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P21cip1 Expression is a Prognostic Biomarker in Esophageal Squamous Cell Carcinoma

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Abstract

Esophageal Squamous Cell Carcinoma (ESCC) is a highly incident and fatal disease and represents more than 90% of all esophageal cancer worldwide. It has a poor prognosis mainly due to late diagnosis and ineffective treatment. *TP53* mutations are the most common genetic alterations found in ESCC, affecting the proper function of a complex network involved in cellular homeostasis, such as cell cycle regulation. The present study evaluated the mRNA expression of key cell cycle regulators, *p14ARF, p16INK4a, p21CIP1, TP53,* and *GADD45A*, in paired ESCC and esophageal non-tumor mucosa. Our goal was to understand whether cell cycle control related-genes could be disrupted by mechanisms other than *TP53* mutations and the impact of these alterations on patients' prognosis. Only *p21CIP1* was over expressed in ESCC compared to matched non-tumor mucosa. Also, we observed a heterogeneous expression of *p14ARF* and *p16INK4a,* which was not associated with DNA methylation patterns on their gene promoter regions. Finally, multivariate analysis revealed that *p21CIP* mRNA expression is an independent prognostic factor for ESCC patients, with low *p21CIP* expression predicting a poorer overall survival.

Keywords: Esophageal squamous cell carcinoma; Cell cycle; *p21CIP*; Prognostic biomarkers.

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Introduction

Esophageal Cancer (EC) is the eighth most incidence tumor and is the sixth leading cause of cancer-related mortality worldwide, indicating a high EC-associated lethality [1]. The areas with the highest EC incidence comprise Asia, Southern and Eastern Africa, the United Kingdom, and some countries of South America, such as Brazil [2]. This tumor is classified into two main histological subtypes, Esophageal Adenocarcinoma (EAC) and Esophageal Squamous Cell Carcinoma (ESCC), the latter accounting for more than 90% of all cases worldwide [2].

Several studies have applied Next-Generation Sequencing (NGS) to describe the main molecular alterations in ESCC. Recently, the Cancer Genome Atlas Consortium (TCGA) published the integrated genomic characterization of EC, showing both mutations and copy number variations affecting cell cycle regulators in this tumor. In concordance with other studies, this study also observed that *TP53* is mutated in 70-90% of ESCC samples [3-6]. *TP53* encodes the p53 protein, that can bind to specific promoter sequences and transactivate a wide range of genes such as *CDKN1A* (*p21CIP1*) and *GADD45A*and has a central role in a very complex network involved in cell cycle regulation [7]. For example, p21inhibits the activity of cyclin-CDK2 or CDK4 complexes and thus plays a role as a p53 effect or in cell cycle control [8-10]. In addition, GADD45A is often induced by p53 in response to DNA damage and other stress signals, triggering cell growth arrest, DNA repair, and apoptosis [7,11].

Regarding copy number variations, CGH (Comparative genomic hybridization) analysis has identified *CDKN2A* deletion in 20% to 76% of ESCC cases evaluated [6,12]. On the other hand, recently, in a meta-analysis, which included data from 41 case-control studies, including Asian, Caucasian, and African patients, *CDKN2A* promoter methylation was significantly higher in EC samples than in healthy controls [13]. *CDKN2A* locus codes for two proteins, p14 and p16, which are involved in the p53-dependent regulation of cell cycle progression [14]. Altogether, these studies have shown that cell cycle homeostasis disruption is fundamental to promoting and progressing ESCC carcinogenesis, with a pivotal role of *TP53* and its molecular partners. However, the impact of the differential expression of these genes onpatients' prognosis is not yet clear and overall controversial.

Therefore, this study aimed to (i) evaluate the gene expression profile of key cell cycle regulators related to p53 function in ESCC and (ii) to determine their potential as prognosis biomarkers.

Materials and methods

Human Samples

Seventy-five matched biopsies were collected from patients diagnosed with ESCC (tumor tissue and non-tumor surrounding mucosa, collected 4 inches from the tumor border) who underwent surgery or endoscopy between 2000-2007. In total, 46 patients from the Southeast region of Brazil (Rio de Janeiro and São Paulo) were included in this study: Three from Hospital Universitário Pedro Ernesto, UERJ, Rio de Janeiro; 22 from INCA, Rio de Janeiro; and 21 from Surgery Department, UNICAMP, São Paulo. The remaining 29 samples were collected in Hospital das Clínicas dePorto Alegre, HCPA/UFRGS, Rio Grande do Sul, in the South of Brazil. Patients enrolled in this study had not undergone prior chemotherapy or radiotherapy. Patients' habits regarding smoking and alcohol consumption, socio-demographic characteristics, tumor differentiation, and esophageal location were collected by a standardized questionnaire and from hospital records. The institutions' Ethics Committees approved this study, and all procedures followed the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients signed written informed consent.

RNA extraction, reverse transcription (RT) and quantitative PCR (qPCR)

Total RNA was extracted from biopsies using TRIzol® (Invitrogen, USA), following the protocol described by the manufacturer. Next, cDNA was synthesized using Super Script™ First-Strand Synthesis System and random hexamers, according to the manufacturer's instruction. qPCR was used to evaluate *p14ARF, p16INK4a, p21CIP1, TP53,* and *GADD45A* mRNA expression (Supplementary Table S1).*GAPDH* and *ACTB*(Supplementary Table S1) were used as housekeeping genes. The number of samples analyzed for each gene varied due to RNA availability. qPCR was performed with the ABI 7700 detection system (Applied Biosystems, USA) as previously described [15].

DNA extraction and bisulfite treatment: *p14ARF* and *p16INK4a* mRNA expression levels were categorized into low ($\log_2$ fold-change<-1), no change (-1≤$\log_2$ fold-change≤1) and high ($\log_2$ fold-change>1). Next, we randomly selected 10 ESCC samples (tumor and non-tumor surrounding mucosa) from each category (high, no change, and low) to perform DNA extraction, followed by bisulfite treatment and pyrosequencing. DNA was extracted from thirty frozen ESCC sample pairs by SDS/proteinase K protocol [16]. Then, 1.0 µg of DNA was treated using the EpiTect® Bisulfite kit (Qiagen, Germany) according to the manufacturer's instructions to convert unmethylated cytosine residues to uracil, leaving the methylated cytosines unchanged.

Pyrosequencing

The methylation status of ten selected CpG sites in *p14ARF* and *p16INK4a* promoters was analyzed by pyrosequencing. Bisulfite-treated DNA (25 ng) was used to amplify the regions of interest with primers designed with the PSQ TM24MA System software (Qiagen, Germany) (Supplementary Table S1). Taq platinum DNA polymerase (Invitrogen, USA) was used for PCR reaction following manufactures protocol. Amplification was performed with 5 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 45 sec at the specific temperature for each pair of primers, and 30 sec at 72°C, followed by one hold at 72°C for 10 min. Pyrosequencing was performed according to the manufacturer's protocol (Qiagen, Germany). The target CpGs were evaluated by converting the resulting pyrograms into numerical values for peak heights and calculating the mean of all CpG sites analyzed at a given gene promoter. Samples that showed low-quality peaks were excluded from the analysis.

Statistical analysis

The Wilcoxon matched pairs test or Kruskal Wallis test was used to assess mRNA expression or methylation percentage differences between tumors and non-tumor surrounding mucosa using GraphPad 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered statistically significant when $p<0.05$. Overall survival was analyzed 24 months after diagnosis. Patients who were alive at the end of the follow-up period were censored. For those who were lost to follow-up, the date of the last information obtained was considered for purposes of censorship.

Furthermore, the impact of mRNA expression of each gene on overall survival was evaluated and this molecular variable was categorized into tertiles. Patients falling in the lower and middle tertiles were grouped and compared to patients falling in the highest tertile. The Kaplan-Meier method was used to assess univariate survival, while statistical significance between groups was calculated with the log-rank test, assuming a statistical significance level of 5%. The Cox proportional hazards regression model was used for univariate and multivariate analyses to explore the relationship between the mRNA expression of each gene analyzed and the prognostic value for survival. Variables that showed $p<0.20$, age, and tumor stage were used to adjust the association between mRNA expression and global survival. All data were analyzed using the statistical package SPSS for Windows 20.0.

Results

Clinicopathological characteristics

The median age of patients included in this study was 56 years [34-83], and most were male (82.7%), alcohol drinkers (88.0%), and tobacco smokers (86.6%), with 81.3% presenting both habits. Most tumors were in the middle or distal thirds of the esophagus (66.6%), showed moderate or well differentiation status (61.4%) and were diagnosed in the advanced clinical stage (66.7%) (Table 1).

Gene expression profile

We evaluated the expression of *GADD45A*, *p14ARF*, *p16INK4a*, *p21CIP1*, and *TP53* in ESCC and non-tumor surrounding mucosa, and tumors samples showed *p21CIP1*overexpression (fold-change: 2,0; p = 0.0011), while the other genes showed no statistically significant expression differences (Figure 1B). Furthermore, it is noteworthy that ESCC samples presented heterogeneity in the *p14ARF* and *p16INK4a* expression compared to matched non-tumor adjacent mucosa (fold-change), which was not observed in the other analyzed genes (Figure 1A).

Table 1: Clinicopathological and socio-demographic data of ESCC patients.

SOCIO-DEMOGRAPHIC DATA	*TOTAL ($n^{\#}$=75)*
Age	
Median (min-max)	56.0 (34-83)
Gender	
Men	62 (82.7%)
Women	13 (17.3%)
Origin	
Southeast	46 (61.3%)
South	29 (38.7%)
Alcohol Consumption	
Never	7 (9.3%)
Ever	66 (88.0%)
Missing	2 (2.7%)
Tobacco Consumption	
Never	8 (10.7%)
Ever	65 (86.6%)
Missing	2 (2.7%)
CLINICAL DATA	
Tumor Location	
Proximal esophagus	5 (6.7%)
Middle esophagus	33 (44.0%)
Distal esophagus	17 (22.6%)
More than one region affected	15 (20.0%)
Missing	5 (6.7%)
Tumor Differentiation	
Well and Moderately	46 (61.4%)
Poorly and Undifferentiated	19 (25.3%)
Missing	10 (13.3%)
Stage	
I + II	21 (28.0%)
III + IV	50 (66.7%)
Missing	4 (5.3%)
T stage	
T1 + T2	6 (8.0%)
T3 + T4	61 (81.3%)
Missing	8 (10.7%)
Lymph node invasion	
No	34 (45.3%)
Yes	33 (44.0%)
Missing	8 (10.7%)
Survival (months)	
Median (min-max)	12 (1-99)

$^{\#}$ number of patients.

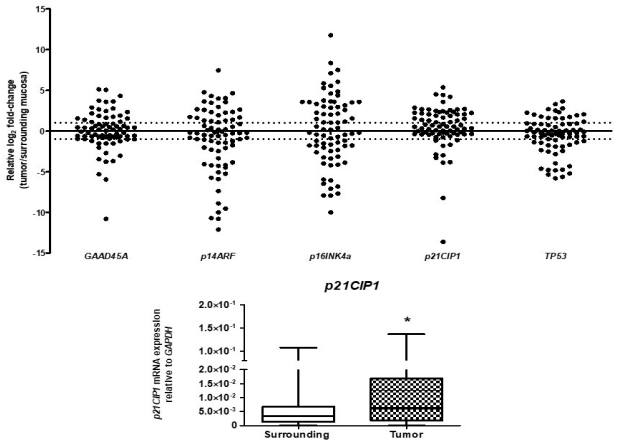

Figure 1: Gene expression of cell cycle regulators in ESCC. (A) Dot-plot showing the expression log_2 fold-change of *GADD45A*, *p14ARF*, *p16INK4a*, *p21CIP1* and *TP53* in ESCC relative to non-tumor surrounding mucosa. Each dot represents a patient. (B) Box-plot showing *p21CIP1* (*CDKN1A*) mRNA levels relative to *GAPDH* in ESCC and non-tumor surrounding tissue. Upper dotted line: log_2 fold-change = 1; lower dotted line: log_2 fold-change = -1; * $p < 0.05$.

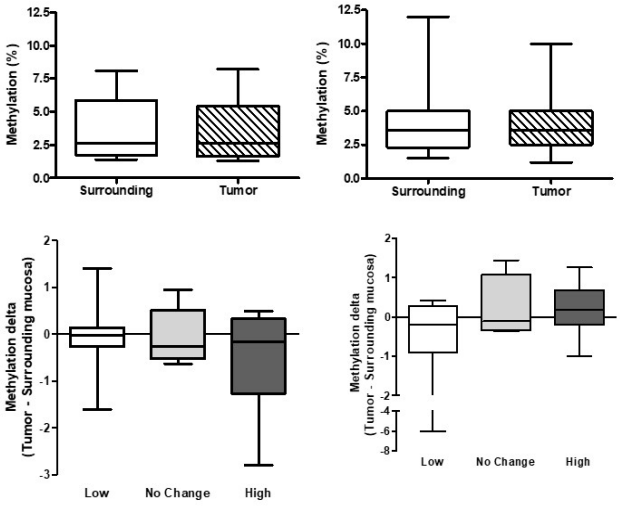

Figure 2: Methylation profile of *p14ARF* and *p16INK4a* promoter region in ESCC. Box-plots showing the mean methylation of all CpG sites evaluated in*p14ARF* (A) and *p16INK4a* (B) promoter regions in non-tumor surrounding tissue and ESCC. Box-plots showing the mean methylation delta (methylation in tumors minus the methylation in surrounding tissues) in patients categorized according to their gene expression profile for *p14ARF* (C) and *p16INK4a* (D). low: expression log_2 fold-change < -1; no change: -1 ≤ expression log_2 fold-change ≤ 1; high: Expression log_2 fold-change > 1.

p16INK4a and *p14ARF* DNA methylation and expression analysis

The methylation status of *p14ARF* and *p16INK4a* promoters was evaluated in to address its correlation with gene expression as a possible explanation for the heterogeneity observed in ESCC samples. First, pyrosequencing revealed that *p16INK4a* and *p14ARF* promoters present similar methylation levels in tumors and surrounding mucosa without significant differences in the mean methylation of the 10 CpG sites analyzed (Figure 2 A & B) or in CpG sites analyzed individually (data not shown). Next, samples were subcategorized into three groups according to the expression levels of *p14ARF* and *p16INK4a* in ESCC in comparison to matched non-tumor adjacent mucosa. So, we assessed whether there would be an association between the delta methylation (tumor - surrounding mucosa) and the mRNA expression levels according to the three subcategorized groups described above, but no statistically significant difference was observed (Figure 2 C & D). Finally, we investigated the association between *p14ARF* and *p16INK4a* up and down regulation and clinical or socio-demographic parameters, including 2-year overall survival. We found no significant association between their expression and the evaluated parameters (Supporting information Table S2).

Impact of differential *p21CIP1* expression on ESCC patient's overall survival

Following the observed *p21CIP1* deregulation in ESCC, we evaluated the association between its findings and clinical or socio-demographic parameters, and no significant associations between *p21CIP1* expression and these parameters were found (Supporting information Table S3). Next, we investigated the impact of *p21CIP1* over expression on ESCC overall survival. Multivariate analysis revealed that patients with low *p21CIP1* expression presented a median 2-year survival rate of 12%, the patients with high *p21CIPI* levels group did not reach the 50% survival mark in the evaluated period, indicating that *p21CIP1* expression is an independent prognostic factor in ESCC Brazilian patients HR: 2.61 (95% CI, 1.33-5.14); p = 0.005) (Figure 3).

Discussion

In the present study, we determined the mRNA expression and methylation changes in genes that encode components of the p53-dependent cell cycle regulation pathway in paired samples of ESCC and non-tumor adjacent mucosa. We found high heterogeneity in *p14ARF* and *p16INK4a* expression; however, the methylation status of the promoter region of these genes was not correlated with this phenomenon. Interestingly, among all investigated genes, only *p21CIP1*(*CDKN1A*) was found to be overexpressed in ESCC and showed to be an independent prognostic factor.

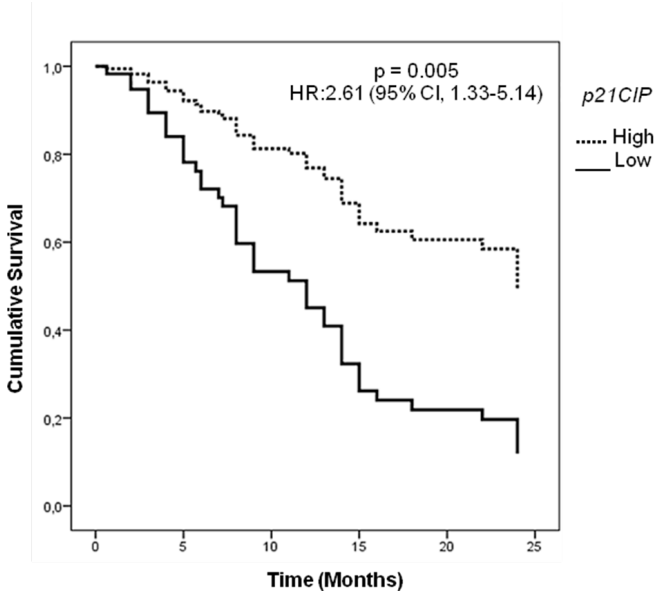

Figure 3: *p21CIP1* high mRNA expression levels were associated with 2-year overall survival of ESCC patients. Kaplan-Meier curve showing ESCC patients' survival according to *p21CIP1* (*CDKN1A*) expression fold-change in tumors relative to paired non-tumor adjacent mucosa, with 24 months of follow-up. The analysis was adjusted for age and tumor stage. high: Highest expression $\log_2$ fold-change tertile; low: lower and middle expression $\log_2$ fold-change tertiles.

In this study, we did not observe associations between *p14ARF* and *p16INK4a* expression and promoter methylation status in ESCC samples. This data agrees with our previous results, showing no differences in the methylation levels of *CDKN2A* locus in ESCC samples compared with non-tumor surrounding mucosa and esophageal mucosa from healthy individuals [17]. However, other studies have detected promoter hyperm ethylation in these genes in ESCC [12,13,18]. This apparent discrepancy could be explained by the different methods used to analyze the methylation profile, like methylation-specific PCR (MSP), or it could reflect differences between the populations studied. The etiological factors associated with ESCC development vary worldwide and could impact the expression or activity of cell cycle regulators, especially *p14ARF* and *p16INK4a*, by distinct molecular mechanisms, including homozygous deletions, intragenic mutations, and complex regulatory nets of non-coding RNAs [6,17,19-21]. Recently, the TCGA report that *CDKN2A* inactivation is a common trait for ESCC (76%) and is usually associated with deep gene deletion [6]. Therefore, although different inactivating molecular alterations have been reported in the *CDKN2A* locus, their impact on gene and protein expression in ESCC needs to be further evaluation.

Among the cell cycle and p53-regulated genes evaluated in this study, only *p21CIP1*was upregulated in ESCC compared to-non-tumor adjacent mucosa. Furthermore, ESCC patients with low *p21CIP1* expression had a shorter 2-year overall survival. The impact of p21 expression on patients' prognosis has been previously reported for several tumors, including colorectal, bladder, and gastric cancer [22-24]. Furthermore, such impact seems to be dependent on the age at diagnosis. For example, the loss of this tumor suppressor was associated with a worse prognosis in younger colorectal cancer patients (HR 4.09, 95% CI, 1.13-14.9) but showed the opposite trend in older individuals (HR 0.37; 95% CI, 0.24-0.59) [22]. Meanwhile, in gastric cancer, increased levels of p21 protein were associated with improved overall survival in older patients [24]. Thus, we did not observe any significant association between age, *p21CIP1* expression, and ESCC patients' overall survival.

In ESCC, previous studies have shown discordant associations between p21 expression and 5-year overall survival, with reports suggesting a lack of association [25,26] and others showing a negative association between p21 over expression and survival of patients [27,28]. Nonetheless, different authors have shown that the over expression of this protein has a positive impact on prognosis [26,29,30], similar to our findings. Therefore, the impact of p21 mRNA and protein levels on overall survival should be further explored in ESCC since it might be dependent on patients' age and the studied population.

Unfortunately, there are no drugs in clinical practice that target p21 [31]. However, drugs tested in ESCC cell cultures, such as Obatoclax (an inhibitor of Bcl-2 family members) and diallyl disulfide (an organosulfur compound derived from garlic), have shown as apparent antitumor effect on these cells [32,33]. Furthermore, treatment with these drugs resulted in cell cycle arrest, reduced cell viability, induction of apoptosis, and increased p21 expression. Thus, studies that assess the potential of p21 as a therapeutic target could improve the prognosis of patients with low expression of this protein.

TP53 expression did not show differences between ESCC and non-tumor surrounding mucosa. Since *p21CIP1* regulation is mediated at least in part by *TP53*, we evaluated the possible correlation between *p21CIP1* and *TP53* expression, observing a positive correlation between their mRNA levels ($r = 0.55$; $p < 0.0001$, data not shown). This moderate correlation may indicate that other factors could be involved in *p21CIP1* regulation in ESCC, such as *TP53* mutational status. Although we did not perform mutation screening in the present study, no correlation was observed between *p21CIP1*expression and *TP53* mutational status in the ESCC TCGA dataset (p=0.2015, data not shown). This could be explained by the plethora of p53-independent pathways and transcription factors capable of inducing *p21CIP1* expressions, such as the Ras-Raf-Mapk oncogenic pathway, and major transcriptional regulators, such as SP1 and STAT [34,35].

Although the prognostic value of p21 expression has been previously explored in cancer, it is essential to notice that this is the first study to address its association with ESCC survival by evaluating mRNA expression by qPCR, a quantitative method. Other studies in ESCC and different tumors have focused on p21 protein expression using IHC, a semi-quantitative method. Besides, these studies used different criteria to evaluate and stratify the immune staining pattern, with controversial results. Therefore, we suggest that future studies should be performed to evaluate the impact of *p21CIP1* mRNA levels in ESCC by quantitative methods to better stratify prognosis and potentially intervene to improve patients' survival.

Conclusion

In conclusion, we showed an up regulation of *p21CIP1* in ESCC, although low *p21CIP1* mRNA levels independently predict a poorer 2-year overall survival of ESCC patients.

Declarations

Conflicts of interest: The authors declare that they have no conflicts of interest.

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Supplementary table S1: Specific sequences of forward and reverse primers used in RT-qPCR or pyrosequencing.

GENE	PRIMERS	PRODUCT SIZE (bp[#])	ASSAY	REFERENCES
p14ARF	F: 5' TCCTAGAAGACCAGGTCATGATG 3' R: 5' ACCACCAGCGTGTCCAGGAA 3'	194	qPCR	Designed by the authors
p16INK4a	F: 5' CAACGCACCGAATAGTTACG 3' R: 5' ACCAGCGTGTCCAGGAAG 3'	171	qPCR	Haller *et al*., 2005
p21CIP1	F: 5' ACCTGTCACTGTCTTGTACCCTTGT 3' R: 5' TGGTAGAAATCTGTCATGCTGGT 3'	121	qPCR	Designed by the authors
TP53	F: 5' TAAGCGAGCACTGCCCAACA 3' R: 5' TCACGCCCACGGATCTGAAG 3'	96	qPCR	Designed by the authors
GADD45A	F: 5' AGAGCAGAAGACCGAAAGGATG 3' R: 5' TCGACGTTGAGCAGCTTGGC 3'	123	qPCR	Designed by the authors
GAPDH	F: 5' CAACAGCCTCAAGATCATCAGCAA 3' R: 5' AGTGATGGCATGGACTGTGGTCAT 3'	123	qPCR	Designed by the authors
β-actin	F: 5' CCAGATCATGTTTGAGACCTT 3' R: 5' CGGAGTCCATCACGATGCCAG 3'	107	qPCR	Designed by the authors
p16INK4a	F: 5 ' GGGTGGGGGAGTATATAGGG 3´ R: 5' biotin TCCCACCCCAACCTCCAAAATCT 3´ S: 5` AGGAGGGAGGGAGAGG 3`	163	Pyrosequencing	Designed by the authors
p14ARF	F: 5' GGGATATGGAGGGGGAGAT 3´ R: 5' biotin TCCCCTCCCCTACTAACC 3´ S: 5` GAGAAAGTAAGTAGAGGAGTTAGG 3	183	Pyrosequencing	Designed by the authors

[#] base pair; F: forward; R: reverse.

Supplementary table S2: Association between *p14ARF* and *p16INK4a* mRNA expression and clinical and socio-demographic parameters.

SOCIO-DEMOGRAPHIC DATA	*P14ARF*				*P16INK4a*			
	TOTAL (n#=54)	*High (n#=25)*	*Low (n#=29)*	*p value**	*TOTAL (n#=63)*	*High (n#=34)*	*Low (n#=29)*	*p value**
Age								
Median (min-max)	56.0 (34-83)	56.0 (34-76)	56.0 (47-83)	0.9515	56.0 (34-83)	55.5 (40-75)	57.0(34-83)	0.6586
Gender								
Men	43 (79.6%)	21 (84.0%)	22 (75.8%)	0.5166	52 (82.5%)	29 (85.3%)	23 (79.3%)	0.7406
Women	11 (20.4%)	4 (16.0%)	7 (24.2%)		11 (17.5%)	5 (14.7%)	6 (20.7%)	
Origin								
Southeast	34 (63.0%)	18 (72.0%)	16 (55.0%)	0.2628	38 (60.3%)	23 (67.6%)	15 (51.7%)	0.3015
South	20 (37.0%)	7 (28.0%)	13 (45.0%)		25 (39.7%)	11 (32.4%)	14 (48.3%)	
Alcohol Consumption								
Never	4 (7.4%)	0 (0%)	4 (13.8%)	0.1116	5 (7.9%)	3 (8.8%)	2 (6.9%)	1.000
Ever	48 (89.0%)	25 (100%)	23 (79.3%)		56 (88.9%)	31 (91.2%)	25 (86.2%)	
Missing	2 (3.7%)	-	2 (6.9%)		2 (3.2%)	-	2 (6.9%)	
Tobacco Consumption								
Never	5 (9.3%)	2 (8.0%)	3 (10.3%)	0.6848	6 (9.5%)	4 (11.8%)	2 (6.9%)	0.6848
Ever	47 (87.0%)	23 (92.0%)	24 (82.8%)		55 (87.3%)	30 (88.2%)	25 (86.2%)	
Missing	2 (3.7%)	-	2 (6.9%)		2 (3.2%)	-	2 (6.9%)	
CLINICAL DATA								
Tumor Location								
Proximal esophagus	4 (7.4%)	3 (12.0%)	1 (3.5%)		5 (7.9%)	4 (11.8%)	1 (3.4%)	
Middle esophagus	25 (46.3%)	11 (44.0%)	14 (48.3%)	0.6837	26 (41.3%)	14 (41.2%)	12 (41.5%)	0.6211
Distal esophagus	9 (16.7%)	4 (16.0%)	5 (17.2%)		16 (25.4%)	8 (23.5%)	8 (27.6%)	
More than one region affected	12 (22.2%)	5 (20.0%)	7 (24.1%)		13 (20.6%)	6 (17.6%)	7 (24.1%)	
Missing	4 (7.4%)	2 (8.0%)	2 (6.9%)		3 (4.8%)	2 (5.9%)	1 (3.4%)	
Tumor Differentiation								
Well and Moderately	33 (61.0%)	15 (60.0%)	18 (62.1%)	1.000	38 (60.3%)	21 (61.8%)	17 (58.6%)	1.000
Poorly and Undifferentiated	14 (26.0%)	7 (28.0%)	7 (24.1%)		17 (27.0%)	10 (29.4%)	7 (24.1%)	
Missing	7 (13.0%)	3 (12.0%)	4 (13.8%)		8 (12.7%)	3 (8.8%)	5 (17.3%)	
Stage								
I + II	18 (33.3%)	8 (32.0%)	10 (34.5%)	1.000	19 (30.1%)	10 (29.4%)	9 (31.0%)	1.000
III + IV	32 (59.3%)	15 (60.0%)	17 (58.6%)		40 (63.5%)	22 (64.7%)	18 (62.1%)	
Missing	4 (7.4%)	2 (8.0%)	2 (6.9%)		4 (6.4%)	2 (5.9%)	2 (6.9%)	
T stage								
T1 + T2	5 (9.2%)	2 (8.0%)	3 (10.3%)	1.000	5 (7.9%)	2 (5.9%)	3 (10.3%)	0.6586
T3 + T4	41 (76.0%)	20 (80.0%)	21 (72.4%)		50 (79.4%)	27 (79.4%)	23 (79.4%)	
Missing	8 (14.8%)	3 (12.0%)	5 (17.3%)		8 (12.7%)	5 (14.7%)	3 (10.3%)	
Lymph node invasion								
No	25 (46.3%)	13 (52.0%)	12 (41.4%)	0.5613	31 (49.2%)	18 (52.9%)	13 (44.8%)	0.7880
Yes	22 (40.7%)	9 (36.0%)	13 (44.8%)		25 (39.7%)	13 (38.3%)	12 (41.4%)	
Missing	7 (13.0%)	3 (12.0%)	4 (13.8%)		7 (11.1%)	3 (8.8%)	4 (13.8%)	
Overall survival (24 months)								
Mean (min-max)	13 (0.2-99.0)	7 (0.2-99.0)	15.0 (0.6-82.8)	0.0743	12.5 (0.2-99)	7.7 (0.2-75.7)	15 (0.6-99.0)	0.2409

*calculated with known numbers; # number of patients. Low: expression log2 fold-change < -1; High: expression log2 fold-change > 1.

Supplementary table S3: Association between *p21CIP1* mRNA expression and clinical and socio-demographic parameters.

SOCIO-DEMOGRAPHICDATA	*p21CIP1*			
	TOTAL (n#=75)	*High (n#=26)*	*Low (n#=49)*	*p value**
Age				
Median (min-max)	56.0 (34-83)	55.0 (34-76)	56.0 (40-83)	0.6924
Gender				
Men	61 (81.3%)	22 (84.6%)	39 (79.6%)	0.7590
Women	14 (18.7%)	4 (15.4%)	10 (20.4%)	
Origin				
Southeast	46 (61.3%)	17 (65.4%)	29 (59.2%)	0.2628
South	29 (38.7%)	9 (34.6%)	20 (40.8%)	
Alcohol Consumption				
Never	7 (9.3%)	1 (3.8%)	6 (12.2%)	0.4094
Ever	66 (88.0%)	25 (96.2%)	41 (83.7%)	
Missing	2 (2.7%)	-	2 (4.1%)	
Tobacco Consumption				
Never	8 (10.6%)	3 (11.5%)	5 (10.2%)	1.0000
Ever	65 (86.7%)	23 (88.5%)	42 (85.7%)	
Missing	2 (2.7%)	-	2 (4.1%)	
CLINICAL DATA				
Tumor Location				
Proximal esophagus	5 (6.7%)	3 (11.5%)	2 (4.1%)	
Middle esophagus	33 (44.0%)	11 (42.4%)	22 (44.9%)	0.3243
Distal esophagus	17 (22.6%)	3 (11.5%)	14 (28.6%)	
More than one region affected	15 (20.0%)	5 (19.2%)	10 (20.4%)	
Missing	5 (6.7%)	4 (15.4%)	1 (2.0%)	
Tumor Differentiation				
Well and Moderately	46 (61.3%)	16 (61.5%)	30 (61.2%)	1.000
Poorly and Undifferentiated	19 (25.3%)	7 (27.0%)	12 (24.5%)	
Missing	10 (13.4%)	3 (11.5%)	7 (14.3%)	
Stage				
I + II	20 (26.7%)	7 (27.0%)	13 (26.5%)	1.000
III + IV	50 (66.7%)	16 (88.5%)	34 (69.4%)	
Missing	5 (6.6%)	3 (11.5%)	2 (4.1%)	
T stage				
T1 + T2	6 (8.0%)	3 (11.5%)	3 (6.1%)	0.3858
T3 + T4	61 (81.3%)	19 (73.1%)	42 (85.7%)	
Missing	8 (10.7%)	4 (15.4%)	4 (8.2%)	
Lymph node invasion				
No	34 (45.3%)	11 (42.3%)	23 (41.4%)	0.8000
Yes	33 (44.0%)	12 (36.0%)	21 (46.1%)	
Missing	8 (10.7%)	5 (12.0%)	3 (11.5%)	

*calculated with known numbers; # number of patients. High: highest expression log2 fold-change tertile; low: lower and middle expression log2 fold-change tertiles.