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K-ras Mutation, HPV Infection and Smoking or Alcohol Abuse Positively Correlate with Esophageal Squamous Carcinoma

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Abstract The Ras/Raf/MEK/ERK (MAPK) signal transduction cascade is an important mediator of a number of cellular fates including growth, survival and apoptosis. The aim of this study was to determine the incidence of B-raf, Kirsten-ras (K-ras) and Neuroblastoma-ras (N-ras) gene mutations in esophageal squamous cell carcinoma (ESCC) in the Greek population. DNA was extracted from 30 ESCC and 32 normal esophageal specimens and screened for V600E B-raf, and K-ras/N-ras codon 12 mutations, by PCR-RFLP based analysis. Among the genes tested, only the heterozygous K-ras mutation was detected in 5 out of the 30 ESCC specimens (16%), whereas no mutation was found in the normal esophageal tissue ($P<0.022$). The normal samples were screened negative for N-ras and V600E B-raf mutations. The increased risk of esophageal cancer was correlated with tobacco use (OR=3.5, $P<0.023$) and alcohol abuse (OR=7.22, $P<0.001$), accompanied with the high incidence of the k-ras codon 12 mutation (22%, OR=1.77 and 21%, OR=1.52), respectively. A similar positive association was seen in human papilloma virus (HPV)-infected patients (OR=5.66, $P<0.003$). Our overall findings demonstrate that the mutational activation of the K-ras gene, HPV infection and tobacco or alcohol abuse, can be considered independently or in combination as high risk factors for ESCC development.

Keywords Esophageal squamous cell carcinoma · Human Papilloma Virus · B-raf · K-ras · N-ras

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Introduction

Esophageal cancer is the ninth most common malignancy and represents the sixth most frequent cause of death from cancer worldwide [13]. Esophageal squamous cell carcinoma (ESCC) is the predominant histological type, followed by adenocarcinoma, melanoma, leiomyosarcoma, carcinoid, and lymphoma. The majority of ESCC and adenocarcinomas are found in the middle and distal esophagus, respectively [9]. The histopathological changes involved in ESCC include esophagitis, atrophy, mild to severe dysplasia, carcinoma in situ, and invasive cancer [17].

ESCC is characterized by a striking variation in geographical distribution and a high mortality rate. The high-risk countries include Eastern Turkey, the Former Soviet Union, Iraq, Iran, Western and Northern China, Hong Kong, and Japan, named as the Asian esophageal cancer belt. In addition South Africa, Brazil and France have been reported as high-risk areas [13]. In most countries the incidence of ESCC among the population is 1.5–2.5 and 2.5–5/100.000 for women and men, respectively, whereas high-risk countries such as northern China or South Africa have an incidence up to 246/100.000 [23]. The mean age of diagnosis (age of the onset of symptoms) is the seventh decade, and when the cancer is diagnosed, the majority of neoplasms are unresectable or with metastases [9].

Risk factors for esophageal cancer include cigarette smoking, alcohol abuse, a diet deficient in vitamins and antioxidants (vegetables, fruits), chemicals (nitrosamines, mycotoxins) and thermal injuries (hot drinks) [24, 25]. Other risk factors include a history of head and neck cancer, a history of radiotherapy to mediastinum, achalasia, esophageal diverticuli, non-epidermolytic palmoplantar keratosis (tylosis) and Plummer–Vinson syndrome (iron deficiency anemia, dysphagia) [8, 9]. Studies on the viral etiopathogenesis of ESCC have also suggested a role of HPV, findings that we confirmed previously [16, 23].

The Ras/Raf/MEK/ERK signal transduction cascade is an important mediator of a number of cellular fates including cellular responses to growth signals, proliferation and survival. Activation of the above protein kinase cascade finally results in the activation of ERK and MAPK which stimulate gene expression, cytoskeletal rearrangements, and metabolism coordinating responses to extracellular signals like cell proliferation, differentiation, senescence and apoptosis [10, 18]. This pathway is hyperactivated in 30% of cancers with activating mutations in Ras occurring in approximately 15%–30% of cancers [11].

The members of the ras family genes are the Harvey-ras (H-ras), K-ras and N-ras genes, localized on the human chromosomes 11, 12 and 1, respectively. These alterations are either point mutations occurring in codon 12, 13, or 61 for each gene, resulting in the continuous stimulation of cell proliferation, or alternatively a 5- to 50-fold amplification of the wild-type gene [22]. The incidence of ras mutations varies greatly among different human tumors. K-ras mutations have been detected in pancreatic, colon, small intestinal, and stomach cancer [12, 28]. In cancers of the urinary tract and bladder, mutations are primarily found in the H-ras gene, while N-ras mutations are dominant in brain tumors and in leukemia. Overall, mutations most frequently occur in K-ras and rarely in H-ras.

In mammals, the raf family consists of three genes, and these have been named A-raf, B-raf and raf-1 (also known as c- raf or c-raf-1). Raf members encode highly conserved serine/threonine cytoplasmic kinases, playing an important role in proliferation, differentiation and programmed cell death [18]. More than 90% of the reported B-raf mutations are T to A transversions at nucleotide 1799 (T1799A), leading to the substitution of glutamic acid for valine at amino acid 600 (V600E) [11, 29]. Recent data have shown that B-raf is mutated in about 7% of cancers, identifying it as another important oncogene on this pathway [11]. The highest incidence of B-raf mutations is in malignant melanoma (27%–70%), papillary thyroid cancer [15] (36%–53%), colorectal cancer [18] (5%–22%), and ovarian cancer (30%), but they also occur at a low frequency (1%–3%) in a wide variety of other cancers, including stomach cancer [14], squamous cell carcinomas of the head and neck, and lung cancer [7].

The aim of the present study was to determine the incidence and consequently the possible implication of B-raf, K-ras, and N-ras gene mutations in ESCC development. The screening was performed in a homogeneous group of ESCC patients derived from the Mediterranean area, and specifically from the island of Crete, Greece. The genetic alterations found were correlated with clinicopathological features of the patients including gender, HPV infection and habits such as tobacco or alcohol use. Our findings give new insight on potential gene–environment interactions conferring to ESCC.

Materials and Methods

Subjects

We studied 30 histologically confirmed solid ESCC specimens obtained from unrelated patients who were hospitalized

Table 1 Incidence of K-ras, N-ras, and B-raf mutation in association with clinicopathological parameters within the ESCC patient group

	Samples screened (*N*)	K-ras mutation no. (%)	N-ras or B-raf mutation no. (%)
Differentiation			
Well	5	–	–
moderate	15	3 (60%)	0 (0%)
Poor	10	2 (40%)	0 (0%)
Stage disease[a]			
I	–	–	–
II	2	–	–
III	19	3 (60%)	0 (0%)
IV	9	2 (40%)	0 (0%)
Sex			
Male	26	5 (100%)	0 (0%)
Female	4	0 (0%)	0 (0%)
Age group			
40–60	7	1 (20%)	0 (0%)
61–75	14	2 (40%)	0 (0%)
76–100	9	2 (40%)	0 (0%)
Tobacco use			
Smokers	22	4 (80%)	0 (0%)
Non-smokers	8	1 (20%)	0 (0%)
Alcohol use[b]			
Users	23	4 (80%)	0 (0%)
No users	7	1 (20%)	0 (0%)
HPV infection			
Infected	17	3 (60%)	0 (0%)
Non-infected	13	2 (40%)	0 (0%)

[a] The 2002 American Joint Committee on Cancer tumor-node-metastasis (TNM) classification system

[b] More than one glass of beer per day

Table 2 Primer sequences used in PCR assays

	Name	Sequence (5′-3′)	Primer (μM)	Cycling conditions	PCR product size (bp)	RFLP products (bp)
β2 micro globulin	β2-mF	TCCAACATCAACATCTTGGT	0.4	56°C, 36 cycles	123	–
	β2-mR	TCCCCCAAATTCTAAGCAGA	0.4			
B-raf	B-raf R	GGCCAAAAATTTAATCAGTGGA	1	55°C, 39 cycles	224	Mut:211/13
	B-raf F	TCATAATGCTTGCTCTGATAGGA	1			WT:124/87/13
K-ras	K-ras R	TCAAAGAATGGTCCTGGACC	1	58°C, 39 cycles	157	Mut:142/15
	K-ras F	ACTGAATATAAACTTGTGGTAGTTGGACCT	1			WT:113/29/15
N-ras	N-ras A	ATATTCATCTTACAAAGTGGTCCTGGA	0.5	60°C, 39 cycles	83	Mut:60/23
	N-ras S	AACTGGTGGTGGTTGGACCA	0.5			WT:41/23/19

in the General University Hospital of Crete (PAGNH) during the period 1989–2002. Thirty-two esophageal specimens with negative ESCC biopsies served as the control group. We obtained informed consent from all the individuals who participated in the study as well as approval from the hospital's ethics committee. All patients were Greek residents. Ethnic bias within the population studied was minimized by excluding subjects from outside Greece.

All ESCC lesions were located in the upper and middle portion of the esophagus. The differentiation status of the ESCC specimens and the stage of malignancy were determined according to the histopathological evaluations as well, moderate or poorly differentiated [4] and TNM classification criteria [The 2002 American Joint Committee on Cancer Tumor-Node-Metastasis (TNM) classification system], [9] respectively (Table 1).

The patient group consisted of 26 men and 4 women, between 46 and 100 years of age (average age 67 years). Normal specimens were selected to be age and sex matched according to the ESCC cases indicated above. Other clinical parameters including tobacco use, alcohol use, and HPV infection, were also correlated with mutation incidence and ESCC development (Table 1) [16].

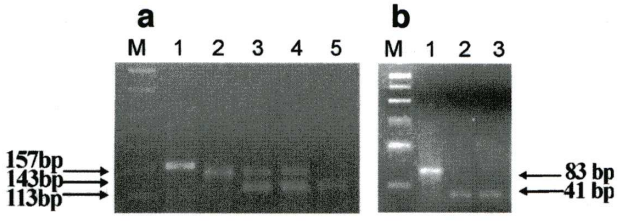

Fig. 1 Determination of K-ras (**a**) and N-ras (**b**) codon 12 mutations in ESCC specimens using PCR detection and RFLP assays. **a** Lane *M*: 50 bp ladder. Lane *1*: undigested PCR product. Lane *2*: Cell line SW480 positive control of K-ras codon 12 homozygous mutation. Lanes *3*, *4*: Heterozygous mutant sample. Lane *5*: Wild-type sample. **b** Lane *M*: 100 bp ladder. Lane *1*: PCR product. Lanes *2*, *3*: Wild-type sample

DNA Extraction

Genomic DNA was extracted from paraffin-embedded esophageal SCC and normal sections after deparaffinization and hydration, according to standard protocols as previously described [26]. DNA purity was assessed by a UV/VIS spectrophotometer estimating the A_{260}/A_{280} ratio.

PCR Amplification and RFLP Analysis

All samples were first examined for the presence of amplifiable DNA using primers for the β2-microglobulin gene. The primers were designed from the complete coding sequences of the appropriate genes using the Primer Express 1.0 software. The sequences chosen from the list generated by Primer Express were purchased from Invitrogen Ltd. UK. All PCR reactions were performed in a total volume of 20 μl containing 5 μM 10X PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl], 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTPs), 0.6 U recombinant Taq DNA polymerase (Invitrogen Ltd., UK) and 100 ng genomic DNA. The primer sequences and concentrations used in the PCR reactions, as well as the PCR conditions and the expected PCR fragment size are summarized in Table 2. Detection of HPV viral DNA was

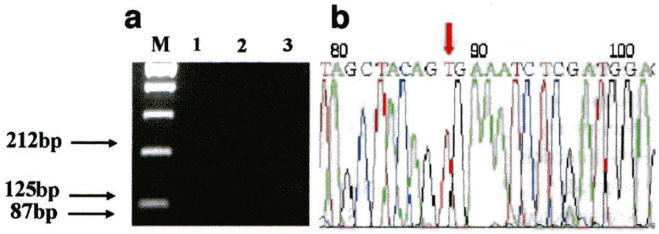

Fig. 2 **a** Screening of B-raf V600E polymorphism in ESCC specimens using PCR and RFLP assays. Lane *M*: 100 bp ladder. Lane *1*: Undigested PCR product. Lanes *2*, *3*: Wild-type sample. **b** Sequencing analysis of BRAF exon 15 reveals only wild-type sequence at codon 600 (GTG). The *arrow* shows the wild-type 1799T nucleotide residue at codon 600 from a representative sample

Table 3 Risk estimates of cases and controls

	Cases[a] (n=30)	Control[b] (n=32)	M–H (χ^2)[c]	*df*	*P* value	OR[d] (95% CI)
Sex						
Male	26	25	0.76	1	0.51	1.82 (0.47–6.98)
Female	4	7				
Tobacco use						
Smokers	22	14	5.47	1	0.023	3.53 (1.21–10.29)
Non-smokers	8	18				
Alcohol use						
Users	23	10	12.62	1	0.001	7.22 (2.33–22.35)
Non-users	7	22				
HPV infection						
Infected	17	6	9.38	1	0.003	5.66 (1.80–17.79)
Non-infected	13	26				

[a] Cases include ESCC samples
[b] Controls include normal samples
[c] M–H χ^2 :Mantel–Haenszel chi-square
[d] OR: odds ratio

performed by polymerase chain reaction (PCR) using the general HPV primers (HPVGP) GP5+/GP6+, while HPV subtypes (6, 11, 16, 18 and 33) were determined as we have previously described [16]. Ten microliters of each reaction mixture were electrophorized on a 2% agarose gel and analyzed for confirmation of the presence of the correct DNA amplimere.

The intermittent restriction digestion of 5 μl of the PCR products was carried out with 4 U TspRI (New England Bio Labs, Beverly, MA) for 8 h at 65°C for the B-raf mutation, and with 4 U MvA1 (Eco RII, Fermentas, Life Science) for 8 h at 37°C for the K-ras and N-ras mutations, followed by analysis on electrophoresis in a 2% agarose with 1% low melting point gel containing ethidium bromide. TspRI digestion of the PCR fragment yielded three major bands at 124 base pairs (bp), 87 bp, and 13 bp in the wild-type allele. In the mutated sequence, one TspR1 site is lost, and digestion yields fragments of 211 and 13 bp. Also, MvA1 restriction digestion analysis for the K-ras mutation, revealed one band at 142 bp in the mutated samples, and one band at 113 bp in the wild-type samples. The cell line SW480 that has been found to harbour a homozygous mutation in codon 12, was used as the positive control for the detection of K-ras mutations. Similarly, we used MvA1 restriction digestion analysis for the N-ras mutation, with expected bands at 60, 23 bp for the mutant type, and 41, 23, 19 bp in the wild-type allele (Table 2).

Sequencing

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). A representative sample was sequenced for BRAF exon 15, using the BigDye™ Terminator Kit, and the Abi Prism 3100 Analyzer (Applied Biosystems).

Table 4 Risk estimates of cases and controls in the population of smokers

	Cases[a] (n=22)	Control[b] (n=14)	M–H (χ^2)[c]	*df*	*P* value	OR[d] (95% CI)
Alcohol use						
Users	19	9	2.34	1	0.21	3.51 (0.68–18.07)
Non-users	3	5				
HPV infection						
Infected	14	3	5.94	1	0.019	6.41(1.37–30.05)
Non-infected	8	11				
K-ras mutation						
Mutant	4	0	2.78	1	0.14	1.77(1.31–2.41)
Non-mutant	18	14				

[a] Cases include ESCC smokers population samples
[b] Controls include normal smokers population samples
[c] M–H χ^2 :Mantel–Haenszel chi-square
[d] OR odds ratio

Statistical Analysis

Chi-squared statistics (Pearson and Mantel–Haenszel's chi-square test) were performed for the statistical analysis of the results. Besides Pearson's test we performed Mantel–Haenszel's chi-square test because it is considered more reliable when small numbers of cases are statistically analyzed; The association between sex, age of the patients, tobacco or alcohol use, HPV infection, K-ras gene mutation, and risk of esophageal cancer, were analyzed by calculating the odds ratio (OR), and 95% confidence intervals (95% CI) using the chi-square test. For statistical purposes, cases were categorized according to the patient's age in three groups corresponding to: (1) 40–60, (2) 61–75 and (3) 76–100 years.

P values less than 0.05 were considered to be statistically significant. All analyses were performed using the Statistical Package for Social Sciences SPSSv14 (SPSS Inc., Chicago, IL, USA).

Results

B-raf, K-ras and N-ras Mutation Frequencies in ESCC and Controls

This study analyzed the frequency of B-raf, K-ras, and N-ras gene mutations, in a group of 62 individuals including 30 ESCC samples, and 32 normal controls. Table 1 summarizes the mutation incidence for all genes studied within the normal and ESCC groups, as well as the various clinicopathological parameters which were taken into consideration in this study. The heterozygous mutation of K-ras codon 12 had a prevalence in 5 out of the 30 ESCC samples (16%), whereas no mutation was detected in the normal esophageal tissues (Fig. 1, $P<0.022$). The frequency of the K-ras mutation was more pronounced among the male patients, tobacco users, and alcohol abusers. Furthermore, no N-ras codon 12 or B-raf V600E gene mutations were detected (Fig. 2).

Effect of Gene–Environment Interplay in ESCC Development

The combined effects of sex, smoking, alcohol abuse, and HPV infection, on the estimates of the risk of esophageal cancer are shown in Table 3. Increased risk and statistical significance was seen in the group of tobacco users (OR= 3.5, $P<0.023$) and alcohol abusers, respectively (OR=7.22, $P<0.001$). A similar positive association was observed in HPV-infected patients (OR=5.66, $P<0.003$). However, no correlation was established between gender and ESCC, giving a borderline risk of OR=1.82.

Furthermore, the groups of tobacco and alcohol users were then categorized into cases, and the normal population was ranked as the controls in order to assess the cumulative risk of all these conditions together. When the individual risk of ESCC smoking patients was studied against the normal case smoking population (Table 4), it was shown that only the presence of HPV infection was statistical significant ($P<0.019$).

In the case of the ESCC alcohol abusers group (Table 5), our findings show that HPV infection significantly associates with an increased risk of ESCC, in contrast to the normal population (OR=5.2).

Discussion

Squamous cell cancer of the esophagus is a serious health problem in Asian belt countries, as a consequence of long-term tobacco and alcohol use. The major reason for high mortality rates is late diagnosis with lesions that are deeply invasive and often metastatic to regional lymph nodes.

Table 5 Risk estimates of cases and controls in the population of alcohol users

	Cases[a] (n=23)	Control[b] (n=10)	M–H (χ^2)[c]	*df*	*P* value	OR[d] (95% CI)
Tobacco use						
Smoker	19	9	0.28	1	0.9	0.52 (0.05–5.42)
Non smokers	4	1				
HPV infection						
Infected	13	2	3.63	1	0.07	5.2 (0.89–30.07)
Non infected	10	8				
K-ras mutation						
Mutant	4	0	1.91	1	0.28	1.52 (1.17–1.98)
Non-mutant	19	10				

[a] Cases include ESCC alcohol users population samples
[b] Controls include normal alcohol users population samples
[c] M–H χ^2 :Mantel–Haenszel chi-square
[d] OR odds ratio

Tumorigenesis arises owing to the accumulation of mutations in critical genes, which alter the normal program of cell proliferation, differentiation and apoptosis. In the present study we examined the B-raf, K-ras, and N-ras genes as candidate genes for esophageal cancer in a population based control study derived from Southern Greece.

Ras genes are involved in a wide range of human tumours. K-ras mutations have been reported frequently in literature for different esophageal neoplasms. Sommerer et al. [21] identified the K-ras mutation in 4 of 19 (21%) Barrett's esophageal neoplasms, and also Lord et al. [15] detected it in 7 of 23 (30%) esophageal adenocarcinomas. Arber et al. [1] performed a study to identify the K-ras mutation in human gastrointestinal tumors, with no detected mutation found in 27 ESCC specimens. In two other studies evaluating head and neck squamous cell cancer specimens (HNSCC), the K-ras mutation was detected in 5 of 89 samples (6%), and in 2 of 26 (7.6%) samples [20, 27]. Our study revealed a presence of the K-ras codon 12 mutation in 5 out of 30 (16%) ESCC samples, whereas no mutation was detected in normal esophageal specimens ($P<0.022$). This is the first report on the K-ras mutation in esophageal squamous cancer, suggesting a potential role of K-ras mutations in esophageal squamous cell carcinomas.

N-ras gene mutations are dominant in brain tumors, leukemia, myelodysplastic syndrome, liver, skin, and thyroid tumors. In accordance with previously published reports, we found no mutations in codon 12 of the N-ras gene in primary ESCC cancers and normal esophageal tissues [1, 15].

The B-raf gene has been recently reported to be mutated in a variety of neoplasms. Sommerer et al. [21] identified the B-raf mutation in 2 of 19 Barrett's esophageal neoplasms (11%), and also Lee et al. [14] detected B-raf mutations in seven stomach carcinomas (2.2%). In two other studies performed to elucidate a possible function of B-raf in squamous cell carcinomas of the head and neck (HNSCC), Weber et al. [27] identified activating B-raf mutations in 3 of 89 HNSCC (3%), and Cohen et al. [6] identified a B-raf mutation in six (4.8%) of 128 head and neck cancers (6 of 77 head and neck squamous call carcinomas). However, in our study we did not identify any B-raf mutation in our study population. This is the first study screening for the presence of the T1799A B-raf mutation in ESCC samples, providing evidence for the lack of association between the B-raf mutation and ESCC development.

This study also investigated potential gene–environment interactions. Cigarette smoking and alcohol consumption are associated with the production of free radical intermediates that induce base damage and single stranded breaks. Tobacco smoke contains an array of potent carcinogens including polycyclic aromatic hydrocarbons, aromatic amines and tobacco specific nitrosamines, which form DNA adducts [19]. The risk of esophageal cancer is strongly related to tobacco and alcohol consumption, with relative risks (RR) over 100 in heavy smokers and heavy drinkers [2, 3, 5]. Our study was able to show evidence for gene–environment interactions in ESCC development, with a threefold increased risk for smokers, sevenfold for alcohol abusers, and fivefold for HPV-infected individuals, respectively.

Summarizing, the advantage of the present study is that, despite the small number of patients, the population was more homogeneous, well-balanced, and dispersed over a small geographical area, and the results are likely to provide accurate estimates of the frequency of the studied mutations. Our findings demonstrate a positive association between HPV-infection, smoking or alcohol abuse in patients carrying the K-ras mutation with an increased risk for esophageal cancer. However, our results are preliminary and larger cohorts are needed for better understanding of the contribution of genetic alterations such as the K-ras mutation, smoking, alcohol, and HPV infection in esophageal cancer. Moreover, the necessity of early detection, prevention with identification of high risk groups via improved screening interventions is essential for better ESCC management.

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