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Protein expression of CYP1A1, CYP1B1, ALDH1A1, and ALDH2 in young patients with oral squamous cell carcinoma

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Abstract. The purpose of this study was to evaluate the expression of the enzymes involved in the biotransformation of tobacco and alcohol. A study group of 41 young patients (≤40 years old) with oral squamous cell carcinoma (OSCC) was compared to 59 control subjects (≥50 years old) with tumours of similar clinical stages and topographies. The immunohistochemical expression of CYP1A1, CYP1B1, ALDH1A1, and ALDH2 was evaluated using the tissue microarray technique. There was a predominance of males, smokers, and alcohol drinkers in both groups. Most tumours were located in the tongue (43.9% vs. 50.8%), were well-differentiated (63.4% vs. 56.6%), and were in clinical stages III or IV (80.5% vs. 78.0%). No difference was observed in the expression of CYP1A1, ALDH1A1, or ALDH2 between the two groups. CYP1A1 and ALDH2 protein expression had no influence on the prognosis. The immunoexpression of CYP1B1 was significantly higher in the control group than in the young group ($P < 0.001$). The 5-year relapse-free survival was better in patients with CYP1B1 overexpression vs. protein underexpression (64% vs. 25%; $P < 0.05$), regardless of age. ALDH1A1 expression improved relapse-free survival in young patients. These results suggest a lower risk of recurrence with increased metabolism of carcinogens by CYP1B1. Further studies involving other genes and proteins are necessary to complement the results of this research.

Key words: oral cancer; squamous cell carcinoma; aldehyde dehydrogenase; cytochrome P450; CYP; ALDH; young age.

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Oral squamous cell carcinoma (OSCC) mainly affects men between the fifth and sixth decades of life and is uncommon in young patients (age ≤40 years).[1] However, the incidence in this young age group has increased in several countries over the last two decades.[2,3] In older patients, the dominant risk factors for OSCC are tobacco and alcohol abuse, which are strongly synergistic.[1] Nevertheless, there remains doubt regarding the role of these factors in young people due to the short time of exposure.[4,5]

Cigarettes contain potentially carcinogenic substances such as heterocyclic amines, *N*-nitrosamines, and polycyclic aromatic hydrocarbons (PAH).[6] The metabolic activation of PAH into epoxide intermediates is catalyzed by the P450 cytochrome (CYP) family.[7] CYPs and microsomal epoxide hydrolases act together in the metabolic activation of benzo[a]-pyrene (BaP), a typical promutagenic PAH found in cigarette smoke.[8] BaP generates a final product that can degrade different molecules, including DNA.[9,10] CYP1A1 and CYP1B1 are the two main enzymes involved in the activation of carcinogenic PAH into electrophilic reagents that trigger the transformation of cells.[10,11] CYP1A1 is considered a classical isoform in the activation of PAH; however, recent studies have indicated that the expression of CYP1B1 plays a more important role in carcinogenesis through PAH metabolism than CYP1A1.[11,12]

The penetration of carcinogens through the mucosa is facilitated by the ingestion of ethanol, which serves as a solvent. Furthermore, ethanol induces CYP2E1, an enzyme that generates reactive oxygen species (ROS).[13] The alcohol is metabolized by alcohol dehydrogenase, which oxidizes ethanol to acetaldehyde, a highly reactive compound that can form DNA adducts.[13] The oxidation of acetaldehyde to acetate is catalyzed by aldehyde dehydrogenases (ALDH), especially ALDH1A1 and ALDH2.[13,14] A reduction in the activity of these enzymes in tissues leads to elevated levels of acetaldehyde, increasing the risk of developing head and neck cancer.[14]

There appear to be no studies focusing on the protein expression of tobacco- and alcohol-metabolizing enzymes in young patients with oral cancer in the English language literature. If differences exist between the young group and subjects older than 50 years of age, treatment strategies may be individualized. Therefore, the aim of the present study was to evaluate the expression of enzymes involved in the biotransformation of tobacco and alcohol in patients aged $\leq$40 years (young group) and to compare the results with those obtained from patients aged $\geq$50 years (control group). The hypothesis was that the expression of these proteins would be similar in the young and control groups.

Materials and methods

A retrospective study was conducted on 100 patients with OSCC treated from 1970 to 2004 at the department of head and neck surgery and otorhinolaryngology of AC Camargo Cancer Center in São Paulo, Brazil. Demographic (age, sex, and race), lifestyle (smoking habit and alcohol consumption), clinical (tumour site and clinical stage), treatment, and pathological (histological grade) factors were analyzed. The clinical characteristics of the patients were obtained from their medical records. The tumours were restaged according to the 2002 version of the American Joint Committee on Cancer (TNM) classification and classified as early clinical stage (clinical stage I–II) or advanced clinical stage (clinical stage III–IV).[15] The cases studied were followed up after treatment. All cases with loco-regional recurrence were confirmed microscopically. The histopathological diagnoses were reviewed and the histological grade was determined on the basis of the World Health Organization classification as well-differentiated (grade I), moderately differentiated (grade II), or poorly differentiated (grade III).[1] Patients with squamous cell carcinoma (SCC) of the lip and oropharynx and those who had received any previous treatment were excluded from the study. The number of cases was determined by the number of young patients diagnosed and treated in this service. The control group was matched to the young group by tumour topography and clinical stage. Only cases with complete clinical information and paraffin blocks available were included in the study.

The study was approved by the necessary human research ethics committee.

Immunohistochemistry

Construction of the tissue microarray has been described previously by Kaminagakura et al.[5] The sections were pre-heated for 12 h at 60 °C, deparaffinized in xylene, and hydrated in decreasing alcohol solutions. For each antibody, the slides were subjected to antigen retrieval with citrate buffer, pH 6.0, in a Pascal pressure cooker (DakoCytomation, Dako, Carpinteria, CA, USA). Endogenous peroxidase was blocked by incubating the slides in a solution of 3% hydrogen peroxide (Merck, Brazil). The slides were then incubated with the primary antibodies (Table 1) for 18 h at 4 °C. All antibodies were diluted in phosphate-buffered saline containing 1% bovine serum albumin (Sigma–Aldrich, Saint Louis, MO, USA) and 0.1% sodium azide. The reactions were detected using the streptavidin–biotin–peroxidase system in accordance with the manufacturer's specifications (LSAB, DakoCytomation). Diaminobenzidine (DAB) was used as the chromogenic substrate (DakoCytomation). The primary antibody was omitted from the reactions in the negative control. All experiments were performed in duplicate.

Statistical analysis

The sections on the slides were scanned with the ScanScope GL System (Leica Biosystems, Nussloch, Germany). The scanned images were analyzed with Aperio ImageScope Viewer software using the Positive Pixel Count algorithm (version 9). Strong and medium-brown pixels were considered as positive staining, while weak-brown pixels and negative pixels were considered as negative staining. Only epithelial malignant neoplastic cells were counted. The percentage of positive staining, expressed as the ratio between positive and total (positive + negative) staining, was considered for statistical analysis.

The χ^2 test was used to verify the homogeneity of the groups in terms of clinical and pathological variables. The cytoplasmic expression of CYP1A1, CYP1B1, ALDH1A1, and ALDH2 proteins was compared between the groups using the non-parametric Mann–Whitney test. For the survival analysis, protein expression was dichotomized according to the median. The follow-up time was defined as the interval between the beginning of treatment and the date of death or the last information for censored observations. The disease-free interval was measured from the date of treatment to the date when the first recurrence was diagnosed. Disease-free survival and overall survival probabilities were estimated by Kaplan–Meier method and the log-rank test was applied to assess the significance of differences among actuarial survival curves. The statistical analysis was

Table 1. List of primary antibodies.

Antibody[a]	Clone	Dilution	Positive control
CYP1A1	6G5	1:75	Ovarian carcinoma
CYP1B1	Polyclonal	1:600	Brain
ALDH1A1	EP1933Y	1:400	Liver
ALDH2	EPR4493	1:200	Colon

[a] *Source*: Abcam, Cambridge, MA, USA.

performed using R version 2.13 (R Development Core Team (2010), Vienna, Austria; www.R-project.org).

Results

One hundred specimens were available for histopathological and immunohistochemical analysis: 41 for the young group and 59 for the control group. The mean age of the young patients was 34.7 (range 19–40) years and of control patients was 62.6 (range 50–90) years. The male to female ratio was 1:1.9 for the young patients and 1:3.9 for the control patients. In both groups, the tongue was the most frequently involved site (43.9% vs. 50.8%), followed by the floor of the mouth (29.3% vs. 22.0%). Thirty-one of the young patients had tobacco exposure (75.6%), while 45 of those in the control group were smokers (76.3%) ($P = 0.63$). Alcohol consumption was similar in the two groups ($P = 0.96$). An advanced clinical stage was observed in 33 patients in the young group (80.5%) and 46 patients in the control group (78.0%) ($P = 0.76$). There was no statistically significant difference between the young and the control groups regarding the treatment approach ($P = 0.15$). The demographic and clinical data are summarized in Table 2.

Table 2. Demographic and clinical characteristics of 41 young patients (≤40 years old) and their matched controls (≥50 years old).

Characteristics	Young (≤40 years), *n* (%)	Control (≥50 years), *n* (%)	*P*-value
	41 (100)	59 (100)	
Age, years			
Mean	34.7	62.6	
Range	19–40	50–90	
Sex			0.12
Male	27 (65.9)	47 (79.7)	
Female	14 (34.1)	12 (20.3)	
Race			0.53
White	32 (78.0)	49 (83.1)	
Other	9 (22.0)	10 (16.9)	
Tobacco use			0.63
Tobacco exposure	31 (75.6)	45 (76.3)	
No tobacco exposure	8 (19.5)	9 (15.2)	
Not mentioned	2 (4.9)	5 (8.5)	
Alcohol use			0.96
Alcohol exposure	24 (58.5)	33 (55.9)	
No alcohol exposure	15 (36.6)	21 (35.6)	
Not mentioned	2 (4.9)	5 (8.5)	
Sub-site			ND
Tongue	18 (43.9)	30 (50.8)	
Floor of the mouth	12 (29.3)	13 (22.0)	
Retromolar area	5 (12.2)	10 (17.0)	
Other	6 (14.6)	6 (10.2)	
T classification			0.25
T1–T2	13 (31.7)	25 (42.4)	
T3–T4	28 (68.3)	34 (57.6)	
N classification			
N0	18 (43.9)	25 (42.4)	0.87
>N0	23 (56.1)	34 (57.6)	
M classification			NA
M0	41 (100)	59 (100)	
Clinical stage			0.76
I–II	8 (19.5)	13 (22.0)	
III–IV	33 (80.5)	46 (78.0)	
Histological grade[a]			0.02
Well-differentiated	26 (63.4)	30 (56.6)	
Moderately differentiated	7 (17.1)	20 (37.7)	
Poorly differentiated	8 (19.5)	3 (5.7)	
Treatment[a]			0.15
Surgery	15 (36.6)	22 (37.9)	
Surgery + RxT	23 (56.1)	24 (41.4)	
RxT alone	1 (2.4)	9 (15.5)	
Surgery + RxT + ChT	2 (4.9)	3 (5.2)	

ND, not determined; NA, not applicable; RxT, radiotherapy; ChT, chemotherapy.
[a] Some values were not available.

In both groups, CYP1A1 expression was only detected in peripheral cells of the tumour nests, while the central areas were negative (Fig. 1A and B). Cytoplasmic immunoexpression of CYP1A1 was frequently observed in areas of tumour invasion, near vessels, muscles, and glands. A significant correlation was observed between CYP1A1 overexpression and male sex ($P = 0.007$), tobacco use ($P = 0.019$), and alcohol consumption ($P = 0.01$). There was no significant difference in CYP1A1 expression between the young and control groups ($P = 0.31$) (Fig. 2A).

CYP1B1 was more expressed in the epithelial malignant cells of control group patients than young group patients and this difference was statistically significant ($P < 0.001$) (Fig. 2B). This protein was overexpressed in both groups (Fig. 1C and D). In the control group, overexpression of this enzyme was correlated with T1 + T2 tumours ($P = 0.001$), an early clinical stage ($P = 0.01$), and tumour-free surgical margins ($P = 0.02$). In the young group, overexpression of CYP1B1 was correlated with T1 + T2 tumours ($P = 0.019$) and early clinical stage ($P = 0.043$).

ALDH1A1 was expressed in few tumours cells and mostly at the invasion front (Fig. 1E and F). It was underexpressed in the two groups ($P = 0.11$) and was not correlated with any of the clinicopathological parameters (Fig. 2C).

ALDH2 was overexpressed in the two groups, in almost all tumours cells (Fig. 1G and H); the difference between the groups was not statistically significant ($P = 0.13$) (Fig. 2D). Overexpression of this enzyme was correlated with tumours located in the floor of the mouth ($P = 0.01$), smoking ($P = 0.01$), and chronic alcohol consumption ($P = 0.016$). Only in the young group was ALDH2 overexpression correlated with well-differentiated tumours ($P = 0.025$).

Overall survival

The first death occurred at 1.3 months after the primary treatment in the young group and at 1.0 months in the control group. The last death occurred at 330.4 months after the primary treatment in the young group and at 277.6 months in the ≥50 years control group. No differences in overall survival were observed between the young and control groups in terms of the expression of CYP1A1 (log-rank $P = 0.14$ in the young group vs. $P = 0.13$ in the control group), CYP1B1 (log-rank $P = 0.40$ vs. $P = 0.72$), ALDH1A1 (log-rank $P = 0.08$ vs. $P = 0.88$), or ALDH2 (log-rank

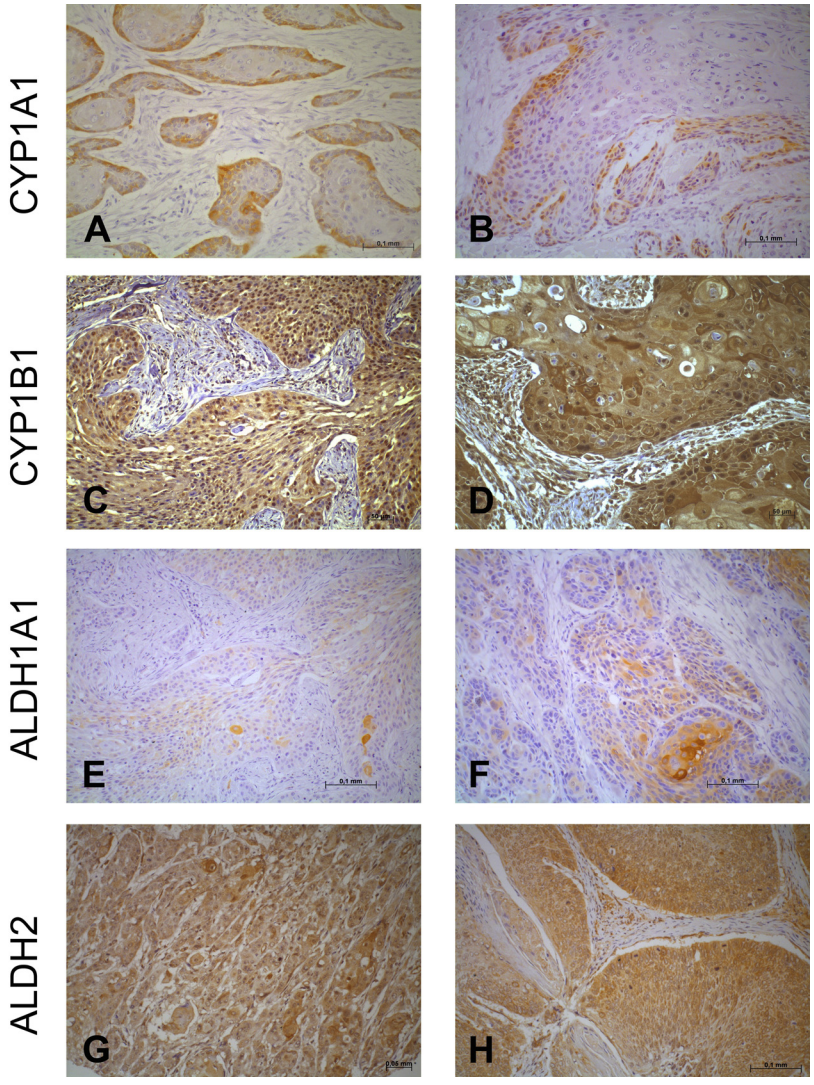

Fig. 1. CYP1A1 expression by peripheral cells of the invasive front: (A) young group, (B) control group. CYP1B1 was overexpressed in the cytoplasm of tumour cells: (C) young group, (D) control group. ALDH1A1 was expressed in few tumour cells: (E) young group, (F) control group. ALDH2 was overexpressed in tumour cells: (G) young group, (H) control group (DAB stain and Mayer's haematoxylin counterstaining).

$P = 0.16$ vs. $P = 0.23$). Protein expression was not associated with reduced overall survival in either group.

Relapse-free survival

The first relapse occurred at 1.2 months after the primary treatment in the young group and at 1.9 months after the primary treatment in the control group. The last relapse was observed at 168.0 months after the primary treatment in the young group and 179.1 months after the primary treatment in the control group.

The probability of 5-year relapse-free survival was 64% for positive CYP1B1 overexpression and 25% for underexpression for all patients in the two groups ($P = 0.008$). However, when CYP1B1 expression was stratified by age, overexpression improved relapse-free survival only in the control group (log-rank $P = 0.03$), while it had no influence on relapse-free survival in the young group (log-rank $P = 0.202$) (Fig. 3A–C).

The probability of 5-year relapse-free survival was 59% for positive ALDH1A1 overexpression and 31% for underexpression for all patients in the two groups ($P = 0.039$). In the young group, ALDH1A1 overexpression improved relapse-free survival (log-rank $P = 0.042$), while it had no influence on relapse-free survival in the control group (log-rank $P = 0.413$) (Fig. 3D–F).

No differences in relapse-free survival were observed between the young and control groups in terms of expression of CYP1A1 (log-rank $P = 0.08$ in the young group vs. $P = 0.06$ in the control group) or ALDH2 (log-rank $P = 0.25$ vs. $P = 0.13$). Expression of CYP1A1 (log-rank $P = 0.94$) or ALDH2 (log-rank $P = 0.49$) was not associated with reduced relapse-free survival in either group.

Discussion

Sample size is an important feature of any study. The sample in this study consisted of 41 young patients, representing one of the largest samples reported in the English language literature. Aiming to eliminate the influence of clinical features between groups, the control group was matched to the young patients according to their clinical stage and tumour topography. Treatment is usually considered an important prognostic factor; consequently, the treatment approach was similar in the two groups.

The components of tobacco smoke, including PAH, are potentially carcinogenic substances and are associated with oral cancer.[11,16] Exposure to PAH stimulates the synthesis of CYP1A1 and CYP1B1.[10–12] Shatalova et al. observed the expression of CYP1B1 in 99.4% of head and neck SCC cases,[17] similar to the results of the present study. Expression of this enzyme has been correlated with the motility and proliferation of cells derived from dysplastic leukoplakia lesions (MSK-Leuk1), suggesting that this protein plays an important role in the clonal expansion of these lesions and facilitates cancer progression in the head and neck region.[17] In OSCC, overexpression of CYP1B1 and CYP1A1 has been correlated with the overexpression of proliferating cell nuclear antigen (PCNA), placental glutathione S-transferase (GST-P), and nuclear factor kappa B (NF-κB), which are proteins involved in cell proliferation, and with the down-regulation of BCL2-associated X protein (BAX), Fas cell surface death receptor (FAS), apoptotic protease activating factor 1 (APAF1), cytochrome c, caspases, and poly-(ADP-ribose) polymerase (PARP), which are apoptotic proteins.[12]

In a tongue SCC cell line, BaP was found to induce its own metabolism through the overexpression of CYP1B1 and consequently to produce more DNA adducts.[16] This finding demonstrates the greater importance of this isoform in the metabolism of PAH, which may be a marker of exposure to tobacco carcinogens.[12] In the present study, overexpression of CYP1B1 was observed in all cases. Expression of this enzyme was higher in

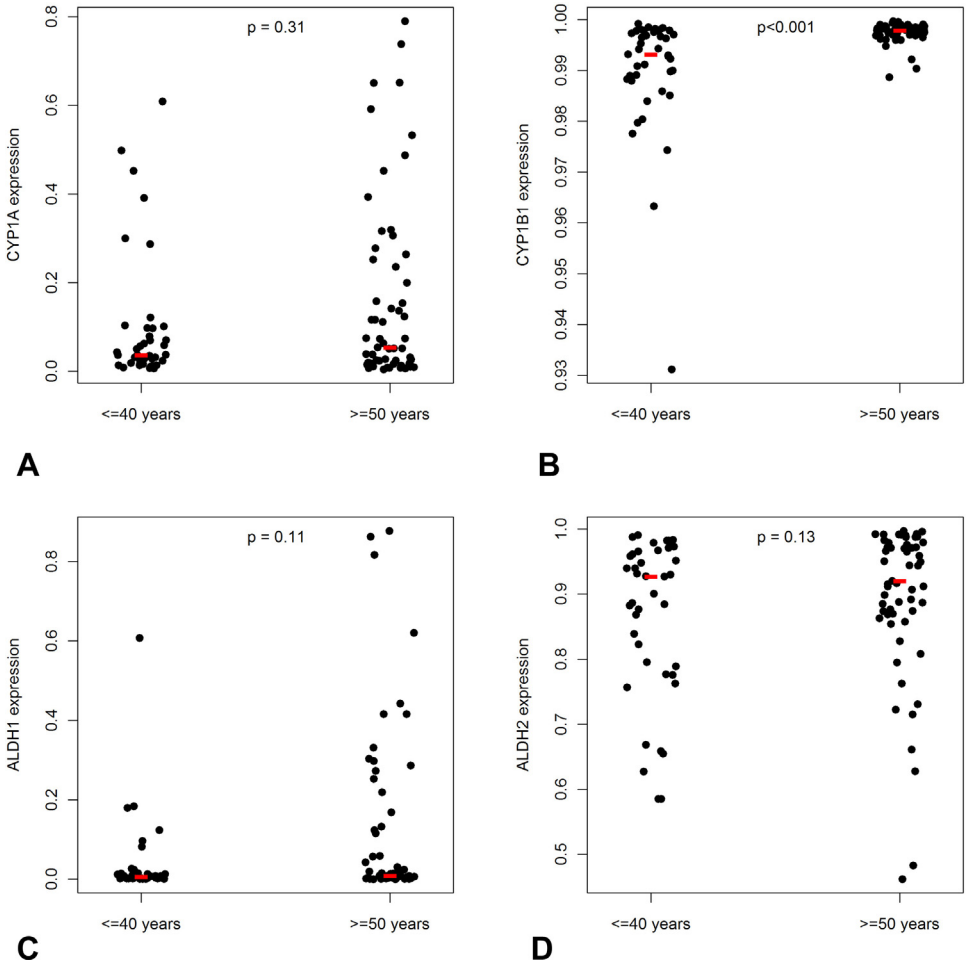

Fig. 2. Comparisons of (A) CYP1A1, (B) CYP1B1, (C) ALDH1A1, and (D) ALDH2 expression in the two groups.

the group older than 50 years, a finding that might be explained by the longer exposure to tobacco carcinogens that could stimulate the expression of this protein.

BaP and heterocyclic amines have been shown to induce the gene and protein expression of CYP1A1 in tongue SCC cells and in the tongues of rats, respectively.[8] However, its expression has been found to be significantly lower when compared to liver samples[8] and to CYP1B1.[12] The lower expression of CYP1A1 may result in lower metabolic activation of BaP in the tongue of rats despite the expression of ethoxyresorufin-O-deethylase, an enzyme on which the activity of CYP1A1 seems to depend.[8] The induction of the expression of drug-metabolizing enzymes accelerates the detoxification of toxic components and, at the same time, increases the activation of xenobiotic metabolites and consequently the risk of DNA damage.[8] The self-oxidation or metabolic activation of components of areca nut extract by CYP1A1 can produce ROS which, in turn, activate different cell signalling pathways such as Src, Ras/Raf, and Mek/Merk. The aberrant growth, differentiation, and inflammation of gingival keratinocytes induced by these pathways may contribute to the pathogenesis of oral submucous fibrosis and OSCC.[18] In the present study, CYP1A1 was only expressed in peripheral cells of the invasive front near blood vessels and nerve fibres, suggesting that this enzyme contributes to tumour aggressiveness in both groups.

Ingested ethanol is metabolized by alcohol dehydrogenase, cytochrome P450 2E1 (CYP2E1), and catalase to form acetaldehyde, which in turn is degraded primarily into acetate by mitochondrial ALDH2.[13,19] Acetaldehyde increases the formation of ROS, which can cause lipid peroxidation of oral leukoplakia and cancer cells in alcoholics.[20] Furthermore, the accumulation of acetaldehyde can lead to the formation of acetaldehyde–DNA adducts, *N*2-ethyl-deoxyguanosine (*N*2-Et-dG), and α-methyl-γ-hydroxy-1-*N*2-propanodeoxyguanosine (α-Me-γ-OH-PdG), which can cause chromosome aberrations and sister chromatid exchanges.[21] Acetaldehyde inhibits the activity of O6-methyl guanyltransferase, an enzyme responsible for the removal of adducts resulting from alkylating agents.[19,22]Genetic variations can cause differences in enzymatic activity; for example, individuals carrying the *ALDH2*1/2** and *ALDH2*2/2** alleles encode deficient ALDH2.[19] The loss of the catalytic activity of ALDH2 is considered to be crucial for the development of SCC of the upper aerodigestive tract in Asian individuals.[19,23] In the present study, although ALDH2 was overexpressed in both groups, no correlation was observed with the clinicopathological parameters.

ALDH modulates carcinogenesis by metabolizing acetaldehyde and retinaldehyde. The latter is produced from retinol by alcohol dehydrogenase and is converted into retinoic acid by ALDH1A1. Retinoic acid exerts anti-apoptotic activity through binding to cellular retinoic acid binding protein (CRABPII), which inhibits cell growth.[24] Furthermore, retinoic acid can bind to fatty acid protein binding

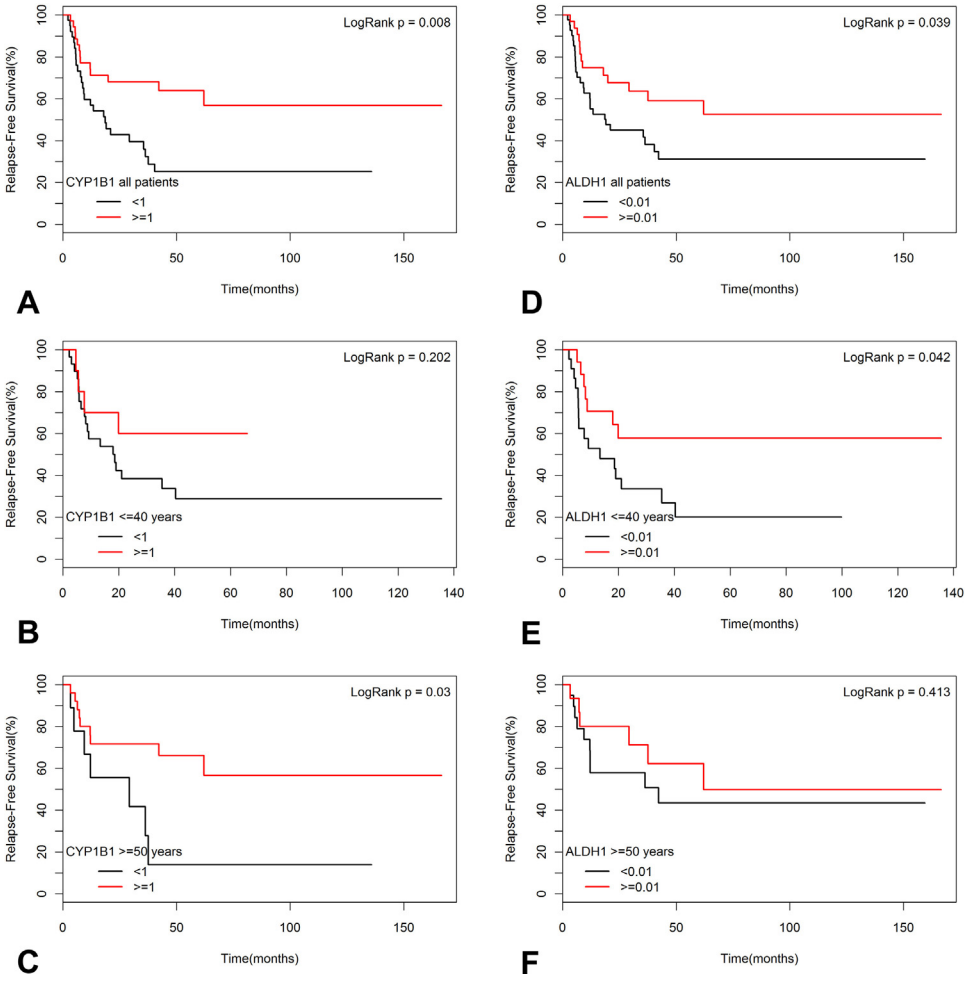

Fig. 3. Relapse-free survival stratified by (A) CYP1B1 expression in the two groups (log-rank $P = 0.008$); (B) CYP1B1 expression only in the young group (log-rank $P = 0.202$); (C) CYP1B1 expression only in the control group (log-rank $P = 0.03$); (D) ALDH1A1 expression in the two groups (log-rank $P = 0.039$); (E) ALDH1A1 expression only in the young group (log-rank $P = 0.042$); (F) ALDH1A1 expression only in the control group (log-rank $P = 0.413$).

protein 5 (FABP5), promoting the survival and proliferation of pancreatic and colon tumour cells.[13] In the present study, few tumours expressed ALDH1A1. If expressed by tumour cells, ALDH1A1 was correlated with better relapse-free survival in young patients. Hwang et al. hypothesized that increased expression of ALDH1 increases the metabolism of acetaldehyde and reduces the risk of developing SCC.[25] An increase in the concentration of acetaldehyde may be due to the loss of ALDH1A1 activity, which increases the risk of developing head and neck SCC since acetaldehyde is also metabolized, to a lesser extent, by cytosolic ALDH1A1.[14]

In SCC of the head and neck, ALDH1 immunoexpression has been found to be correlated with poorly differentiated tumours, lymph node metastasis, and a poor prognosis.[26] However, Ota et al. found no correlation between overexpression of ALDH1 and lower overall survival or relapse-free survival, but did find a correlation with local recurrence *in vivo*.[27] Cisplatin-surviving cells have exhibited high protein expression of ALDH1 and ATP-binding cassette, a high invasive capacity, and the ability to self-renew. These features confer the ability to initiate and drive primary tumour growth, invasion, metastasis, resistance to chemotherapy,[27] and local recurrence.[13] Head and neck SCCs that express little or no ALDH1A1 may be more responsive to chemotherapy due to the deficiency in the metabolism of retinaldehyde into retinoic acid.[14] Moreover, ALDH1A1 has been used as a marker of cancer stem cells and of cancer-initiating cells.[26,27]

In the present study, overall survival was not affected by the expression of the proteins evaluated. ALDH1A1 was underexpressed and, when detected, improved relapse-free survival in young patients. Patients older than 50 years expressed more CYP1B1 than young patients and overexpression of this enzyme improved relapse-free survival. This finding suggests that the longer cells are exposed to tobacco, the higher their expression of this enzyme, with a consequently better detoxification of carcinogens in this age group.

Tobacco and alcohol are metabolized through a series of pathways that are complex, dynamic, and integrate with other pathways, such as those of cell proliferation, cell differentiation, apoptosis, and biotransformation of other substances. Such metabolic processes involve a great number of proteins that can play other roles in addition to metabolization; for example ALDH1, which identifies stem cell in OSCC. Further studies involving other genes and proteins are necessary in order to complement the results of this research.

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Competing interests

None.

Ethical approval

The study was approved by the Human Research Ethics Committee of the Institute of Science and Technology – UNESP (process 208.406).

Patient consent

Not required.

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