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ANALYSIS OF RAS-ONCOGENE MUTATIONS IN LARYNGEAL SQUAMOUS CELL CARCINOMA CYTOLOGICAL SAMPLES OF NORTHERN ROMANIAN DESCENT

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Abstract: *The applicability of the Ras-gene family isoforms in cancer research has been documented for several malignancies but remains limited in head and neck cancers. This is the first study to attempt an examination of Ras-gene mutations in laryngeal squamous cell carcinoma (LSCC) subjects of Romanian descent. Analysis using Real-time PCR techniques identified Ras-mutations in only 11 of the 56 cytological samples, while the prevalent form of mutation was the K-Ras isoform (73%). Both K-ras and N-ras isoforms were more frequent in males (P=0.015064) and tended to favour advanced clinical stages (T3/T4) while having a strong preference for better histological outcomes (P=0.000057 K-ras/ P= 0.010362 N-ras). In conclusion, the results of the study suggest that mutations within the Ras-gene protooncogene family appear to favour male subjects and more advanced clinical stages of LSCC, but with the possibility of a better long-term prognostic in terms of supplementary histological risk criteria.*

INTRODUCTION

The predominant form of malignant tumors of the larynx originates from the squamous epithelium (95%), though a smaller percentage may occur from other tissue within the layers of the larynx.(1,2) The involvement of oncogenes has been proven in a wide range of human malignancies, including cancer of the larynx. The Ras-family genes are considered important oncogenes implicated in the pathogenesis of various cancer forms as they were found to be one of the more frequently mutated oncogenes in human malignancies (3,4) to the point of being considered a "hallmark gene" in carcinogenesis.(5,6) The three known isoforms consist of Harvey-Ras (H-Ras), Kirstan-Ras (K-Ras), and neuroblastoma-Ras (N-Ras). The genes were first identified and characterized using the Harvei and Kirsten stains of acutely transforming retroviruses and subsequently isolated from human cells with the help of transfection-based assays.(5,7)

Under normal circumstances, Ras-genes activate upon stimulation and induce transduction, after which they become inactive.(8) When considering mutations of the Ras-genes, the process occurs due to single-point mutations within their coding sequence, which causes GAP-mediated GTP hydrolysis and locks the mutated Ras isoform in an active Ras-GTP state, forcing it to continue stimulating cellular growth or differentiation. This transformation from proto-oncogenes to active oncogenes happens when a glycine residue is substituted by different acid residues and usually involves the 12th codon on chromosome 12, while codons 13 and 61 are more rarely afflicted.(4,9,10)

The incidence rate of mutation within the Ras-genes has been reported to range greatly in human cancers (up to 30%), with various results regarding the isoform most susceptible to neoplastic influence.(3) Articles assessing the prevalence of Ras-mutations, including works performed by The Catalog of Somatic Mutations in Cancer (COSMIC), have identified the K-ras as the most frequently mutated isoform of the Ras-gene family (86%) in human cancers (11,12), while N-Ras (11%) and H-Ras (3%) show a far lower incidence rate (6,9,13). Concurrently, division by cancer site highlights adenocarcinomas (K-ras mutations within the pancreas 90%, colon 50%, and lung 50%), hematological malignancies of the myelomonocytic lineage (N-ras mutation being most common), and bladder tumors/HNC (high H-ras frequency) as opposed to tumors of the neuroectodermal origin or lymphoid malignancies.(5,14)

For head and neck cancers (HNC), however, the exact frequency rate of Ras- mutations remains uncertain as assessments performed by pathological sites consider that both the presence and the role Ras-genes pose in laryngeal squamous cell carcinoma (LSCC) to be exceptional with mostly inconsistent results.(5,9,15)

AIM

This study aimed to investigate the implications of Ras-gene mutations within cytological specimens of LSCC and correlate them with the clinical and pathological characteristics.

MATERIALS AND METHODS

Blood samples and tumour specimens

We conducted a genetic study on 56 archival formalin-fixed paraffin-embedded (FFPE) tissue samples of LSCC from the Department of Pathology, Cluj County Emergency Clinical Hospital, Romania. Data regarding gender, age, and TNM (Classification of Malignant Tumours) were recorded for each sample from medical records. Tumour staging and classification followed the WHO Classification of Head and Neck Tumours and the NCCN (National Comprehensive Cancer Network) guidelines for Head and Neck cancers.(1,16)

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DNA extraction and PCR analysis

Genetic analysis of the Ras-mutations was performed using a specific Mutation Analysis Kits is intended for detecting mutations on exons 2,3,4 (codons 12, 13, 59, 61, 117 and 146) of the three isoforms of the Ras-gene family. Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples using a commercial DNA extraction kit (Bioline ISOLATE II Genomic DNA kit) according to the manufacturer's protocol. The Ras mutation analysis real-time assay is based on allele-specific PCR and specific primers that are 100% complementary to mutant variants of the gene. Secondary detection of amplification products requires the use of fluorescent hydrolysis probes; each probe containing contains a fluorophore (FAM or VIC) at the 5-prime terminus and a quencher at the 3-prime terminus. For a mutation to be detected at the stated allelic frequency a minimum of 10 ng of DNA must be present in the PCR reaction. After DNA isolation, the concentration requires measuring using spectrophotometric analysis (UV spectrophotometer) and the following thermocycling conditions: cycle X1 10 min at 95°C, cycle X40 15 sec la 95°C then 60 sec at 60°C.

The final results were put through real-time PCR instrument software (Applied Biosystems 7500, SDS version 2.0 and above) analysis for interpretation.

Statistical analysis

The obtained data on Ras-gene expression according to predetermined categories such as gender, age groups (according to median value), tumour classification criteria, and histological grade were assessed in univariate analysis. We then deferred to statistical examination (Chi-square test, Chi-Square for Goodness of Fit, and Fisher's exact test) to assess the possible difference with the above-mentioned variables. Analysis was carried out using Epiinfo 7 and the Excel Data Analysis package with P values <0.05 considered significant.

RESULTS

Ras-gene mutations could only be identified in 11 (20%) of the 56 cytological LSCC samples. The samples belonged to 10 male subjects and 1 female subject with ages ranging between 45-76 years with a mean age of 58.45 ±8.86 years. The K-Ras isoform appeared in 8 cases (73%), the N-Ras isoform in 3 (27%), and no mutations were found for the H-Ras isoform (table no. 1).

Table no. 1. Distribution of identified Ras-mutations within cytological sample of LSCC

Number of specimens	Ras isoform	Point-mutations in codons
3	K-ras mutation	G12D
1	K-ras mutation	G12V
2	K-ras mutation	G13D
1	K-ras mutation	G12S
1	K-ras mutation	G13C
1	N-ras mutation	G12D
2	N-ras mutation	Q61R
0	H-ras mutation	None

[a]: *Abbreviation: K-Ras (Kirstan-Ras); N-Ras (Neuroblastoma-Ras); Codon 12 (G12D, G12V, G12S); Codon 13 (G13D, G13C), Codon 61 (Q61R)*

Point-mutations were mostly present in codons 12 and 13 of K-Ras (6 vs 2) and codons 61 and 12 (2 vs 1) of N-ras. K-Ras mutations in codon 13 only expressed amino acid residue G13D, while in codon 12 the most common occurrence was G12D (49%) followed by 1 case of G12V, G12S, and G12C each. In N-Ras mutation we found the order of amino acid residue to be 2 cases Q61R (67%) and one case G12D (33%).

As shown in table no. 2, distribution according to tumour classification criteria for the 11 samples positive for Ras-mutation highlights the advanced clinical stages T3-T4 with 8 cases (73%) as opposed to T1-T2 with 3 cases (27%) with nodal metastasis N0 in 7 cases (64%) and N1-N2 in 4 (36%). However, reveres distribution according to supplementary histological risk criteria (SHC) was noted for both isoforms (table no. 2, figure no. 1). Histological grade consisted of 5 (46%) moderately differentiated carcinoma (G2), 3 (27%) well-differentiated (G1), 2 (18%) poorly differentiated (G3), and 1 case of mixed grade (G1/G2).

Table no. 2. Isoform distribution according to predefined study groups

Criteria	Category	K-Ras	N-Ras	P-value K-Ras	P value N-ras
Age groups	<58 years	4	0		
	>58 years	4	3	0	0.1069
Gender	Male	8	2		
	Female	0	1	**0.01** [d]	**0.0151** [d]
T	T4	4	0		
	T3	2	2		
	T2	1	0		
	T1	1	1	0.57241	0.95365
N	N0	5	2		
	N1	1	1		
	N2	2	0	0.7788	0.8465
HP	G1	2	1		
	G2	3	2		
	G3	2	0	0.25684	0.08327
	mixed G1/G2	1	0		
SHC	L0	5	2		
	V0	5	2		
	R0	4	2		
	R1	1	0		
	PN1	1	0		
	ENE-	0	1	0.1336	0.5637

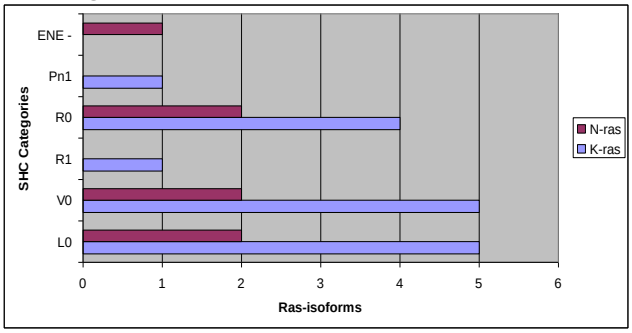
[a]: *Abbreviation: T (primary tumour); N (regional node metastasis); HG (histological grade such as G: well-differentiated squamous cell carcinoma, G2: moderately differentiated squamous cell carcinomaand, G3: poorly differentiated squamous cell carcinoma); SHC (supplementary histological risk criteria)*

[b]: *SHC parameters: negative lymphatic invasion (L0), negative venous invasion (V0), negative resection margin (R0), positive resection margin (R1), perineural invasion (PN1), extranodal extension (ENE-)*

[c]: *SHC major vs minor categories: (R1 and ENE-) vs (V1, L1, Pn1, N1-2)*

[d]: Bold values as statistically significant after application of FDR correction, p<0.05.

Figure no. 1. Distribution of K-Ras an N-Ras isoforms according to SHC criteria



Statistical analysis

Statistical analysis was performed after dividing the samples of LSCC according to the following predetermined groups (table no. 2: tumour classification (T1-T2 vs. T3-T4), histological grade (well/moderately differentiated vs. poorly differentiated), nodal metastasis (N0 vs N1-N2), gender (male vs. female), age groups in years (<58 vs >58). Chi-Square for

Goodness of Fit test was initially applied to establish which of the identified isoforms had the highest incidence rate as well the most commonly affected codon within the cytological samples. The results were not statistically significant in terms of both isoform (K-ras vs N-ras, P=0.13167) and codon domination (K-ras codon type, P=0.73576/ N-ras codon type, P=0.5637).

Analysis of the positive sample for Ras-mutation according to gender found a statically significant predominance in males as opposed to females (P=0.000911). At the same time, secondary distribution by isoform confirmed this predominance in males for both K-ras (P= 0.009992) and N-ras (P= 0.015064) according to the Chi-Square for Goodness of Fit test. Similar distribution according to age mean did not reveal a statically significant association.

Analysis performed on the Ras-mutations according to the tumour classification criteria using Fisher's test (Table II) did not reveal any statistically significant result for either K-Ras (T1-T2 vs. T3-T4, P= 0.572407; N0 vs. N1-N2, P=0.778801; G1-G2 vs. G3, P= 0.256842) or N-Ras (T1-T2 vs. T3-T4, P= 0.953649; N0 vs. N1-N2, P=0.846496; G1-G2 vs. G3, 0.083265). Examination according to SHC found positive associations between the two isoforms and the lower risk factors (L0, V0, and R0): P= 0.000057 for K-ras/ P= 0.010362 for N-ras. However, when grouping the mentioned data according to the major and minor SCH risk categories, no statistically significant association could be found for either isoform. As the H-Ras expression was not found in any of our samples, no such analysis was carried out for the respective isoform.

DISCUSSIONS

Mutation within the three isoforms of the Ras-gene proto-oncogene family remains, to this day, an important step in the activation mechanism of human cancers. The purpose of this study was to identify the presence of mutations within the Ras-gene family to understand whether these known oncogenes can influence laryngeal cancer development. This is the first study to investigate the Ras-family gene isoforms within cytological specimens of LSCC of Romanian descent and correlate them with clinical and pathological characteristics. Given the wide availability of neoplastic cytological specimens, on par with simple blood samples, the genetic analysis of such specimens proves to be an important tool for early detection and evolution.

HNC cancer incidence rates in the Western world (5%) were found to be far lower than in Eastern societies (5%), depending on the anatomical site.(14) In a review, Kodaz et al. (13) reported rates of 4.8-11.5% and 3.3% for K-Ras mutations in laryngeal and oropharyngeal cancer, respectively, while nasopharyngeal cancer reports incidence rates of 4% for H-Ras mutations and < 1% for H-Ras. Further studies assessing the incidence rate of individual point-mutations within each isoform have identified distinct conformation states, of which positions 12, 13, and 61 appear to be the most affected in human cancers.(3,6) Lu et al. (10) and Vatansever et al. (17) found that the highest incidence rate takes place in codon 12 of the K-Ras isoform (G12 89% > G13 9% > Q61 1%) and 61 of N-Ras (Q61 (60%) > G12 (25%) > G13 (14%)]. At the same time, mutations in G12 of K-ras show a prevalence of transforming into aspartate (G12D 36%), followed by valine (G12V, 23%) and cysteine (G12C, 14%). Our results corroborate not only the low incidence rate of ras oncogene mutations reported for Western populations (20% of the tested laryngeal cytological specimens showed Ras mutations) but also the same predominance for codons 12 of K-Ras and 61 of N-Ras along with the G12D mutation within the former isoform.

Although the subjects carrying the K-Ras isoform also showed prevalence towards moderately differentiated squamous cell specimens and advanced clinical stages (T3/T4) without lymph node metastasis, the results could not be statistically confirmed. In particular, we observed a reverse preference of the K-ras mutations towards supplementary histological criteria such as L0 (negative lymphatic invasion), V0 (negative venous invasion), and R0 (negative resection margin), respectively. These statistically significant results tend to indicate better prognostic rates for clinically advanced cases. In other articles about LSCC, Papanikolaou et al. (18) also correlated high expression of the K-ras isoform with an advanced histological grade of dedifferentiation in LSCC, while Rizos et al. (19) found a fairly low mutation rate for the K-Ras isoform in LSCC specimens and could not conclude any relevant statistical analysis. Ruíz-Godoy et al. (20) could not replicate those same results but did report high Ras-protein expression in the more advanced clinical stages, well-differentiated histological grade, and tumor recurrence. For N-Ras, the number of identified specimens in this study was too low to conclude any specific association with tumor classification criteria.

Our results also identified an association between the isoforms in terms of gender, where male subjects with LSCC appear to have a higher chance of presenting Ras-mutations compared to female subjects. However, given the small number of samples that showed positive ras-mutation within this study, we cannot conclusively affirm that these traits are a common occurrence in LSCC.

The observed lack of H-Ras isoform expression in the studied cytological specimens partially resonates with the work done by Clark et al.(21) However, other studies have found positive expression of the H-ras isoform in HNC. For example, Saranath et al. (22) noted a rise in the incident rate of H-ras mutations in HNCs, oral carcinoma especially, in patients who chewed tobacco as opposed to those who did not. Literature reports a particularity of head and neck squamous cell carcinoma when speaking of ras-mutations and carcinogen consumption (primarily tobacco), thus underlying the potential for induced environmental-linked carcinogenic properties for this gene family.(14,23)

Given the fairly low and inconclusive expression rates amongst the Ras-gene family in both HNC and LSCC to date, including the variable results of this study, further research should be considered to prove the presence of these oncogenes has a significant influence on the progression and prognostic of LSCC.

CONCLUSIONS

The results of our study show that the obtained low incidence rates for the two Ras-gene isoforms corroborates literature data on the subject and although we could not conclusively affirm positive involvement within laryngeal cancer development, the results are not negligible.

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Ethics Committee Approval:

This study was approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania (approval number: 162, 02.04.2018/ 296, 01.09.2021).

Conflicts of Interest:

The authors declare that there are no conflicts of interest.

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