Research Article

Emine H. Karagedik, Saim Pamuk, Merve N. Ataş, Murat Ulsan, Levent Aydemir and Arzu Ergen*

Investigation of RASSF4 gene in head and neck cancers

Baş Boyun Kanserlerinde RASSF4 Geninin Araştırılması

<https://doi.org/10.1515/tjb-2021-0026>

Received February 4, 2021; accepted August 11, 2021;
published online September 15, 2021

Abstract

Objectives: RASSF gene family can inhibit the growth of RAS oncogene. This gene family is suggested to have a role in cell cycle control, apoptosis, cell migration, and mitosis control. This study evaluated RASSF4 gene expression levels, SNPs and serum levels in tissues dissected from both healthy individuals and patients diagnosed with head, and neck cancer.

Methods: RASSF4 gene expression levels were determined using the RT-PCR. Serum levels of RASSF4 were tested using the Enzyme-Linked Immuno Sorbent Assay technique in study groups. RASSF4 rs7896801 and rs884879 genotypes were identified using by the RT-PCR.

Results: No statistical difference was observed between study groups according to RASSF4 gene expression levels. According to SNP results, rs7896801 revealed a 2.4 fold increase of G-allele presence in patients ($p=0.015$). The increase in the presence of AA genotype was statistically significant for the control group ($p=0.015$). Distribution of

genotypes and alleles for rs884879 showed a 2.2 fold increase in CC genotype for healthy group ($p=0.031$) however, the presence of T allele showed a significant increase in the patients ($p=0.048$).

Conclusions: We suggest that this study will play a pioneering role for the next studies on RASSF4 gene, especially on SNPs.

Keywords: ELISA; gene expression; head and neck; polymorphism; RASSF4.

Öz

Giriş: RASSF ailesi RAS onkogeninin büyümesini engelleyici özellikler gösterir. Bu gen ailesinin hücre döngüsü kontrolü, apoptoz, hücre göçü ve mitoz kontrolünde görev aldıkları tahmin edilmektedir.

Amaç: Bu çalışmada RASSF4 gen anlatımı, SNP'ler ve serum düzeyinin baş boyun kanseri hastaları ve sağlıklı bireylerden alınan örneklerde incelenmesi amaçlanmıştır.

Materyal Ve Metod: RASSF4 gen anlatımı düzeyleri RT-PCR kullanılarak tayin edilmiştir. Çalışma gruplarında RASSF4 serum düzeyleri ELISA tekniği ile ölçülmüştür. RASSF4 rs7896801 ve rs884879 genotip tayinleri ise RT-PCR kullanılarak gerçekleştirilmiştir.

Bulgular: Çalışma grupları arasında RASSF4 gen anlatımları açısından istatistiksel bir farklılık gözlemlenmemiştir. SNP sonuçlarına göre ise rs7896801 G alleli varlığının 2.4 kat hastalarda arttığı gösterilmiştir ($p=0.015$). Kontrol grubunda ise AA genotip taşıma sıklığı istatistiksel olarak artmıştır ($p=0.015$). rs884879 için genotip ve allel dağılımına bakıldığında sağlıklı grupta CC genotipi taşımak 2.2 kat artarken ($p=0.031$), hastalarda ise T alleli görülme sıklığı anlamlı olarak yükselmiştir ($p=0.048$).

Tartışma: Çalışmamız özellikle RASSF4 SNP'leri açısından gelecek çalışmalar için öncü rol oynayacak niteliktedir.

*Corresponding author: Prof. Dr. Arzu Ergen, Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey, E-mail: aergen@istanbul.edu.tr.
<https://orcid.org/0000-0001-5736-8453>

Emine H. Karagedik and Merve N. Ataş, Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey. <https://orcid.org/0000-0002-0905-1977> (E.H. Karagedik). <https://orcid.org/0000-0001-5045-5095> (M.N. Ataş)
Saim Pamuk, Murat Ulsan and Levent Aydemir, Department of Otorhinolaryngology, Faculty of Medicine, Istanbul University, Istanbul, Turkey. <https://orcid.org/0000-0002-4376-5893> (S. Pamuk). <https://orcid.org/0000-0002-5836-4304> (L. Aydemir)

Anahtar Kelimeler: ELISA; gen anlatımı; baş boyun; polimorfizm; RASSF4.

Introduction

Head and neck cancers are of complex nature that require extensive diagnosis, and treatment. These cancers involve the oral cavity, larynx, pharynx, salivary glands, and nasal cavity [1]. Alcohol and tobacco use are the two most important factors of head and neck cancers. HPV is also one of risk factors for head and neck cancer [2, 3].

RAS has been identified to have the ability to activate both growth-enhancing and growth-inhibiting pathways [4–6]. These adverse activities suggest that activation of potent oncogenes such as RAS can control biological processes in a potential tumor cell. The discovery of the RASSF family allows for the disclosure of some of the growth inhibitory effects of RAS [7].

RASSF proteins act as tumor suppressors, and are inactivated by hypermethylation of the promoter region in tumor subgroups. RASSF proteins are suggested to be involved in cell cycle control, apoptosis, cell migration, and mitosis control [8–10]. RASSF 7–10 lacks this motif [11]. The presence of the SARAH zone separates RASSF 1–6 from RASSF 7–10. The RA range is close to the C-Terminal area in RASSF 1–6, close to the N-Terminal area in RASSF 7–10. Therefore, RASS1-6 C-RASSF “classic RASSF” is called RASSF7-10 N-RASSF proteins [12].

RASSF4 is expressed in various human tissues. The C-terminal has the RASSF4 SARAH area in the RASSF group [13]. The expression of RASSF4 inhibits the growth of human tumor cells. This effect is enhanced by the addition of a RAS CAAX membrane localization signal to the –COOH terminus of RASSF4. RASSF4 were shown to play a role in apoptosis, and cell cycle arrest, and often reduced in many cancers [7, 14].

The aim of this study was to investigate the effect of RASSF4 gene on head and neck cancers at expression, SNPs and serum levels.

Materials and methods

The samples used in the study consist of tumor tissues, peripheral blood tissues, and tumor tissues of 68 patients who were diagnosed with head and neck cancer in the Department of Otorhinolaryngology, Istanbul Faculty of Medicine, Istanbul University. The system prescribed by the “American Joint Committee on Cancer (AJCC)” and “The International Union for Cancer Control (UICC)” was used for tumor staging.

The samples in control group, was obtained from 70 healthy individuals with no diagnosis of head and neck cancer. Informed consent forms were obtained from all participants according to the

Helsinki Declaration. Ethics committee approval was obtained from the medical ethics committee of Istanbul Faculty of Medicine (1138/2017).

The clinical evaluations were performed, and the tissues, and blood samples were collected in the Department of Otorhinolaryngology, Istanbul Faculty of Medicine, Istanbul University. The blood, and tissue samples were preserved under proper conditions until the study was performed.

Total RNA, and DNA were isolated from tissue samples taken for expression, and polymorphism. Serum was obtained from the blood samples, and RASSF4 levels were determined.

DNA isolation

INVITROGEN PureLink® Genomic DNA Kit was used for DNA isolation from tissues. The contents of the kit were prepared in accordance with the instructions, and the isolation was performed.

RNA isolation

Easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Korea) was used for RNA isolation from tissues. The contents of the kit were prepared in accordance with the instructions, and the isolation was performed.

Reverse transcription

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA) was used to obtain cDNA from isolated total RNAs from head and neck tissues

RNA contents were added to the solution mixture in different concentrations to obtain a 100 ng/μL mixture.

The TaqMan Gene Expression Master Mix Kit (Applied Biosystems, USA) with probes was used to determine RASSF4 assay primers and their properties while TaqMan Gene Expression Assay, GAPDH was used for the control.

Evaluation of RASSF4 serum levels

Serum samples, which were previously separated and stored at –80 °C for RASSF4 serum determination were determined using the BT Labs Elisa kit (Cat No:E4368Hu) (Standard Curve Range: 15–3000 ng/L; Sensitivity: 7.52 ng/L; Precision: CV(%)=SD/mean × 100, Precision: Intra-Assay: CV<8%, Inter-Assay: CV<10%) procedure in Multiskan Spectrum (Thermo Electron Corporation, USA).

SNPs genotyping

The Taqman SNPs genotyping assay kit (Applied Biosystems, USA) was used to detect the rs884879 and rs7896801 in Real Time PCR Instrument (Applied Biosystems 7500 Fast Instrument, Applied Biosystems StepOnePlus™). Probes for RS884879; FAM-labeled probe was used to detect the C allele. The VIC-labeled probe was used to determine the T allele. The probes for RS7896801 were used to detect the FAM-labeled probe A allele. The VIC-labeled probe was used to determine the G allele.

Statistical analysis

The statistical analysis was performed using the SPSS 21.0 Package program. The limit of significance was taken as $p < 0.05$. χ^2 and Fisher's Exact tests were used to evaluate the differences between the genotype, and allele incidence among the groups. Student's t-test, One-way Anova, and Mann Whitney U-tests were used to compare the demographic data between the groups.

Results

The study group consisted of 68 patients diagnosed with head and neck cancer, and 70 healthy individuals. There was no statistical difference regarding the age between the study groups ($p > 0.05$).

No significant difference was observed in terms of RASSF4 gene expression in tumor and healthy tissue ($p > 0.05$) (Table 1).

The RASSF4 rs7896801 and rs884879 genotype and allele distributions were examined between the patients and controls, the frequency of rs7896801 G allele carrying was 2.4 fold higher in the patients ($p = 0.015$, $\chi^2 = 5.95$, OR = 2.4, 95% CI = 1.18–5). The frequency of carrying AA genotype increased significantly in the control group ($p = 0.015$, $\chi^2 = 5.95$, OR = 1.8, 95% CI = 1.10–3.18) The examination of the

Table 3: RASSF4 Δ CT levels and serum RASSF4 levels according to tumor staging.

Tumor stage	RASSF4 Δ CT levels	p-Value	Serum RASSF4 levels, ng/L	p-Value
1–2	4.89 $\pm$ 3.09	0.89	186.47 $\pm$ 99.98	0.07
3–4	4.74 $\pm$ 3.52		449.90 $\pm$ 303.32	

RASSF4 rs884879 genotype and allele distributions between study groups showed that the frequency of carrying CC genotype increased 2.2 times in the healthy group compared to the patient group ($p = 0.031$, $\chi^2 = 4.64$, OR = 2.2, 95% CI = 1.03–5.23). Significant increase was detected in the frequency of carrying T allele in the patient group ($p = 0.048$, $\chi^2 = 3.92$, OR = 2.5, 95% CI = 0.98–6.74) (Table 2).

No significant result was detected in accordance with the RASSF4 rs7896801 and rs884879 genotype distributions in the examination of RASSF4 serum levels ($p > 0.05$). Also, there was no significant difference was detected in accordance with the tumor staging between early, and advanced stage tumors in examination of the RASSF4 gene expressions and serum RASSF4 levels ($p > 0.05$) (Table 3).

Discussion

Various aspects of head and neck cancer have been extensively studied, however, the role of mutations and mRNA expression levels were included in a small number of studies. Oncogenes such as PIK3CA, KIT, RAS were most susceptible to mutations; BRAF, PDGFRA, ABL1 and EGFR mutations were found to occur less frequently in the nasopharyngeal carcinoma study of Zhang et al. The comparison of the frequency of mutations in subgroups of patients with or without recurrence or metastasis showed that the frequency of recurrent and developing mutations were increased in nasopharyngeal cancer patients [15].

RAS/RAF/ERK pathway is known to have an important role in tumor development. KRAS, HRAS and NRAS mutations occur at least in one third of all human cancers. KRAS mutations are the most common mutations [16–18]. However, Zhang et al. found KRAS mutations less than expected in nasopharyngeal cancer compared to NRAS, and HRAS mutations [15].

Studies with RASSF4 are usually epigenetic inactivation studies caused by methylation or deletions. Decreasing RASSF4 mRNA expression and the hypermethylated promoter region of RASSF4 was found in head and neck squamous cell carcinoma, and nasopharyngeal carcinoma [19, 20]. We did not find any significant differences in RASSF4 expression

Table 1: RASSF4 Δ CT values in patients and controls.

RASSF4	Δ CT	95% CI	p-Value
Tumor tissue, n=68	4.78 $\pm$ 3.59	0.18–2.19	0.098
Healthy tissue, n=68	3.78 $\pm$ 3.45		

Table 2: Distribution of RASSF4 rs7896801 and rs884879 genotype and alleles in study groups.

rs7896801	Patients, n=68	Control, n=70	p-Values
Genotype			
AA	15 (22.1%)	29 (41.4%)	$p = 0.015$
AG	35 (51.5%)	31 (44.3%)	$p = 0.398$
GG	18 (26.5%)	10 (14.3%)	$p = 0.075$
Allel			
A	65 (47.8%)	89 (63.5%)	$p = 0.075$
G	71 (52.2%)	51 (36.5%)	$p = 0.015$
rs884879	Patients, n=68	Control, n=70	
Genotype			
CC	7 (10.3%)	16 (22.9%)	$p = 0.031$
CT	47 (69.1%)	38 (54.3%)	$p = 0.076$
TT	14 (20.6%)	16 (22.9%)	$p = 0.913$
Allel			
C	61 (44.85%)	70 (50%)	$p = 0.903$
T	75 (55.15%)	70 (50%)	$p = 0.048$

levels in head and neck carcinoma. Zhang et al. showed that RASSF4 overexpression inhibits proliferation, invasion, EMT, and Wnt signaling pathway in osteosarcoma cells [21].

Although RAS protein was detected in a nasopharyngeal cancer study, Porter et al. found no structural change in the RAS gene [22]. Herman et al. reported that promoter methylation plays an important role in function loss of some tumor suppressor genes [23]. Loss of RASSF1 expression in human cancer has been associated with abnormal promoter methylation in at least 37 tumor types. RASSF1A has the potential to be an ideal cancer biomarker because the methylation of RASSF1A occurs significantly in various tumor types compared to normal tissues [24–26]. RASSF2 promoter methylation is seen to correlate with lymph node metastasis in lung cancer cell lines, gastric cancer and nasopharyngeal carcinoma [27–29]. In a recent study, it was demonstrated that loss of RASSF4 promotes Multiple Myeloma progression by disconnecting RAS from its proapoptotic pathways [30]. Eckfeld et al. found no association in examining the coding sequence of RASSF4 in terms of inactivating mutations [7]. In our study, We observed that rs7896801 G and rs884879 T alleles play as a risk factor for head and neck cancer. Besides, we did not show any association among tumor staging and RASSF4 expression and serum RASSF4 levels.

This is the first study which examined the RASSF4 gene expression, serum levels, and SNP variants in Turkish patients with head and neck cancer. In our study, no significant significance was observed in terms of gene expression, but SNPs that may be risk factors for head and neck cancer were suggested.

We suggest that these preliminary data will lead to further studies for RASSF4 protein, especially in SNP studies.

Research funding: This study was funded by Scientific Research Projects Coordination Unit of Istanbul University. Project Number: 29262.

Author contribution: E.H.K, laboratory studies; S.P., sample collection; M.N.A., laboratory studies; M.U., clinical evaluation; L.A., sample collection; A.E., statistical evaluation, supervisor.

Competing interests: The authors declare that there is no conflict of interests regarding the publication of this article.

Çıkar Çatışması: Yazarlar, bu makalenin yayınlanması ile ilgili herhangi bir çıkar çatışması olmadığını beyan eder.

Ethical approval: This study was conducted with the approval of the Ethical Committee of the Istanbul Faculty of Medicine, Istanbul University (1138/2017).

References

1. Markman M. Head and neck cancer questions & answers | what to ask your doctor | CTCA. <https://www.cancercenter.com/cancer-types/head-and-neck-cancer/questions> [Accessed 30 Sep 2020].
2. Steenbergen RD, Snijders PJ, Heideman DA, Meijer CJ. Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. *Nat Rev Canc* 2014;14:395–405.
3. Bruni L, Albero G, Mena M, Gomez D, Munoz J, Bosch FX, et al, ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human papillomavirus and related diseases in the world. In: Summary Report. Barcelona, Spain: HPV Information Centre; 2019.
4. Downward J. Ras signalling and apoptosis. *Curr Opin Genet Dev* 1998;8:49–54.
5. Cox AD, Der CJ. The dark side of Ras: regulation of apoptosis. *Oncogene* 2003;22:8999–9006.
6. Hueber AO, Evan GI. Traps to catch unwary oncogenes. *Trends Genet* 1998;14:364–7.
7. Eckfeld K, Hesson L, Vos MD, Bieche I, Latif F, Clark GJ. RASSF4/AD037 is a potential Ras effector/tumor suppressor of the RASSF family. *Canc Res* 2004;64:8688–93.
8. Hesson LB, Cooper WN, Latif F. Evaluation of the 3p21.3 tumour-suppressor gene cluster. *Oncogene* 2007;26:7283–301.
9. Lerman MI, Minna JD. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Canc Res* 2000;60:6116–33.
10. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 2000;25:315–9.
11. Sherwood V, Recino A, Jeffries A, Ward A, Chalmers AD. The N-terminal RASSF family: a new group of Ras-association-domaincontaining proteins, with emerging links to cancer formation. *Biochem J* 2010;425:303–11.
12. Richter AM, Pfeifer GP, Dammann RH. The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochim Biophys Acta* 2009;1796:114–28.
13. Scheel H, Hofmann K. A novel interaction motif, SARAH, connects three classes of tumor suppressor. *Curr Biol* 2003;13:899–900.
14. Han Y, Dong Q, Hao J, Fu L, Han X, Zheng X, et al. RASSF4 is downregulated in nonsmall cell lung cancer and inhibits cancer cell proliferation and invasion. *Tumour Biol* 2016;37:4865–71.
15. Zhang ZC, Fu S, Wang F, Wang HY, Zeng YX, Shao JY. Oncogene mutational profile in nasopharyngeal carcinoma. *Onco Targets Ther* 2014;7:457–67.
16. Kratz CP, Schubert S, Bollag G, Niemeyer CM, Shannon KM, Zenker M. Germline mutations in components of the Ras signaling pathway in Noonan syndrome and related disorders. *Cell Cycle* 2006;5:1607–11.
17. Ma BB, Lui VW, Poon FF, Wong SCC, To KF, Wong E, et al. Preclinical activity of gefitinib in non-keratinizing nasopharyngeal carcinoma cell lines and biomarkers of response. *Invest N Drugs* 2010;28:326–33.
18. Hui ABY, Lo KW, Teo PML, To KF, Huang DP. Genome wide detection of oncogene amplifications in nasopharyngeal

- carcinoma by array based comparative genomic hybridization. *Int J Oncol* 2002;20:467–73.
19. Chow LSN, Lo KW, Kwong J, Wong AYH, Huang DP. Aberrant methylation of RASSF4/AD037 in nasopharyngeal carcinoma. *Oncol Rep* 2004;12:781–7.
 20. Steinmann K, Sandner A, Schagdarsurengin U, Dammann RH. Frequent promoter hypermethylation of tumor-related genes in head and neck squamous cell carcinoma. *Oncol Rep* 2009;22:1519–26.
 21. Zhang M, Wang D, Zhu T, Yin R. RASSF4 overexpression inhibits the proliferation, invasion, EMT, and Wnt signaling pathway in osteosarcoma cells. *Oncol Res* 2017;25:83–91.
 22. Porter MJ, Field JK, Leung SF, Lo D, Lee JC, Spandidos DA, et al. The detection of the c-myc and ras oncogenes in nasopharyngeal carcinoma by immunohistochemistry. *Acta Otolaryngol* 1994;114:105–9.
 23. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042–54.
 24. Yegnasubramanian S, Kowalski J, Gonzalgo ML, Zahurak M, Piantadosi S, Walsh PC, et al. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Canc Res* 2004;64:1975–86.
 25. Xing M, Cohen Y, Mambo E, Tallini G, Udelsman R, Ladenson PW, et al. Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. *Canc Res* 2004;64:1664–8.
 26. Lehmann U, Länger F, Feist H, Glöckner S, Hasemeier B, Kreipe H. Quantitative assessment of promoter hypermethylation during breast cancer development. *Am J Pathol* 2002;160:605–12.
 27. Kaira K, Sunaga N, Tomizawa Y, Yanagitani N, Ishizuka T, Saito R, et al. Epigenetic inactivation of the RAS-effector gene RASSF2 in lung cancers. *Int J Oncol* 2007;31:169–73.
 28. Endoh M, Tamura G, Honda T, Homma N, Terashima M, Nishizuka S, et al. RASSF2, a potential tumour suppressor, is silenced by CpG island hypermethylation in gastric cancer. *Br J Canc* 2005;93:1395–9.
 29. Vos MD, Ellis CA, Elam C, Ülkü AS, Taylor BJ, Clark GJ, et al. RASSF2 is a novel K-Ras-specific effector and potential tumor suppressor. *J Biol Chem* 2003;278:28045–51.
 30. De Smedt E, Maes K, Verhulst S, Lui H, Kassambara A, Maes A, et al. Loss of RASSF4 expression in multiple myeloma promotes RAS-driven malignant progression. *Canc Res* 2018;78:1155–68.