Alcohol metabolizing gene polymorphisms and their relationship with oral cancer risk and clinicopathological features

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ABSTRACT. Oral cancer incidence is higher in individuals between the fifth and seventh decades of life, but some studies indicate a decreasing age trend. From the epidemiological point of view, alcohol consumption is associated with the emergence of oral cancer by interfering with mechanisms of DNA synthesis and repair. From a genetic standpoint, variant alleles in genes encoding the enzymes of alcohol (CYP2E1 and ADH) and acetaldehyde (ALDH2) metabolism may play an important role in the genesis of oral cancer. This study aimed to assess the relation of polymorphisms ADH1B (rs1229984 and rs2066702), ADH1C (rs698), ALDH2 (rs671) and CYP2E1 96bp insertion and the risk of squamous cell carcinoma of the mouth floor, as well as its clinicopathological and prognostic characteristics in relation to alcohol consumption. Our sample group was made of 301 patients, with 159 controls without a previous history of cancer and 142 patients with oral cancer. Genomic DNA was extracted from peripheral blood samples and genotypes were determined by PCR-RFLP. Our results suggest that the presence of ALDH2 Lys504 allele and 96bp insertion CYP2E1 were significantly associated with oral cancer risk. ADH1C gene Ile350 allele was associated with the presence of positive lymph nodes, and lymphatic invasion was related to the presence of polymorphic alleles ADH1B*1, ADH1C Ile350 and ALDH2 Lys504. In conclusion, these results reveal potential markers of oral cancer risk and behavior.

KEY WORDS: ADH, ALDH, polymorphism,

lymph node metastasis, oral cancer.

INTRODUCTION

Alcohol alone can have an indirect role in oral carcinogenesis, working as an oral mucosa solvent, promoting molecular changes in cell membranes, increasing tissue permeability and exposure to environmental carcinogens (Poschl and Seitz, 2004). Acetaldehyde, an alcohol metabolite, can form DNA adducts and directly interfere with mechanisms of DNA synthesis and repair (Brooks and Theruvathu, 2005). Direct or indirect actions of alcohol on DNA increase oral cancer risk according to the length of exposure time, and risk is significantly reduced after cessation of the exposure (Huang et al., 2003; Castellsague et al., 2004). Head and neck cancer (HNC) is an important cause of mortality and morbidity worldwide, presenting approximately 600,000 new cases yearly (Bauman et al., 2012). Considering only tumours of the oral cavity, 389,000 new cases are diagnosed per year, with a mortality rate of 50% (Ferlay et al., 2010). Tumor incidence is higher in individuals aged 50-70 years, however, some studies have suggested that the age of onset has decreased (Hoogsteen et al., 2007).

The epidemiologic literature has extensively documented an association between alcohol consumption and oral cancer, as well as a genetic heritable susceptibility (Han et al., 2010). Approximately 80% of oral cancers are attributed to isolated tobacco consumption or alcohol and tobacco use (Hashibe et al., 2009). The relation between oral cancer and tobacco is explained by the presence of 50 or more carcinogens in tobacco, such as polycyclic aromatic hydrocarbons and nitrosamines that can damage cellular DNA (Chen et al., 2013).

The presence of regional lymph node metastasis is still the most important predictive factor for oral cancer and correlates with a 50% reduction in life expectancy (Ferlay et al., 2010; Zhen et al., 2004). However, micro metastasis cannot be detected by routine histological evaluation (Pentenero et al., 2005). The major alcohol elimination pathway in humans involve the action of ADH oxidative enzymes, leading to the production of acetaldehyde. Approximately 10% of ingested alcohol may be metabolized via alternative routes, such as via

cytochrome P450 2E1 (CYP2E1), considered the second main route, which is activated especially in chronic alcohol consumption (Agarwal, 2001). In a second step, ALDH enzymes (aldehyde dehydrogenase) oxidate acetaldehyde, which is eliminated through respiration, transpiration, and urine (Brennan et al., 2004).

The most common genetic variations are found in ADH1B and ADH1C genes (Chen et al., 2009). Isoenzymes ADH1B differ in the transcription of 2 amino acids due to 2 polymorphisms which generate haplotypes ADH1B*1, ADH1B*2 e ADH1B*3, corresponding to forms (Arg47; Arg369), (His47; Arg369) and (Arg47; Cys369) (Osier et al., 2002). Individuals with the haplotypes ADH1B*2 e ADH1B*3 have greater ability to oxidate alcohol into acetaldehyde, when compared to ADH1B*1 (Marichalar-Mendia et al., 2010). In the ADH1C gene, a polymorphism located in the enzyme activation region causes the exchange of an isoleucine for a valine in amino acid 350, reducing its activity 2.5 times in comparison to the wild-type allele (Ile350) (Ho et al., 2007).

Alcohol oxidation is NAD-dependent and the presence of polymorphisms in the NAD-binding domain can modify the rate of NADH dissociation and therefore alcohol oxidation (Niederhut et al., 2001). Because the same oxidation cascade is used to metabolize retinol (vitamin A), the accumulation of NAD resulting from alcohol oxidation may alter retinol oxidation (by competition) and promote cancer (Chase et al., 2009). Retinoic acid (RA) modulates mucosal T-helper cell responses altering local immunity (Mielke et al., 2013) and cell cycle control by regulation of AP-1 transcription factor (Hsu et al., 2004). Additionally, a polymorphic 96-bp insertion in the promoter region of CYP2E1 reflects in a significant increase in enzyme quantity, augmenting conversion of alcohol in acetaldehyde during chronic alcohol consumption (McCarver et al., 1998).During the second phase of alcohol metabolism, ALDH2 converts acetaldehyde in acetate (Crabb et al., 2004) and the presence of polymorphism G1510A, which promotes the substitution of a glutamate for a lysine in amino acid 504, generates an inactive enzyme, incapable of converting acetaldehyde into acetate (Hashibe et al., 2006). Heterozygote individuals (Glu504/Lys504) present an acetaldehyde blood concentration 6 times greater than wild-type ones (Boccia et al., 2009).

Based on these facts, the present work aimed to evaluate the relation between polymorphisms ADH1B*1/*2/*3 (Arg47Hisrs1229984 e Arg369Cys rs2066702), ADH1C Ile350Val (rs698), ALDH2 Glu504Lys (rs671) and CYP2E1 96-bp insertion (CYP2E1*D) and the risk for squamous cell carcinoma of the lower mouth, as well as its clinicopathological and prognostic features in relation to alcohol consumption status.

MATERIALS AND METHODS

Ethics

This study was approved by Research Ethics Committee of the Heliopolis Hospital (reference number 621/2008) and the School of Medicine, University of São Paulo (reference number 167/10 – 2010), and a written informed consent was obtained from all patients enrolled.

Samples

Our sample group was made of 301 patients attended at the Heliopolis Hospital and the Medical School Hospital, during years 2002-2009. Among these, 159 patients were controls without a previous history of cancer and 142 were patients with carcinomas of the lower mouth, with a minimum follow up of 2 years. Patients with incomplete clinical or epidemiological information or previously treated for their oral cancer were excluded from the present study, as well as chemotherapy treated patients and patients with metastatic disease.

The gender and age data showed a predominance of males (84.7%) and mean age of 55 years (df±11.2). Tobacco use was declared by 85.7% of individuals and 14.3% were never-smokers. As for alcohol drinking, 46.4% reported current alcohol consumption, 25.2% have stopped drinking for more than a year and 13.6% were never-drinkers (Table 1).

Table 1. Epidemiology analysis of alcohol metabolizing gene polymorphisms and oral squamous cell carcinoma risk.

					General			
Characteristics	Case	Control	p		Multivariate Analysis			
	No.	(%)	No.	(%)		OR (CI 95%)¥	p¥	
Gender								
Female	26	18.3	20	12.6	0.168		1	
Male	116	81.7	139	87.4		0.31 (0.14-0.72)		0.006
Age, years								
≤ 55	70	49.3	88	55.3	0.294	–	–	
> 55	72	50.7	71	44.7		–	–	
Smoking								
No	10	7	33	20.8	0.001		1	
Yes	132	93	126	79.2		4.02 (1.69-9.59)		0.002
Alcohol Consumption								
Never	20	14.1	33	20.8	< 0.001		1	
In the past	31	21.8	60	37.7		1.01 (0.43-2.38)		0.976
At present	91	64.1	66	41.5		2.69 (1.20-6.05)		0.017
ADH1B haplotype								
***1/*1**	120	84.5	131	82.4	0.622	–	–	
Variant	22	15.5	28	17.6		–	–	
ADH1C Ile350Val								
Ile/Ile	99	69.7	97	61	0.113		1	
ILe/Val+Val/Val	43	30.3	62	39		0.68 (0.40-1.14)		0.145
ALDH2 Glu504Lys								
Glu/Glu	110	77.5	102	64.2	0.012		1	
Glu/Lys+Lys/Lys	32	22.5	57	35.8		0.48 (0.28-0.84)		0.01
CYP2E196-bp ins								
Wild type	123	86.6	117	73.6	0.005		1	
Insertion	19	13.4	42	26.4		0.40 (0.21-0.77)		0.006

p – Significance value; OR – Odds ratio; CI – Confidence interval.

¥ Values adjusted by multivariate logistic regression.

Individuals with alcohol drinking habit have done so for an average 32.6 years (df±11.0). Patients that quit drinking have been alcohol consumers for 26.7 years (df ±13.3) and have stopped 12.7 years ago (df±10.2) on average. As for the amount of alcohol consumed, 26.8% of individuals reported drinking up to 30g/day, 29.9% drank 31-100g/day and 43.3% consumed over 100g/day. In addition, 23.1% of past drinkers consumed up to 30g/day, 15.4% drank 31-100g/day and 61.5% drank over 100g/day (Table 2).

Table 2. Epidemiology analysis and alcohol metabolizing gene polymorphisms and their relation with oral squamous cell carcinoma according to drinking status.

	Alcohol Consumers							Past Alcohol Consumers						
Characteristics	**Case**	**Control**	***p***	**Multivariate Analsysis**				**Case**	**Control**	***p***	**Multivariate Analysis**			
	No.	**(%)**	**No.**	**(%)**		**OR (CI 95%)¥**	***p*¥**		**No.**		**No.**	**(%)**		**OR (CI 95%)¥**	***p*¥**
Gender															
Female	7	7.7	5	7.6	0.978	—	—	4	12.9	4	6.7	0.266	—	—	
Male	84	92.3	61	92.4		—	—	27	87.1	56	93.3		—	—	
Age, years															
≤ 55	50	54.9	49	74.2	0.013	1		14	45.2	23	38.3	0.53	—	—	
> 55	41	45.1	17	25.8		2.06 (0.88-4.87)	0.097	17	54.8	37	61.7		—	—	
Smoking															
No	3	3.3	7	10.6	0.065	1		0	0	11	18.3	0.007	1		
Yes	88	96.7	59	89.4		2.47 (0.47-13.04)	0.286	31	100	49	81.7		undefined		
Duration of drinking habit, years															
≤ 10	2	2.2	2	3	0.065	1	—	4	12.9	8	13.3	0.504	—	—	
Nov-30	28	30.8	32	48.5		0.31 (0.03-3.08)	0.315	14	45.2	34	56.7		—	—	
> 30	61	67	32	48.5		0.39 (0.04-3.98)	0.428	13	41.9	18	30		—	—	
Abstinence from drinking, years															
≤ 5	—	—	—	—		—	—	10	32.3	16	26.7	0.935	—	—	
6 – 10	—	—	—	—		—	—	7	22.6	14	23.3		—	—	
11 – 20	—	—	—	—		—	—	10	32.3	20	33.3		—	—	
> 20	—	—	—	—		—	—	4	12.9	10	16.7		—	—	
Amount consumed, g/Day															
≤ 30	16	17.6	26	39.4	0.01	1		6	19.4	15	25	0.139	1		
31 – 100	31	34.1	16	24.2		3.60 (1.32-9.86)	0.013	2	6.5	12	20		0.38 (0.06-2.40)	0.305	
> 100	44	48.4	24	36.4		2.71 (1.07-6.87)	0.036	23	74.2	33	55		1.53 (0.48-4.85)	0.473	
ADH1B haplotype															
***1/*1**	75	82.4	59	89.4	0.227	—	—	28	90.3	46	76.7	0.093	1		
Variant	16	17.6	7	10.6		—	—	3	9.7	14	23.3		0.34 (0.09-1.34)	0.123	
ADH1C Ile350Val															
Ile/Ile	66	72.5	41	62.1	0.167	1		20	64.5	38	63.3	0.911	—	—	
ILe/Val+Val/Val	25	27.5	25	37.9		0.49 (0.22-1.06)	0.071	11	35.5	22	36.7		—	—	
ALDH2 Glu504Lys															
Glu/GluG	69	75.8	40	60.6	0.041	1		23	74.2	38	63.3	0.296	—	—	
Glu/Lys+Lys/Lys	22	24.2	26	39.4		0.44 (0.20-0.95)	0.037	8	25.8	22	36.7		—	—	

CYP2E196-bp ins														
Wild type	79	86.8	47	71.2	0.015	1		26	83.9	45	75	0.33	–	–
Insertion	12	13.2	19	28.8		0.35 (0.14-0.87)	0.024	5	16.1	15	25		–	–

Genotyping

Genomic DNA of 301 patients was extracted from peripheral blood samples as previously described (Miller et al., 1988). Genotypes were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). PCR conditions were: a 25□L reaction mixture containing 100ng of genomic DNA, 10 mMTris-HCl (pH 8.3), 50 mMKCl, 200 □M of each deoxyribonucleoside 5´ triphosphates, 1.5 mM de MgCl2, 1 U Taq DNA polymerase (Life Technologies, Inc®, Rockville, MD, USA) and 25 pmol of each primer. PCR initiated with a melting step of 5 minutes at 94□C, followed by 35 cycles of 1 minute at 94□C, 1 minute at 58°C and 1 minute at 72ºC. PCR products were digested with enzymes specific for each polymorphism, following manufacturer's recommendations. Digested products were separated and visualized on 3% agarose gels, stained with ethidium bromide. ADH1B haplotype for polymorphisms Arg47His and Arg369Cys were considered *1 for the wild-type allele (Arg47 + Arg369) and *2 and *3 for variants His47 and Cys369, respectively. PCR primers used to amplify ADH1B Arg47His (rs1229984) polymorphism were: 5' GGG ATT AGT AGC AAA ACC CTC A 3' and 5' TCA CCC CTT CTC CAA CAC TC 3', yielding a 243bp product, which as digested with RseI (Fermentas Life sciences®) enzyme and genotyped accordingly: Arg/Arg (243bp), Arg/His (243, 145 and 98bp) or His/His (145 and 98bp). For polymorphism ADH1B Arg369Cys (rs2066702) primers were: 5' ACC

TGT ATG GGG ATG GA 3' and 5' ATT GCC TCA AAA CGT CAG GA 3', yielding a product of 173bp, which was digested with AlwNI (Fermentas Life sciences®) enzyme and genotyped as follows: Arg/Arg (173bp), Arg/Cys (173, 145 and 28bp) or Cys/Cys (145 and 28bp). Primers for polymorphism ADH1C Ile350Val (rs698) were: 5´ TTG TTT ATC TGT GAT TTT TTT TGT 3' and 5´ CGT TAC TGT AGA ATA CAA AGC 3', generating a product of 378bp, which was digested with SspI (Fermentas Life sciences®) enzyme and genotype as follows: Ile/Ile (274 and 104bp), Ile/Val (378, 274 and 104bp) or Val/Val (378bp). Polymorphism ALDH2 Glu504Lys (rs671) was amplified with primers: 5' AAC CCA TAA CCC CCA AGA GT 3' and 5' CAG GTC CCA CAC TCA CAG TTT 3', generating 180bp product, which was digested with TspRI (Fermentas Life sciences®) enzyme and genotyped accordingly: Glu/Glu (155 and 25bp), Glu/Lys (180, 155 and 25bp) or Lys/Lys (180bp). For the 96bp insertion in CYP2E1 gene, we used primers 5' GTG ATG GAA GCC TGA AGA ACA 3' and 5' CTT TGG TGG GGT GAG AAC AG 3', resulting in a product of 633bp in wild-type alleles and 729bp in alleles with the insertion.

Statistical analysis

Genotypic frequencies were tested for Hardy-Weinberg Equilibrium (HWE) in the control population. The chi-square test was used for association analysis and confirmation was obtained by the Lilliefors test (statistical significance was set at $p<0.05$). Variables with significance levels $p<0.20$ were used in multivariate logistic regression models to obtain adjusted odds ratio (OR) and confidence intervals (CI 95%). Statistical calculations were performed using the Epi Info® v3.4.3 software.

RESULTS

Oral cancer risk

Observed genotypic frequencies for all polymorphisms were in Hardy-Weinberg equilibrium in controls ($p>0.05$). Case-control analysis showed that alcohol addiction ($p<0.001$) and polymorphisms ALDH2 Glu504Lys ($p=0.012$) and CYP2E1 96-bp ins ($p=0.005$) were significantly related with oral cancer risk. Multivariate analysis showed a 2.5 higher risk of developing oral cancer in alcohol consumers, when compared with individuals that never drank (OR=2.69, CI=1.20-6.05), and that the presence of alleles ALDH2 Lys504 and the 96-bp insertion in gene CYP2E1 reduced oral cancer risk by a half when compared to wild-type alleles (OR=0.48, CI=0.28-0.84, and OR=0.40, CI=0.21-0.77, respectively; Table 1).

The amount of alcohol consumed was related with cancer risk ($p=0.010$), but the length of time in years showed no relation with cancer risk ($p=0.065$). Multivariate analysis showed that individuals that consumed 31-100g/day or >100g/day had a risk of developing oral cancer 3 times higher than the ones that used less than 30g/day (OR=3.60, CI=1.32-9.86, and OR=2.71, CI=1.07-6.87, respectively). Presence of

polymorphisms ALDH2 Glu504Lys and CYP2E1 96-bp ins presented significant differences among cases and controls (p=0.041 and p=0.015, respectively), and risk analysis showed that presence of allele ALDH2Lys504 and CYP2E1 gene 96-bp insertion decreased risk in a half (OR=0.44, CI=0.20-0.95, and OR=0.35, CI=0.14-0.87, respectively). When we evaluated risk in past drinkers, no significant relation was found (Table 2).

Tumor size (T) and lymph node metastasis (N)

Polymorphisms in alcohol metabolizing genes did not present a significant relation with tumor size, neither for alcohol consumers or not consumers (Supplementary Table 1). Lymph node metastasis was more frequent in alcohol users (p=0.010), showing a 4.5 higher risk in the multivariate analysis, when compared with never-drinkers (OR=4.62, CI=1.12-19.10). However, no polymorphism was related with lymph node metastasis (Table 3).

Table 3. Analysis of clinicopathological tumor features, drinking status and alcohol metabolizing gene polymorphisms and their relationship with metastatic cervical lymph node status.

	Lymph node Status (N)[§]						
Characteristics	**Negative**		**Positive**			**Multivariate Analysis**	
					p		
	No.	**(%)**	**No.**	**(%)**		**OR (CI 95%)[¥]**	***p*[¥]**
Tumor Size (T)[§]							
pT1, pT2	30	45.5	18	23.7	< 0.001	1	
pT3	18	27.3	8	10.5		0.58 (0.17-1.96)	0.378
pT4	18	27.3	50	65.8		5.58 (2.04-15.30)	< 0.001
Differentiation Grade							
Well differentiated	32	48.5	25	32.9	0.140	1	
Moderately differentiated	30	45.5	47	61.8		1.51 (0.62-3.64)	0.361
Poorley differentiated	4	6.1	4	5.3		0.89 (0.14-5.49)	0.901
Lymphatic invasion							
Absent	46	70.8	20	27.4	< 0.001	1	
Present	19	29.2	53	72.6		4.85 (2.06-11.42)	< 0.001
Drinking status							
Never	14	21.2	6	7.9	0.010	1	
Past drinker	18	27.3	13	17.1		1.71 (0.36-8.07)	0.497
Current drinker	34	51.5	57	75.0		4.62 (1.12-19.10)	0.034
ADH1B haplotype							
*1/*1	58	87.9	62	81.6	0.301	–	–
Variante	8	12.1	14	18.4		–	–
ADH1C Ile350Val							
Ile/Ile	41	62.1	58	76.3	0.066	1	
Ile/Val+Val/Val	25	37.9	18	23.7		0.43 (0.16-1.16)	0.095

ALDH2 Glu504Lys								
Glu/Glu	51	77.3	59	77.6	0.984	–	–	
Glu/Lys	11	16.7	12	15.8		–	–	
Lys/Lys	4	6.1	5	6.6		–	–	
CYP2E1 96-bp ins								
Wild type	56	84.8	67	88.2	0.563	–	–	
Insertion	10	15.2	9	11.8		–	–	

p – Significance value; OR – Odds ratio; CI – Confidence interval.

[¥] Values adjusted by multivariate logistic regression. [§] TNM classification 7th edition (Sobin et al., 2009).

When only alcohol consumers were evaluated, none of the addiction characteristics showed a relation with lymph node metastasis. However, the homozygous genotype ADH1C Ile350 was more frequent in node-positive cases (p=0.024). Multivariate analysis showed patients with homozygous ADH1C Ile350 genotype had 4 times higher metastasis risk when compared with individuals that presented at least one allele ADH1C Val350 (OR=0.24, CI=0.07-0.83). Past alcohol consumers did not show significant relations with node metastasis or alcohol metabolizing gene polymorphisms (Table 4).

Table 4. Analysis of clinicopathological tumor characteristics and alcohol metabolizing gene polymorphisms and their relation with metastatic cervical lymph node status according to drinking profile.

Characteristics	**Lymph node status (N)[§]**													
	Current alcohol consumers							**Past alcohol consumers**						
	Negative		**Positive**		***p***	**Multivariate Analysis**		**Negative**		**Positive**		***p***	**Multivariate Analysis**	
	Nº.	**(%)**	**Nº.**	**(%)**		**OR (CI 95%)[¥]**	***p*[¥]**	**Nº.**	**(%)**	**Nº.**	**(%)**		**OR (CI 95%)[¥]**	***p*[¥]**
Tumor Size (T)[§]														
pT1, pT2	11	32.4	12	21.1	0.003	1		10	55.6	4	30.8	0.007	1	
pT3	13	38.2	8	14		0.53 (0.13-2.08)	0.363	3	16.7	0	0		undefined	0.962
pT4	10	29.4	37	64.9		4.34 (1.15-16.35)	0.03	2	11.1	9	69.2		7.46 (1.13-49.44)	0.037
Differentiation Grade														
Well differentiated	17	50	18	31.6	0.195	1		8	44.4	5	38.5	0.616	–	–
Moderately differentiated	15	44.1	36	63.2		1.78 (0.63-5.04)	0.28	9	50	8	61.5		–	–
Poorly differentiated	2	5.9	3	5.3		1.05 (0.09-12.52)	0.97	1	5.6	0	0		–	–
Lymphatic invasion														
Absent	23	67.6	16	28.6	< 0.001	1		12	66.7	4	33.3	0.078	1	
Present	11	32.4	40	71.4		3.47 (1.25-9.64)	0.017	6	33.3	8	66.7		5.18 (0.80-33.61)	0.085
Duration of drinking habit, years														
≤ 10	0	0	2	3.5	0.538	–	–	2	11.1	2	15.4	0.564	–	–
#######	11	32.4	17	29.8		–	–	7	38.9	7	53.8		–	–
> 30	23	67.6	38	66.7		–	–	9	50	4	30.8		–	–
Abstinence from drinking, years														
≤ 5	–	–	–	–		–	–	5	27.8	5	38.5	0.575	–	–
6 – 10	–	–	–	–		–	–	3	16.7	4	30.8		–	–
11 – 20	–	–	–	–		–	–	7	38.9	3	23.1		–	–
> 20	–	–	–	–		–	–	3	16.7	1	7.7		–	–
Amount consumed, g/day														
≤ 30	6	17.6	10	17.5	0.787	–	–	4	22.2	2	15.4	0.879	–	–
31 - 100	13	38.2	18	31.6		–	–	1	5.6	1	7.7		–	–

> 100	15	44.1	29	50.9		–	–	13	72.2	10	76.9		–	–
ADH1B haplotype														
***1/*1**	28	82.4	47	82.5	0.99	–	–	17	94.4	11	84.6	0.376	–	–
Variant	6	17.6	10	17.5		–	–	1	5.6	2	15.4		–	–
ADH1C Ile350Val														
Ile/Ile	20	58.8	46	80.7	0.024	1		12	66.7	8	61.5	0.768	–	–
Ile/Val+Val/Val	14	41.2	11	19.3		0.24 (0.07-0.83)	0.024	6	33.3	5	38.5		–	–
ALDH2 Glu504Lys														
Glu/Glu	26	76.5	43	75.4	0.716	–	–	12	66.7	11	84.6	0.376	–	–
Glu/Lys	5	14.7	11	19.3		–	–	5	27.8	1	7.7		–	–
Lys/Lys	3	8.8	3	5.3		–	–	1	5.6	1	7.7		–	–
CYP2E1 96-bp ins														
Wild type	28	82.4	51	89.5	0.331	–	–	15	83.3	11	84.6	0.659	–	–
Insertion	6	17.6	6	10.5		–	–	3	16.7	2	15.4		–	–

Differentiation grade and lymphatic invasion

Alcohol addiction and polymorphisms in alcohol metabolizing genes were not statistically related with differentiation grade, not even when patients were stratified in current or past alcohol consumers (Supplementary Table 2). Nonetheless, lymphatic invasion frequency was significantly higher in homozygous ADH1B*1 (p=0.037), ADH1C Ile350 (p=0.018) and ALDH2 Lys504 (p=0.050). However, multivariate analysis did not confirm the relation of polymorphism ALDH2 Glu504Lys with lymphatic invasion, but confirmed that homozygous ADH1B*1 and ADH1C Ile350 are independent factors for lymphatic invasion, promoting a 3.5 higher risk when compared with other genotypes (OR=0.27, CI=0.09-0.81, and OR=0.28, CI=0.12-0.67, respectively; Table 5).

Table 5. Analysis of clinicopathological tumor characteristics and alcohol metabolizing gene polymorphisms and their relationship with lymphatic vessel invasion.

	Lymphatic invasion						
Characteristics	**Absent**		**Present**			**Multivariate Analysis**	
					p		
	No.	**(%)**	**No.**	**(%)**		**OR (CI 95%)¥**	**$_p$¥**
Tumor Size (T)§							
pT1, pT2	26	39.4	18	25.0	0.037	1	
pT3	15	22.7	11	15.3		1.15 (0.38-3.46)	0.800
pT4	25	37.9	43	59.7		4.23 (1.70-10.49)	0.002
Differentiation grade							
Well differentiated	33	50.0	23	31.9	0.082	1	
Moderately differentiated	29	43.9	45	62.5		2.05 (0.93-4.51)	0.075
Poorly differentiated	4	6.1	4	5.6		1.39 (0.24-8.17)	0.716
Drinking status							
Never	11	16.7	7	9.7	0.306	–	–
Past drinker	16	24.2	14	19.4		–	–
Current drinker	39	59.1	51	70.8		–	–
ADH1B haplotype							
*1/*1	51	77.3	65	90.3	0.037	1	
Variante	15	22.7	7	9.7		0.27 (0.09-0.81)	0.019
ADH1C Ile350Val							
Ile/Ile	39	59.1	56	77.8	0.018	1	

Ile/Val+Val/Val	27	40.9	16	22.2		0.28 (0.12-0.67)	0.004
ALDH2 Glu504Lys							
Glu/Glu	52	78.8	55	76.4	0.050	1	
Glu/Lys	13	19.7	9	12.5		0.63 (0.22-1.81)	0.387
Lys/Lys	1	1.5	8	11.1		8.44 (0.95-74.88)	0.056
CYP2E196-bp ins							
Wild type	55	83.3	64	88.9	0.344	–	–
Insertion	11	16.7	8	11.1		–	–

p – Significance value; OR – Odds ratio; CI – Confidence interval

¥ Values adjusted by multivariate logistic regression. § TNM classification 7th edition (Sobin et al., 2009).

Among current alcohol consumers, daily consumption (p=0.427) or duration of addiction (p=0.936) was not related to lymphatic invasion. However, a higher frequency of lymphatic invasion was observed in homozygous ADH1B*1 (p=0.024) and ADH1C Ile350 (p=0.048). Multivariate analysis showed 5 times increased risk of lymphatic invasion for homozygous ADH1B*1 (OR=0.19, CI=0.05-0.67), whereas homozygous ADH1C Ile350 increased risk 4 times (OR=0.24, CI=0.08-0.71) when compared with other genotypes. Past drinkers showed no significant relation with lymphatic invasion (Table 6).

Table 6. Analysis of clinicopathological tumor characteristics and alcohol metabolizing gene polymorphisms and their relation with lymphatic vessel invasion, according to drinking profile.

Characteristics	Lymphatic invasion											
	Current drinkers							Past drinkers				
	Absent		Present			Multivariate Analysis		Absent		Present			Multivariate Analysis	
	No.	(%)	No.	(%)	*p*	OR (CI 95%)¥	*p*¥	No.	(%)	No.	(%)	*p*	OR (CI 95%)¥	*p*¥
Tumor Size (T)§														
pT1, pT2	12	30.8	10	19.6	0.176	1		7	43.8	6	42.9	0.870	–	–
pT3	11	28.2	10	19.6		0.92 (0.25-3.34)	0.897	2	12.5	1	7.1		–	–
pT4	16	41.0	31	60.8		3.16 (1.00-9.98)	0.050	7	43.8	7	50.0		–	–
Differentiation Grade														
Well differentiated	18	46.2	16	31.4	0.356	–	–	8	50.0	5	35.7	0.403	–	–
Moderately differentiated	19	48.7	32	62.7		–	–	7	43.8	9	64.3		–	–
Poorly differentiated	2	5.1	3	5.9		–	–	1	6.3	0	0.0		–	–
Duration of drinking habit, years														
≤ 10	1	2.6	1	2.0	0.936	–	–	2	12.5	2	14.3	0.727	–	–
11 - 30	11	28.2	16	31.4		–	–	6	37.5	7	50.0		–	–
> 30	27	69.2	34	66.7		–	–	8	50.0	5	35.7		–	–
Abstinence from drinking, years														
≤ 5	–	–	–	–		–	–	4	25.0	5	35.7	0.275	–	–

6 – 10	–	–	–	–	–	–	2	12.5	5	35.7	–	–
11 – 20	–	–	–	–	–	–	7	43.8	3	21.4	–	–
> 20	–	–	–	–	–	–	3	18.8	1	7.1	–	–
Amount consumed, g/Day												
≤ 30	8	20.5	8	15.7	0.427	–	–	4	25.0	2	14.3	0.765	–	–
31 – 100	15	38.5	15	29.4		–	–	1	6.3	1	7.1		–	–
> 100	16	41.0	28	54.9		–	–	11	68.8	11	78.6		–	–
ADH1B haplotype													
*1/*1	28	71.8	46	90.2	0.024	1		13	81.3	14	100.0	0.138	1	
Variante	11	28.2	5	9.8		0.19 (0.05-0.67)	0.010	3	18.8	0	0.0		undefined	
ADH1C Ile350Val													
Ile/Ile	24	61.5	41	80.4	0.048	1		9	56.3	10	71.4	0.317	–	–
Ile/Val+Val/Val	15	38.5	10	19.6		0.24 (0.08-0.71)	0.010	7	43.8	4	28.6		–	–
ALDH2 Glu504Lys													
Glu/Glu	30	76.9	38	74.5	0.360	–	–	11	68.8	11	78.6	0.103	1	
Glu/Lys	8	20.5	8	15.7		–	–	5	31.3	1	7.1		0.20 (0.02-2.00)	0.171
Lys/Lys	1	2.6	5	9.8		–	–	0	0.0	2	14.3		undefined	
CYP2E196-bp ins													
Wild type	32	82.1	46	90.2	0.260	–	–	13	81.3	12	85.7	0.567	–	–
Insertion	7	17.9	5	9.8		–	–	3	18.8	2	14.3		–	–

p – Significance value; OR – Odds ratio; CI – Confidence interval. ¥ Values adjusted by multivariate logistic regression. TNM classification 7th edition (Sobin et al., 2009).

DISCUSSION AND CONCLUSIONS

We have identified that alcohol addiction increases approximately 2.5 times the risk of developing oral cancer when compared to non-addicted individuals. Consuming over 30g/day of alcohol increases approximately 3 times the risk of oral cancer when compared with a smaller consumption. Our data are in agreement with the literature, pointing towards the amount of consumed alcohol as an important risk factor for several types of cancer (Poschl and Seitz, 2004; Everatt et al., 2012), including oral cancer (Hashibe et al., 2009; Goldstein et al., 2010). Also in concordance with the literature is the fact that addiction time duration showed no relation with cancer risk (Hashibe et al., 2010). Our findings support the hypothesis of acetaldehyde promoted carcinogenesis, increased membrane permeability, and facilitated carcinogen cell entrance, as well as lowered immune surveillance in alcohol consumers.

Our results suggest that alcohol users with polymorphic ALDH2 and CYP2E1 present a smaller risk when compared with wild-type allele bearers. These results are in agreement with other studies that investigated the presence of polymorphism ALDH2 Glu504Lys with oesophageal cancer (Gu et al., 2012) and head and neck cancer risk (Yuan et al., 2013). In contrast, some studies present contradicting results, showing that allele Lys504 increases risk of oesophageal (Wu et al., 2013), stomach (Matsuo et al., 2013) and colorectal cancer (Yang et al., 2009), based on the hypothesis that these individuals accumulate acetaldehyde, increasing cancer risk. In comparison, other studies suggest that polymorphic ALDH2 allele has a protective role in excessive alcohol consumption (Chen et al., 2013) that was not observed in our study.

Polymorphic CYP2E1 decreased cancer risk in alcohol consumers, which was previously observed in the literature for colorectal cancer (Khrunin et al., 2011; Sameer et al., 2011). Therefore, we can propose that some polymorphisms may alter the mechanisms of carcinogenesis promoted by alcohol and acetaldehyde, as no increased risk is observed in past drinkers or non-addicts. We did not find an association between ADH1C

gene polymorphism and oral cancer. Other studies have found an association between this polymorphism and hepatic cirrhosis or pancreatitis, but not with cancer (Seitz et al., 2010).

There is no consensus about the role polymorphisms in genes ADH, CYP2E1 and ALDH2 in relation to oral cancer risk, however, a recent study with Brazilian patients with head and neck cancer identified polymorphic ADH1C as a putative molecular marker (Garcia et al., 2010).

When clinicopathological characteristics were evaluated, alcohol consumption and gene polymorphisms were not related to tumor size or differentiation degree. In contrast, alcohol consumption was significantly related to lymph node metastasis, suggesting an increased risk of 4.5 times, when compared with non-drinkers. Addiction time and daily doses seem not to affect the mechanism of node metastasis, but the presence of homozygous ADH1C Ile350 allele showed a 4-fold increased chance of node metastasis when compared with bearers of allele Val350. In addition, homozygous genotypes ADH1B*1 and ADH1C Ile350 showed a 4-fold and higher risk of lymphatic invasion, when compared with non-homozygous drinkers. Alcohol and acetaldehyde accumulation due to polymorphisms ADH1C Ile350 and ADH1B*1 may alter carcinogen absorption (Niederhut et al., 2001).

Several polymorphisms in oxidative stress genes may interfere with ethanol oxidation, altering cancer risk and behavior. However, this process is not well understood, and unknown genetic or epigenetic mechanisms may result in diverse oral cancer risk and behavior.

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