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Original Article

RhoA, ROCK–1, and ROCK–2 Gene Expression and Polymorphisms in Cholesteatoma Patients

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BACKGROUND: The aim of this study was to evaluate the gene polymorphism and expressions of Rho-A, ROCK-1, and ROCK-2 in cholesteatoma.

METHODS: In this study, 120 healthy control group patients and 120 cholesteatoma patients were enrolled. Venous blood was taken from all of the cholesteatoma and control group patients. The genotyping of ROCK-1(G/T)rs35996865, ROCK-2(A/C)rs10178332, and Rho-A(A/T)rs2177268 polymorphisms was performed using predesigned TaqMan SNP Genotyping Assays. Assays-on-Demand SNP genotyping kit was used for the real-time polymerase chain reaction. The expression levels of Rho-A(Hs00357608_m1), ROCK-1(Hs01127699_m1), and ROCK-2(Hs00178154_m1) genes were determined.

RESULTS: The expression of Rho-A, ROCK-1, and ROCK-2 was lower in cholesteatoma patients than in the control group. There was no difference in Rho-AAT/TT and ROCK-1GT/TT variation in cholesteatoma patients compared to the control. However, ROCK-2 AC/CC variance was lower in cholesteatoma patients.

CONCLUSION: The expression of Rho-A, ROCK-1, and ROCK-2 genes may be decreased in cholesteatoma. Furthermore, since ROCK-2 AC/CC genotype is also lower in cholesteatoma, having C allele seems to decrease the risk of developing this disease.

KEYWORDS: Cholesteatoma, cholesteatoma genetics, cholesteatoma pathogenesis, Rho pathway

INTRODUCTION

Cholesteatoma is a well-demarcated, gradually expanding, destructive, epithelial lesion of the temporal bone. It is produced by the keratinizing squamous epithelium. It is commonly called as skin in the wrong place.[1] Cholesteatoma is histopathologically benign and morphologically characterized by squamous cell proliferation.[2] It consists of 3 layers perimatrix, matrix, and cystic content. Matrix is composed of keratinizing stratified squamous epithelium. Perimatrix contains fibroblasts and collagen fibers. Cystic content is made up of keratinaceous debris. Matrix and perimatrix release several cytokines and enzymes leading a vicious inflammatory cycle which may eventually cause hyperproliferation of keratinocytes and fibroblasts.[3] Inflammatory cell infiltration is commonly seen in the perimatrix, and inflammation exacerbates the development of cholesteatoma. It has been reported that interactions between matrix keratinocytes and perimatrix fibroblasts do not only maintain cell differentiation, proliferation, and tissue homeostasis within cholesteatoma, but it may induce osteoclastogenesis as well.[4,5]

The exact pathogenetic molecular mechanisms behind transformation of the keratinizing squamous epithelium to the cholesteatoma are still unclear. Numerous etiological theories have been proposed; for example, the retraction pocket theory, the proliferation theory and immigration theory, as well as the metaplasia theory.[6-8] It has been reported that an altered control of cellular proliferation exists in cholesteatoma, which affects the balance toward the aggressive and invasive growth of squamous epithelium.[8,9] However, it is yet unclear whether this altered control is due to defects in the mechanisms and underlying genes that control proliferation or to cytokines released from infiltrating inflammatory cells.[10] Cholesteatoma is generally regarded minimally

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Mendelian inheritance.[11] Nevertheless, the reports of familial clustering of disease and of association with genetic syndromes suggest underlying, but as yet unidentified genetic risk factors.[11] It has been reported that several hundred genes are differentially regulated in cholesteatoma samples such as genes involving growth differentiation, signal transduction, cell communication, protein metabolism, and cytoskeleton formation.[10,12] It has been reported that keratinocytes of cholesteatoma are undifferentiated, aggressive, and continuously proliferate. The intercellular communication between keratinocytes and fibroblasts has been an area of focus for keratinocytes differentiation. The exact signaling pathway of this communication, as has been reported in our previous study, that diminished activity and expression of Rho-kinase may be responsible for the hyperproliferation and undifferentiation of keratinocytes which are hallmarks of cholesteatoma.[13] Rho kinase belongs to a family of serine/threonine kinases, which is one of numerous targets and an important downstream effector of Rho proteins.[14] There are 2 known isoforms of Rho kinase, that is, ROCK-1 and ROCK-2, which are encoded by separate genes on human chromosomes 18q11 (ROCK-1) and 2p24 (ROCK-2).[15,16] Rho kinase (ROCK), together with its upstream activator Rho, is a well-known intracellular signaling pathway in the regulation of the actin cytoskeleton and myosin-based contractility. Signaling events downstream of ROCK are responsible for smooth muscle contraction, proliferation, differentiation, migration, apoptosis, changing cell membrane shape, secretory function, extension and retraction of neuron, intercellular divergence, and carcinogenesis.[13,17-20] Chapman et al[21] reported that the presence of a ROCK inhibitor stimulated the proliferation and immortalized human keratinocytes. Existence of a ROCK inhibitor in the medium maintains an indefinite extension of life span, and when the ROCK inhibitor was removed from the medium, cells senescence develops after a few passages.[22]

There are no reports evaluating possible link between the genetic role of Rho/Rho kinase (Rho-A, ROCK-1, and ROCK-2) gene polymorphisms and expressions with cholesteatoma. In our previous study, we demonstrated for the first time that Rho kinase protein could be downregulated in the cholesteatoma, and keratinocyte undifferentiation could be controlled by this pathway.[13] For this reason, in this study, we aimed to investigate any possible genetic association of Rho and ROCK (ROCK-1 and ROCK-2) genes polymorphism, expression, and cholesteatoma. We studied ROCK-1(G/T) rs35996865, ROCK-2(A/C) rs10178332, and Rho-A(A/T) rs2177268 single-nucleotide polymorphisms (SNPs) in the blood samples of cholesteatoma and control group patients due to the relatively high minor allele frequencies of these SNPs in the Caucasian population and due to the location of these SNPs within the promoter region, exonic and intronic sites that could potentially impact on ROCK expression and function.[23-25]

MAIN POINTS

- Cholesteatoma is a non-neoplastic, keratinizing lesion, characterized by the undifferentiation and proliferation of epithelium of the middle ear and mastoid cavity.
- Previously, it was demonstrated that diminished activity and expression of Rho kinase may be responsible for the hyperproliferation and undifferentiation of keratinocytes which are hallmarks of cholesteatoma.
- However, there are no reports evaluating possible link between cholesteatoma and the genes of Rho/Rho kinase signaling.
- In this study, we found that the expression of Rho-A, ROCK-1, and ROCK-2 genes may be decreased in cholesteatoma.
- Furthermore, since ROCK-2 AC/CC genotype is also lower in cholesteatoma, having C allele seems to decrease the risk of developing this disease.

MATERIALS AND METHODS

Patients and Blood Samples

This study was approved by the local ethical committee of our University (Number: 2017/59). The study was conducted in accordance with the principles of the Helsinki Declaration. Written agreement form including the permission for taking 5 mL blood and studying on their blood for the cholesteatoma genetic research was taken from all patients who participated in this study. The patients and controls were from the same geographic region and of the same ethnic origin. From October 2017 to September 2019, 120 consecutive patients who had chronic otitis media with secondary acquired cholesteatoma (study group) and 120 age- and gender-matched healthy individuals without cholesteatoma or without chronic otitis media (control group) were enrolled in this study. All patients in the study group had chronic ear discharge for at least 1 year. There was no cholesteatoma history in the relatives of the patients based on detailed history.

Exclusion Criteria

1. Patients with well-known other genetic diseases,
2. patients with psoriasis or other skin diseases,
3. patients with recurrent or residual cholesteatoma,
4. patients who previously undergone otologic surgery, and
5. patients with congenital cholesteatoma.

After taking a detailed medical history, otorhinolaryngologic, audiologic and radiologic (except control group's patients), and histopathologic examination were performed. Five milliliter venous blood was taken from the patients in study and control groups in a tube containing ethylenediaminetetraacetic (EDTA) and stored at −70°C.

Genotyping DNA Extraction and Analysis

Genetic analyses were conducted at the Advanced Technology Education, Research, and Application Center of our university. DNA isolation of peripheral blood in the tubes containing EDTA was performed by kit method. Genomic DNA was isolated from leukocytes using the Purelink® DNA Mini kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol. The genotyping of the ROCK-1(G/T) rs35996865, ROCK-2(A/C) rs10178332, and Rho-A (A/T) rs2177268 polymorphisms was performed using predesigned TaqMan SNP Genotyping Assays (Thermo Fisher Scientific). The Assays-on-Demand SNP genotyping kit was used for the real-time polymerase chain reaction (PCR, Applied Biosystems). Single-nucleotide polymorphism amplification assays were performed according to the manufacturer's instructions. In brief, 20 μL of reaction solution containing 4 μL of DNA was mixed with 10 μL of 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, Massachusetts, USA) and 0.5 μL of predeveloped assay reagent from the SNP genotyping product containing primers and probes (C_32466393_10 for the ROCK-1 (G/T) (rs35996865), C

3026005_10 for the ROCK-2 (A/C) (rs10178332), and C 16146833_10 for the Rho-A (A/T) (rs2177268) (Applied Biosystems, Thermo Fisher Scientific). Reaction conditions consisted of preincubation at 50 °C for 2 minutes and at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 10 seconds and at 60 °C for 1 minute. Amplifications and analysis were performed in an Applied Biosystems ViiA™ 7 Real-Time PCR System, using the SDS 2.0.6 software for allelic discrimination.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from peripheral blood using the TRIzol™ Reagent (Invitrogen™, Waltham, Massachusetts, USA) according to the manufacturer's protocol with the following modifications. The quality and quantity of extracted RNA were determined by NanoDrop (Nano Drop Technologies, Thermo Scientific, USA). Then, cDNA synthesis was performed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Two microliters of reaction mix included 2.0 µL 10× real time (RT) buffer, 0.8 µL 25× deoxynucleotide triphosphate (dNTP) mix (100 mM), 2.0 µL 10× RT random primers, 1.0 µL MultiScribe™ Reverse Transcriptase, 10.2 µL nuclease-free H_2O, and 4 µL cDNA. The reaction mixture was incubated at 25 °C for 10 minutes, 37 °C for 120 minutes, at 85 °C for 5 minutes, and finally held at 4 °C. The reaction was performed in a thermal cycler from Techne Prime.

Real-Time Polymerase Chain Reaction Gene Expression

Real-time PCR was performed in a Real-time PCR System (Applied Biosystems ViiA™ 7, USA). A PCR mix of 20 µl included 10.0 µL TaqMan Gene Expression Master Mix (2×), 1.0 µL TaqMan Gene Expression Assay (20×), 2.0 cDNA template, 7.0 Nuclease-free H2O. The thermal cycle conditions for the Uracil DNA Glycosylase (UDG) incubation at 50 °C for 2 minutes, AmpliTaq Gold, UP Enzyme Activation at 95 °C for 10 minutes, followed by 40 cycle denaturing at 95 °C for 15 seconds, and after then Anneal/Extend at 60 °C for 1 minute. Reference sample was made by mixing the control group RNAs. Reactions were incubated in a 96-well plate. Actin Beta (ACTB) (Hs99999903_m1) was used as endogenous control in real-time PCR. ROCK-1 (Hs01127699_m1), ROCK-2 (Hs00178154_m1), Rho-A (Hs00357608_m1) genes expression levels were determined with TaqMan Gene Expression Assays (Thermo Fisher Scientific, USA). All reactions were taken in triplicate. Analyses were performed with ViiA™ 7 Software (Applied Biosystems).

Statistical Analysis

Power analysis was made according to the previous studies concerning the relationship between gene polymorphism and expression and cholesteatoma pathogenesis. In total, 120 patients were needed in each (cholesteatoma and control) group according to the power analysis results with 80% power and 5% type 1 error.

Shapiro–Wilk normality test was conducted for all quantitative variables. Variables with normal distribution are summarized as median [25 percentile-75 percentile], and categorical variables are summarized as number and percentage. The independent-sample *t* test was used to compare the 2 groups when the normality assumption was provided, and the Mann–Whitney *U*-test was used when it was not provided. In the comparison of more than 2 groups, analysis of variance was used in case of assumption of normality, otherwise Kruskal–Wallis test was used. Chi-square test was used to evaluate the relationship between 2 categorical variables. Analyses were made with Statistica v.13.1. A P value $< .05$ was accepted as statistically significant.

RESULTS

In the study group, cholesteatomas were seen at attic region in 84 and posterosuperior quadrant of pars tensa in 26 patients. It surrounded the malleus capitulum and body of incus and invaded the mastoid cavity. Ossicular chain and scutum erosion were detected in 79 patients. Labyrinth erosion was seen in 12 patients. None of the patients in the control group had chronic otitis media with or without cholesteatoma.

In this study, Rho A (A/T), ROCK-1 (G/T), and ROCK-2 (A/C) variations and expressions were examined from cholesteatoma patients' blood samples; 71 (59.17%) of 120 patients with cholesteatoma were males and 49 (40.83%) were females. Cholesteatoma is more common in males (males 73, females 47) ($P = .001$). In the control group, there were 71 males and 49 females. The average age of cholesteatoma patients was 41.51, and the control group was 43.83 ($P = .573$). The control group age distribution was chosen in parallel with the age distribution of cholesteatoma patients.

There were no differences in Rho-A (A/T) variation compared to control group with cholesteatoma patients ($P = .113$) (Table 1). Moreover, there were no differences in ROCK-1 (G/T) variation compared to control group in cholesteatoma patients ($P = .648$). However, ROCK-2 (A/C) variation is lower in cholesteatoma patients than in the control group ($P < .001$) (Figure 1). On the other hand, Rho-A, ROCK-1, and ROCK-2 expression levels were lower in patients with cholesteatoma than in the control group ($P < .001$) (Table 2).

DISCUSSION

In this study, we investigated the relationship between cholesteatoma etiopathogenesis and the Rho/ROCK gene expressions as well as polymorphisms. Cholesteatoma often develops in a small number of patients with chronic otitis media (COM). The certain causative risk factors of transformation from COM to cholesteatoma are still poorly understood. But this transformation could be started by environmental factors, heritable factors, or random effects.[26] Additionally, it has been reported that cholesteatoma can develop between 7% and 20% rate in the contralateral ear.[27,28] Reports of familial clustering and bilateral development of cholesteatoma can be associated with genetic mechanisms.[26] In a recent systematic review, it has been proposed that the pattern of cholesteatoma observed in several families is typical of a monogenic or oligogenic disorder with incomplete penetrance.[29] However, any genetic risk factors have not been detected yet. None of our patients with cholesteatoma was relative based on their history. Cholesteatoma has several well-known features. These are uncontrolled hyperproliferation by the undifferentiating keratinocytes, aggressiveness, migration, invasion, and recidivism, the majority of which may be governed by several cellular signaling cascades. One of those cascades could be the Rho/ROCK pathway, which may contribute to aforementioned characteristics of cholesteatoma.[3,13,21]

The ROCK-1 and ROCK-2 genes contain 33 exons.[30] Overexpression of constitutively active ROCK-1 or ROCK-2 increases cell proliferation.[31] In contrast, inhibition of ROCK activity decelerates cytokinesis.[32] However, Chapman et al[21] reported that the presence of a ROCK inhibitor stimulated the proliferation and immortalized human keratinocytes. Existence of a ROCK inhibitor in the medium maintains an indefinite extension of life span, and when the ROCK

Table 1. ROCK-1, ROCK-2, and Rho-A Genotype and Allele Distribution

Gene Variations	Genotype	Group				*P*	95% CI
		Cholesteatoma		Control			
		N	%	N	%		
ROCK-1	GG	78	65.0	71	59.2	.648	-
	GT	35	29.2	41	34.2		-
	TT	7	5.8	8	6.7		-
Rho A	AA	54	45.0	46	38.3	.113	-
	AT	48	40.0	63	52.5		-
	TT	18	15.0	11	9.2		-
ROCK-2	AA	94	78.3	70	58.3	<.001*	-
	AC	20	16.7	48	40		0.310 (0.169-0.569)
	CC	6	5	2	1.7		
	A	208	86.7	188	78.3		-
	C	32	13.3	52	21.7	.017*	0.556 (0.343-0.901)

*statistically significant.

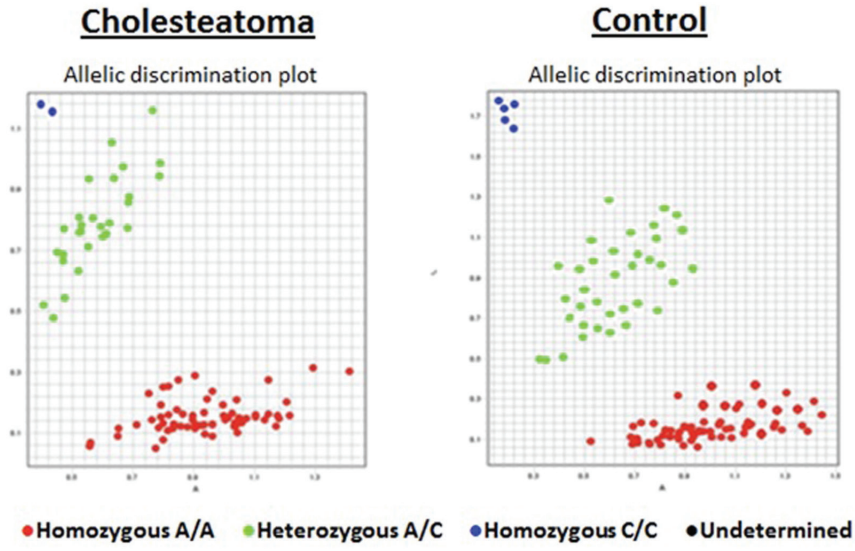

Figure 1. ROCK-2 allelic and genotypical discrimination plot. AA, AC, and CC genotype rates were determined with TaqMan probe in control and cholesteatoma patients. Note that homozygous CC is lower in cholesteatoma than the control.

inhibitor was removed from the medium, cells senescence develops after a few passages.[22]

Although cholesteatoma is generally regarded minimally Mendelian inheritance, reports of familial clustering of disease and of association with genetic syndromes suggest underlying, but as yet unidentified genetic risk factors.[11] However, there are no reports evaluating possible link between the genetic role of Rho/Rho kinase (Rho-A, ROCK-1, and ROCK-2) gene polymorphisms and expressions with cholesteatoma.

Table 2. Comparison of the Mean Values of the Studied Gene Expressions in Cholesteatoma and Control Groups

Genes	Cholesteatoma Group Median [25P-75P]	Control Group Median [25P-75P]	*P*
Rho A	1.22 [0.08-4.73]	15.11 [7.45-24.70]	<.001
ROCK-1	0.85 [0.15-12.15]	90.34 [59.11-168.55]	<.001
ROCK-2	5.13 [0.14-14.38]	45.46 [0.02-95.67]	<.001

In our previous study, we demonstrated for the first time that Rho kinase (ROCK-2) protein could be downregulated in the cholesteatoma as demonstrated by Western blotting.[13] In this study, however, we demonstrated that the expression levels of Rho-A, ROCK-1, and ROCK-2 genes were lower in patients with cholesteatoma than in the control group. There was no difference in Rho-A (A/T) and ROCK-1 (G/T) variations in cholesteatoma patients compared to the control indicating that having these variations of both Rho-A and ROCK-1 was not associated with the expression level of Rho-A and ROCK-1. However, we found that ROCK-2 (A/C) variation was lower in cholesteatoma patients than in the control group. The diminished protein level, which was found in

our previous study, may be due to the downregulated ROCK-2 gene expression as has been demonstrated in this study.[13] ROCK-1 variant is localized in upstream region of gene; however, ROCK-2 and Rho-A variant are localized in intron of the gene. Variation in the upstream and intron regions can affect the amount of synthesis without changing the structure of the corresponding protein. This may explain the expression reduction in ROCK-2 gene. The expression levels of Rho-A and ROCK-1 may be affected from other variations.

The limitation of our study was that we performed our Rho/ROCK genes expression and polymorphism study with a relatively low number of participants for a genetic case–control study. However, we think that our results are meaningful with regard to the genetic basis of the cholesteatoma pathogenesis.

CONCLUSION

The expression of Rho-A, ROCK-1 and ROCK-2 genes may be decreased in cholesteatoma. Furthermore, since ROCK-2 AC/CC genotype is also lower in cholesteatoma, having C allele seems to decrease the risk of developing this disease.

Ethics Committee Approval: This study was performed in line with principles of the Declaration of Helsinki. Approval was granted by the ethics committee of University of Mersin (Number: 2017/59).

Informed Consent: Informed consent was obtained from all individual participants included in the study.

Peer Review: Externally peer-reviewed.

Author Contributions: Concept – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Design – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Supervision – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Resource – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Materials – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Data collection and/or processing – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Analysis and/or Interpretation – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Literature research – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Writing – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.; Critical reviews – K.G., K.B., E.A., O.İ., D.Y.E., A.Ç., C.Ö.

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