGAGGAAAAAGCTGAGTTGC	C/T	203367	6	1(11)	2(7)	1(2)
rs9915104	F1: CAACTACTCGGGAGGCTGAG R1: CAGGCCTTGGTATGTGTGTG	C/G	204137	0	0(0)	0(0)	0(0)
rs11356209	F1: ACACCATCAGCAACCTTTCC R1: CTTGGAACCCAGTTTGTTGG	-/A	203333	0	0(0)	0(0)	0(0)
rs11356210	F1: TCAAACTGGGTTCCAAGGAG R1: CGAGGAAAAAGCTGAGTTGC	-/A	203465	0	0(0)	0(0)	0(0)
rs11383870	F1: TTTGGGTTAGAAAGGGCACA R1: TTCTCTGTGCGTCAATTTGC	-/A	202931	1	0(0)	0(0)	1(0)
rs11650641	F1: TAGTGGGGTTTTGAGGGACA R1: CGGATAAGCAAGGCCATCTA	A/G	61971	0	0(0)	0(0)	0(0)
rs12942009	F1: GTGTGGAGACCCTCTTCAGG R1: CGAGGAAAAAGCTGAGTTGC	C/T	203456	0	0(0)	0(0)	0(0)
rs12942039	F1: CAACAAACTGGGTTCCAAGG R1: TAACCAGCAGCGCATATCAG	C/T	203498	12	1(11)	4(15)	7(12)
rs12949751	F1: CAACAAACTGGGTTCCAAGG R1:TAACCAGCAGCGCATATCAG	A/G	203500	7	1(11)	2(7)	4 (7)
rs28687962	F1:CTGGGAACAAGGGGGTTATT R1:GCTGGGGTTACAGGAGTGAG	G/T	203531	0	0(0)	0(0)	0(0)
SNPs in the coding region of RPH3AL							
Codon 49	F1: GTGCACTTTGGAGACAGCAA R1: GTGGGAGGGGAGGGTAATAA	G/T	171136	0	0(0)	0(0)	0(0)
Codon 62	F1: GTGCACTTTGGAGACAGCAA R1: GTGGGAGGGGAGGGTAATAA	A/C	171097	0	0(0)	0(0)	0(0)
Codon 303	F1: GTGCACTTTGGAGACAGCAA R1: GTGGGAGGGGAGGGTAATAA	C/T	63686	0	0(0)	0(0)	0(0)

[1]No of mut = Number of cases with mutant genotype.

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Table 3

Multivariate Cox proportional hazard analyses for risk of disease recurrence and patient survival

		Recurrence			Survival		
		Hazard Ratio[1]	**95% C. I.[2] (Lower – Upper)**	**p-value**	**Hazard Ratio[1]**	**95% C. I.[2] (Lower – Upper)**	**p-value**
RPH3AL Genotype	C/C vs. C/A	4.64	1.65–13.07	0.004	1.79	0.85–3.78	0.12
	A/A vs. C/A	13.85	3.12–16.43	<0.001	3.88	1.39–10.82	<0.01
	A/A vs. C/C	2.99	0.76–11.75	0.12	2.16	0.81–5.79	0.12
Nodal Metastasis	Pos.[3] vs. Neg.[4]	2.41	1.12–5.18	0.02	2.25	1.30–3.92	<0.01
Tumor Grade	High vs. Low	1.27	0.61–2.68	0.62	1.95	1.14–3.31	0.01
Ethnicity	A-A[5] vs. Caucasians	0.89	0.41–1.95	0.77	1.77	0.97–3.24	0.06
Gender	Men vs. Women	1.98	0.96–4.07	0.06	0.87	0.51–1.48	0.62

[1] Adjusted for the genotype of 5′UTR-25 of the *RPH3AL* gene, age, gender, and ethnicity, and nodal metastasis, depth of tumor grade, tumor location, and tumor size

[2] C.I. = Confidence intervals

[3] Pos. = Positive

[4] Neg. = Negative

[5] A-A = African-Americans.