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Polymorphism Arg290Arg in Esophageal-Cancer-Related Gene 1 (ECRG1) is a Prognostic Factor for Survival in Esophageal Cancer

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Abstract
Background Esophageal cancer is one of the most frequent cancers worldwide and is associated with poor outcome. Besides clinicopathological data, few prognostic molecular markers exist. Esophageal-cancer-related gene1 (ECRG1) short tandem repeats are associated with higher risk for developing esophageal squamous cell carcinoma. The aim of the present study was to evaluate the impact of DNA polymorphisms in the coding region of ECRG1 in esophageal carcinoma.
Methods Genomic DNA of 107 patients with esophageal cancer that underwent complete surgical resection between 1997 and 2005 was extracted. DNA was analyzed for ECRG1 polymorphisms Arg290Arg, Arg290Gln, and Gln290Gln by PCR and gel electrophoresis. Polymorphisms were correlated with survival data by the Kaplan–Meier method, multivariate Cox regression analysis, and odds ratio were determined. For all variables, cross tables were generated, followed by calculation of the p value by using the chi-square test/Fisher-exact test.
Results Follow-up data of 102 patients with esophageal cancer were available after complete surgical resection for a median follow-up time of 24.3 months. Polymorphism Arg290Arg was found in 47 patients (46.1%), Arg290Gln in 48 patients (47.0%), and Gln290Gln in seven cases (6.9%). Arg290Arg polymorphism was significantly associated with reduced overall survival (p=0.01) and tumor-free survival (p=0.01) by the log-rank test. Multivariate regression analysis by Cox revealed polymorphism Arg290Arg to be a significant prognostic factor for survival (p=0.012).
Conclusions Polymorphism Arg290Arg in ECRG1 is associated with poor clinical outcome after complete surgical resection in patients with esophageal cancer.

Keywords Esophageal-cancer-related gene1 · ECRG1 · Prognostic factor · Risk factor · DNA polymorphism · Esophageal cancer

Introduction

Esophageal cancer ranks among the ten most frequent cancers worldwide and has a very aggressive clinical behavior.[1,2] In the United States and Western Europe, esophageal carcinoma is currently the most rapidly increasing cancer.[3–5] Risk factors include smoking, heavy consumption of alcohol, micronutrient deficiency, and dietary carcinogen exposure.[6–8] Usually, the tumor is detected in an advanced stage, and the reported 5-year survival rate ranges from 10% to 36% after surgical resection.[9,10]

The reason for poor outcome after surgical resection is the extensive local invasion and frequent regional lymph node metastasis. Age of the patient, depth of tumor invasion, and presence of metastasis in lymph nodes and peripheral organs are well-known prognostic factors. Besides these clinicopathological data, different molecular markers have been examined for their impact on prediction of development and prognosis in esophageal cancer. However, only few powerful markers currently exist.[11]

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Esophageal-cancer-related Gene1 (ECRG1) is a recently-found tumor suppressor gene that has an impact on the regulation of cell proliferation and cell cycle.[12] Previous studies have shown that ECRG1 is downregulated in esophageal cancer. Additionally in vivo and in vitro overexpression of ECRG1 was found to inhibit tumor cell proliferation.[13,14] Mutation and genetic polymorphisms in coding sequences of a gene may cause functional alterations. Screening the coding region of ECRG1 for single nucleotide polymorphisms, a variant allele in exon 8 resulting in expression of glutamine or arginine in codon 290 was identified by PCR-based SSCP and DNA sequencing.[15,16] The aim of this study was to reveal the impact of these DNA polymorphisms in the coding region of ECRG1 for the prognosis after complete surgical resection of esophageal cancer.

Materials and Methods

Study Design and Patients

This study was approved by the ethics committee of the Hamburg Medical Association (Hamburg/Germany). Patients with esophageal cancer that underwent surgery in the Department of Surgery at the University Medical Center Hamburg–Eppendorf between 1997 and 2005 were included after histopathological confirmation, if complete surgical resection with tumor-free resection margins on histopathological examination of the surgical specimen (R0) was performed. Patients that died during the hospitalization were excluded.

Written informed consent to follow up and genetic analysis on their blood and tumor was obtained from the patients. Overall, 107 consecutive patients meeting the inclusion criteria and signed the consent were included in this trial. All patients were Caucasian. Tumor stage and grade were classified according to the most recent TNM classification of the International Union Against Cancer.[17,18] Five patients had to be excluded in analysis of the survival because they were lost to follow-up.

Clinicopathological Data

All data including sex, histology, depth of tumor invasion, lymph node metastasis, grading, and disease stage were obtained from the clinical and pathological records. Clinical follow-up data were retrieved by reviewing the hospital records, direct communication with patients or the attending physicians, and from the Hamburg Cancer Registry. Tumor-free and overall survival was calculated from the date of surgical resection of the tumor to the date of death or last follow-up. Altogether, the median follow-up period was 24.3 (range 1.7–58.2) months.

Analysis of DNA Polymorphism

A 5-ml sample of peripheral blood was taken in the operation theater preoperatively in all patients. The time of taking blood has no influence on this analysis because the marker is genetically fixed. Genomic DNA was extracted from peripheral blood leukocytes and purified according to established protocols using the QIAamp Blood Tissue Kit (Qiagen, Hilden, Germany). The PCR amplification was accomplished with a 25-μl reaction mixture consisting of 50 ng template DNA, 0.4 μM each primer, 0.2 mM each deoxynuccleotide-triphosphate (dNTP), 2.0 mM $MgCl_2$ and 1.0 U Taq DNA polymerase with 1× reaction buffer (Takara, Japan). PCR primers for amplifying DNA fragment containing the polymorphism were 5-CAGGGCTTAGCGCTCTGTTA-3 and 5-GCTCATATACTTTGGGCAGCTT-3 that produce a 354-bp fragment. The reaction conditions consisted of an initial melting step of 2 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C; and a final elongation step of 7 min at 72°C. The 290 Gln/Gln genotype has a single band representing the entire 354-bp fragment, the variant 290 Arg/Arg genotype results in two fragments of 232 and 122 bp; the heterozygous 290 Arg/Gln genotype has all three fragments of 122, 232, and 354 bp. The restricted product was analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. Three genotypes revealed by RFLP with MspI digestion were confirmed by DNA sequencing (Fig. 1). Positive and negative controls were used in the RFLP-PCR assay. All samples were analyzed in duplicate; the results were consistent in all cases. The agarose gels were read independently by two persons blinded to the study. The concordance of PCR analysis and sequencing was 100%.

Statistical Analysis

We used SPSS® for Windows® (Version 11.5.1; SPSS Inc., Chicago, IL) for statistical analysis. Survival curves of the patients were plotted using the Kaplan–Meier method and analyzed using the log-rank test. Cox regression analysis

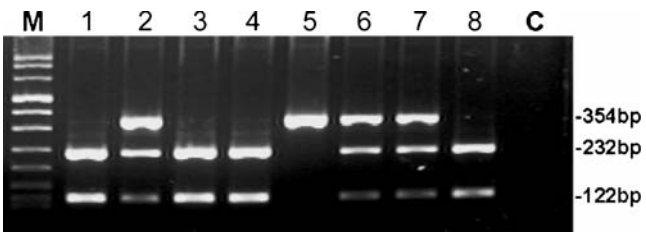

Figure 1 Electrophoresis of ECRG 1. Electrophoresis in a 2% agarose gel stained with ethidium bromide. The 354 bp fragment represents the Gln290Gln genotype; the variant Arg290Arg genotype results in two fragments of 232 and 122 bp; the heterozygous Arg290Gln genotype has all three fragments of 122, 232, and 354 bp.

was used for multivariate analysis to assess the independent influence polymorphism Arg290Arg simultaneously with other covariates. Significance statements refer to p values of two-tailed tests that were less than 0.05. For correlation analysis cross tables were generated for all variables, followed by calculation of the p value by using the chi-square test/Fisher-exact test. Patient's data and blood were collected prospectively, while no data of frequencies of the different alleles in the Caucasian population and impact on prognosis were available. Therefore, no statistical power analysis was performed prior to the study.

Results

Characteristics of the Patients

A total of 107 patients with esophageal cancer were chosen for this study. Characteristics of patients are listed in Table 1. The median age was 62 years; all patients were Caucasian. Eighty-seven patients (81.3%) were male and 20 patients (18.7%) female. Histopathological examination revealed adenocarcinoma in 41 (38.3%) patients and squamous cell carcinoma in 66 (61.7%) patients. Analyzing the distribution of the DNA polymorphism in adenocarcinoma and squamous cell carcinoma with Fisher's exact test showed no significant association (p=0.681). Therefore, both histologies were analyzed together as esophageal cancer. The invasion depth/tumor size was classified in histopathological examination as pT1 in 19 (17.8%) patients, pT2 in 30 (28.0%), pT3 in 46 (43.0%), and pT4 in 12 (11.2%) patients. Thirty-one (29.0%) of the patients had no lymph node metastases (pN0), while lymph node metastases were found in 76 (71.0%) patients. Tumor grading was classified as G1 in ten (9.3%) patients, G2 in 47 (43.9%), and G3 in 50 (46.7%) patients.

Transthoracic esophagectomy was performed in 68 patients, while transhiatal approach was used in 39 patients. A radical lymphadenectomy was performed in all patients. The median numbers of resected lymph nodes was 31 (19–43) in the transthoracic group and 25 (15–34) in the transhiatal group. The distribution of the number of resected lymph nodes and operative approach were comparable concerning the different alleles and had no significant influence on survival. Forty-five patients underwent adjuvant treatment, while no patient received neoadjuvant therapy.

The distribution of DNA polymorphisms in ECRG1 according to age, sex, tumor invasion depth (pT), and presence of lymph node metastasis (pN) is listed in Table 1. No clinical or pathological factor was associated with the polymorphism frequency in ECRG1 (age (p=0.847), sex (p=0.470), adjuvant therapy (p=0.654), tumor size (p=0.564), lymph nodes (p=0.288), grading (p=0.836), and histology of the tumor (p=0.564)) using the chi-squared/Fisher's exact test. Analyzing distribution of the concerning the univariate statistical analysis using the

Table 1 Clinicopathological Characteristics of Esophageal Cancer Patients' DNA Polymorphism in Esophageal Cancer Related Gene 1 (ECRG1)

Variable	No. of Patients		ArgArg		ArgGln		GlnGln		Gln allele	
Sex										
Male	87	81.3%	43	40.2%	40	37.4%	4	3.7%	44	41.1%
Female	20	18.7%	8	7.5%	9	8.4%	3	2.8%	12	11.2%
Age										
≤62	50	46.7%	23	21.5%	23	21.5%	4	3.7%	27	25.2%
>62	57	53.3%	28	26.2%	26	24.3%	3	2.8%	29	27.1%
Tumor depth										
Invading the submucosa (pT1)	19	17.8%	7	6.5%	10	9.3%	2	1.9%	12	11.2%
Invading the muscularis propria (pT2)	30	28,0%	17	15.9%	11	10.3%	2	1.9%	13	12.1%
Invading the adventitia (pT3)	46	43.0%	22	20.6%	22	20.6%	2	1.9%	24	22.4%
Invading contiguous structures (pT4)	12	11.2%	5	4.7%	6	5.6%	1	0.9%	7	6.5%
Lymph nodes										
No lymph node metastasis (pN0)	31	29.0%	12	11.2%	17	15.9%	2	1.9%	19	17.8%
Lymph node metastasis (pN1)	76	71.0%	39	36.4%	32	29.9%	5	4.7%	37	34.6%
Grading										
Well differenciated (G1)	10	9.3%	4	3.7%	5	4.7%	1	0.9%	6	5.6%
Moderate differenciated (G2)	47	43.9%	22	20.6%	21	19.6%	4	3.7%	25	23.4%
Poorly differenciated (G3)	50	46.7%	25	23.4%	23	21.5%	2	1.9%	25	23.4%
Histology										
Adenocarcinoma	41	38.3%	21	19.6%	18	16.8%	2	1.9%	20	18.7%
Squamous cell carcinoma	66	61.7%	30	28.0%	31	29.0%	5	4.7%	36	33.6%
Total	107	100.0%	51	47.7%	49	45.8%	7	6.5%	56	52.3%

log-rank test revealed a significantly poorer prognosis for survival for older patients (>62 years; $p=0.034$), patients with presence of lymph node metastasis ($p=0.024$), increasing invasion depth/tumor size ($p=0.046$), and grading ($p=0.047$). No significant differences were found comparing adenocarcinoma and squamous cell carcinoma ($p=0.175$). No significant impact on survival was found for the number of resected lymph nodes ($p=0.541$) or adjuvant treatment ($p=0.681$). Therefore, these parameters had to be excluded from multivariate Cox regression analysis.

DNA Polymorphism

Peripheral blood taken on the day of surgery was examined in 107 patients. Genomic DNA was extracted and analyzed for polymorphism in ECRG1. Arg290Arg was found in 51 (47.7%) patients, Arg290Gln in 49 (45.8%) and Gln290Gln in seven (6.5%) samples. The median overall survival in patients with Arg290Arg was 17.0 months (95% CI 9.6–24.4). The median survival for Arg290Gln was 30.8 months (95% CI 20.7–40.9), for Gln290Gln 39.9 months (95% CI 6.8–72.9), and for the presence of at least one Gln allele 30.8 months (95% CI 23.0–38. 6). Survival curves plotted by the Kaplan–Meier method for DNA polymorphism ECRG1 for overall survival are shown in Fig. 2 (Arg290Arg vs. Arg290Gln vs. Gln290Gln). In Fig. 3, the survival curves Arg290Arg versus presence of the Gln allele (Arg290Gln and Gln290Gln) in ECRG1 are plotted. Statistical analysis using the log-rank test revealed that patients with polymorphism Arg290Arg in ECRG1 had a significantly poorer prognosis ($p=0.038$); comparing Arg290Arg with Arg290Gln, significantly poorer prognosis was confirmed ($p=0.015$). Analyzing the impact of the presence of the Gln allele (Arg290Gln and Gln290Gln) in ECRG1 versus Arg290Arg, a significantly shorter overall survival was found in univariate analysis ($p=0.01$).

Multivariate analysis using Cox regression revealed Arg290Arg (versus Arg290Gln and Gln290Gln) as prognostic marker for survival ($p=0.012$) with a relative risk of 2.016 (95% CI 1.164–3.493; Table 2). Analyzing the DNA polymorphism (Arg290Arg vs. Arg290Gln vs. Gln290Gln), it was found to be a prognostic marker for survival ($p=0.046$) in multivariate analysis as well. The relative risk was found to be 1.630 (95% CI 1.009–2.633; Table 3). Age, grading, and invasion depth/tumor size (pT) were found to be prognostic factors for survival as well. Presence of lymph node metastasis was found to be a prognostic factor in univariate log rank test ($p=0.024$); it could not be identified as independent prognostic factor ($p=0.610$) in multivariate analysis.

Analyzing the tumor-free survival, DNA polymorphism of the ECRG1 ($p=0.01$), lymph node status (0.03) and tumor invasion depth ($p=0.02$) were identified to be prognostic factors in univariate log rank test. No significant impact on tumor-free survival could be detected for age ($p=0.12$), sex ($p=0.06$), grading ($p=0.12$), and histology ($p=0.39$). Therefore, the polymorphism, lymph node status, and tumor invasion depth were included in multivariate Cox regression analysis. In multivariate analysis, tumor invasion depth ($p=0.03$) and presence of Arg290Arg polymorphism ($p=0.01$) were confirmed as prognostic factors.

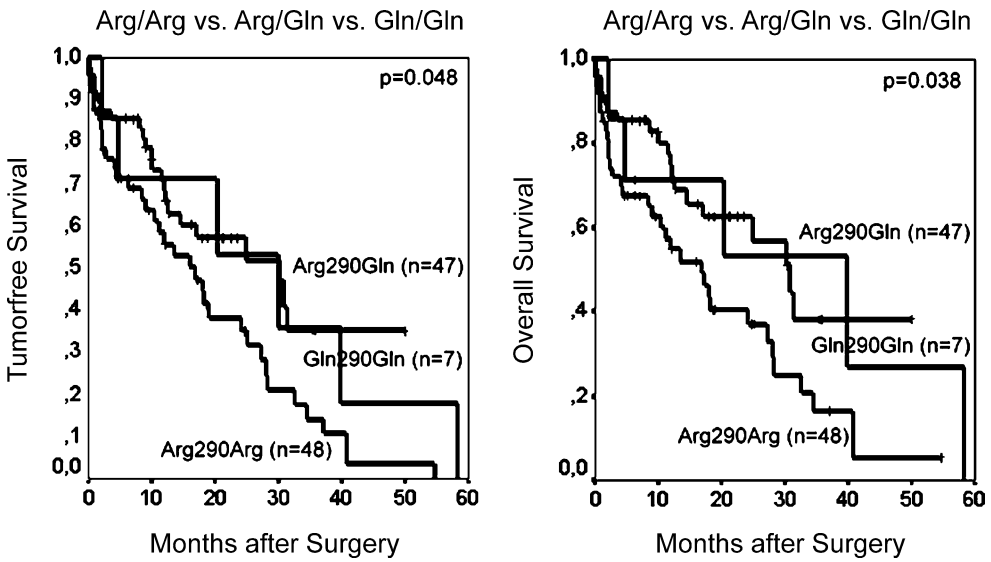

Figure 2 **a**, **b** Kaplan–Meier analysis for overall survival for DNA polymorphism in ECRG1. (Arg290Arg vs. Arg290Gln vs. Gln290Gln) in patients with esophageal cancer after curative esophagectomy. Kaplan–Meier analysis for tumor-free and overall survival for DNA-Polymorphism in ECRG1.P value was calculated with two-sided log-rank test. The median overall survival of patients with 290Arg290 was 17.0 months (95% CI 9.6–24.4). For Arg290Gln the median overall survival was 30.8 months (95% CI 20.7–40.9) and 39.9 months (95% CI 6.8–72.9) for Gln290Gln. The median tumor-free survival of patients with 290Arg290 was 17.0 months (95% CI 13.3–22.7). For Arg290Gln the median survival was 30.1 month (95% CI 15.0–45.3) and 30.0 months (95% CI 2.6–57.4) for Gln290Gln.

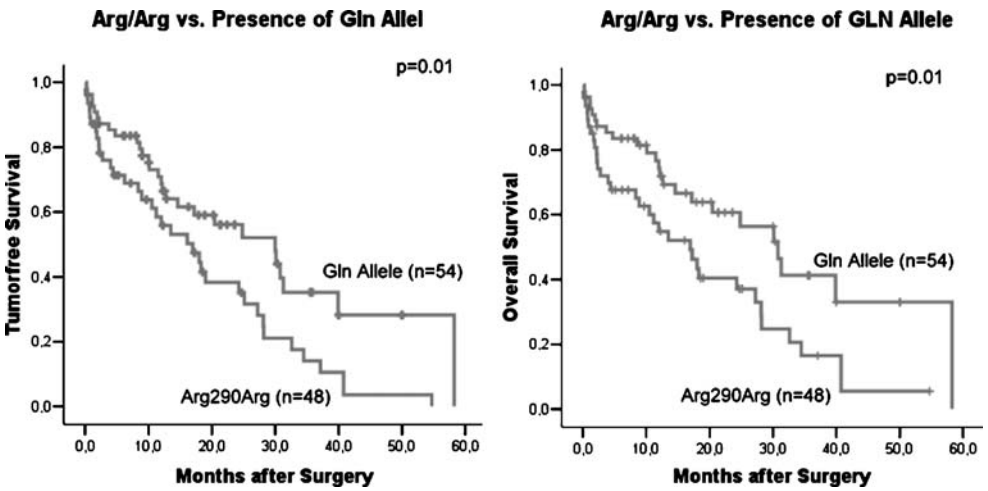

Figure 3 **a**, **b** Kaplan–Meier analysis for overall survival for presence of Gln allele in ECRG1. (Arg290Arg vs. Arg290Gln and Gln290Gln) in patients with esophageal cancer after curative esophagectomy. Kaplan–Meier analysis for tumor-free and overall survival for presence of Gln allele in ECRG1. P value was calculated with two-sided log-rank test. The median overall survival of patients with 290Arg290 was 17.0 months (95% CI 9.6–24.4), 30.8 months (95% CI 23.0–38.6) for presence of at least one Gln allele. The median tumor-free survival of patients with 290Arg290 was 17.0 months (95% CI 13.3.–22.7), respectively 29.1 months (95% CI 22.2–36.1) for presence of at least one Gln allele.

To analyze the impact of the polymorphism, the patients were grouped according to UICC classification. Twelve patients were grouped to UICC I. Due to a limited number of patients and number of deaths (only one patient died), survival analysis including Kaplan-Meier and calculation of log rank test is not possible. Analyzing the 40 patients with UICC II (A+B) stage, no significant impact of the ECRG1 polymorphism on survival was identified. In the 50 patients that were grouped to UICC III, Arg290Arg was found to be a predictor for poorer prognosis concerning tumor-free survival (p=0.008) and overall survival (p=0.025).

Discussion

This study detected ECRG1 polymorphism Arg 290Arg as prognostic factor for survival after complete surgical resection of esophageal cancer. The Arg290Arg polymorphism is associated with significantly poorer prognosis for tumor-free and overall survival. In multivariate analysis, age, grading, and tumor invasion depth were also found to be associated with prognosis concerning overall survival. It has to be mentioned that the size of the study population (n=107) is a limitation of the power of this trial, but even in patients with UICC III, the polymorphism Arg290Arg was identified as poor prognostic factor. In univariate analysis, positive lymph nodes were identified as poor prognostic factor for overall and tumor-free survival, but lymph node status was not significant in a multivariate model with other, more highly associated covariates. This might be caused by the association between tumor size and lymph node metastasis and the limited number of patients in this trial.

Table 2 Multivariate Analysis by *Cox* Regression for Overall Survival for Various Factors (n=102) for Presence of Gln Allele in ECRG1

Overall survival	Odds ratio/95%CI[a]	*P* value
Age(<62 vs. >62)	1.831 (1.047–3.202)	0.034
Tumor invasion depth (pT1/2vs. T3/4)	1.687 (1.135–2.510)	0.010
Lymph node metastasis (pN0 vs. N1)	1.266 (0.511–3.133)	0.610
Grading (G1/2 vs. G3)	1.765 (1.034–3.015)	0.037
AA vs. AG and GG	2.016 (1.164–3.493)	0.012

[a] *CI* Confidence interval. Statistics were done by multivariate Cox regression analysis. All statistical tests were two-sided. Odds ratio presented are for overall survival

Table 3 Multivariate Analysis by *Cox* Regression for Overall Survival for Various Factors (n=102) for DNA Polymorphism in ECRG1

Overall survival	Odds ratio/95%CI[a]	P value
Age (<62 vs. >62)	1.814 (1.035–3.179)	0.037
Tumor invasion depth (pT1/2 vs. T3/4)	1.635 (1.104–2.421)	0.014
Lymph node metastasis (pN0 vs. N1)	1.346 (0.546–3.320)	0.519
Grading (G1/2 vs. G3)	1.716 (1.009–2.917)	0.046
Polymorphism (AA vs. AG vs. GG)	1.630 (1.009–2.633)	0.046

[a] *CI* Confidence interval. Statistics were done by multivariate Cox regression analysis. All statistical tests were two-sided. Odds ratio presented are for overall survival

Besides age, tumor invasion depth, lymph node status, and presence of peripheral metastases, different predictive and prognostic markers in esophageal cancer have been described in the past without proceeding to widespread clinical use. In this trial, adenocarcinoma and squamous cell carcinoma were included and grouped together, because prognosis and surgical treatment are comparable; additionally, the expression of DNA polymorphism of the ECRG1 showed no significant difference between both groups.

Expression of different proteins such as EGFR, COX2, p53, and TGF β was found to be associated with the aggressiveness of tumors or shorter survival.[19–23] Therefore, these might be predictive markers for response to radiochemotherapy.[20,21,24–26]

Genomic DNA polymorphisms are stable and do not change throughout one's lifetime since they are genetically fixed. They can be detected consistently in contrast to protein expression analysis. Only few genetic markers have been evaluated for their clinical impact. Alterations of genes involved in the cell cycle control (p21, p27) are associated with outcome. Cyclin D1 polymorphisms were found to be associated with genomic instability and poorer prognosis in esophageal carcinoma in a recent study.[27] High levels of Bax and low levels of Bcl-X are associated with longer overall survival.[28–31] Recently polymorphism TCA_3/TCA_3 in exon 4 of the esophageal-cancer-related gene 2 (ECRG2) was found to be an independent prognostic factor for poor survival in esophageal and oral squamous cell cancer.[32–34] The use of diagnostic analysis of DNA polymorphisms is increasing rapidly in the last months. Different polymorphisms have been published in patients with cancer[35–37] but also in other diseases such as Parkinson's disease.[38]

ECRG1 is a member of the membrane anchored serine protease domains that play a role in proteolytic activity. In vivo and in vitro assays revealed that overexpression of ECRG1 protein inhibits tumor cell proliferation. ECRG1 was able to induce an arrest of the cell cycle in a cell line in an experimental setting. Therefore, ECRG1 might play a role in development of esophageal cancer.[39]

Li et al. found the polymorphism in codon 290 in exon 8 to be a predictive factor in the Chinese population in development of esophageal cancer in a study including 998 patients and 1,252 controls. The genotype Arg290Gln was associated with slightly higher risk for developing esophageal cancer. In association with smoking, the presence of this genotype Arg290Gln was a significant factor for development of squamous cell carcinoma.[16] No data of impact on survival was provided in this trial. This study is the first to analyze the impact of DNA polymorphism of ECRG1 on prognosis of the patients so no comparable results are available. Further trials are necessary to evaluate the impact of ECRG1 on developing esophageal cancer and its prognosis and potential therapeutic management.

Conclusion

In this trial, we could detect the genotype Arg290Arg to be a prognostic factor for poorer tumor-free and overall survival in esophageal cancer. The evaluation of the impact of the polymorphism of ECRG1 on development and prognosis of cancer has to be explored in the future. Further trials are needed to evaluate the potential of our findings and its possible impact as a new starting point for adjuvant or neoadjuvant therapy.

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