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Original scientific article

EXPRESSION OF CAVEOLIN-2 IN PATIENTS WITH ORAL CANCER AND CORRELATIONS WITH CLINICOPATHOLOGICAL PARAMETERS

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Wan C., F. Zhang, L. Zhu (2021). *Expression of caveolin-2 in patients with oral cancer and correlations with clinicopathological parameters.* - Genetika, Vol 53, No.2, 703-716.
We aimed to analyze the expression of caveolin-2 (CAV2) in patients with oral cancer and its correlations with clinicopathological parameters. The expression of CAV2 in oral cancer and its influence on the survival curves of oral cancer patients were inquired through the Human Protein Atlas Database. The cancer tissue specimens and normal paracancerous tissue specimens (≥2 cm away from cancer tissues) were collected from 173 patients with oral cancer confirmed by pathology. Moreover, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry were performed to detect the messenger ribonucleic acid (mRNA) and protein expressions of CAV2 in oral cancer tissues and corresponding paracancerous tissues, respectively, and their associations with the clinicopathological characteristics and survival conditions of oral cancer patients were analyzed. It was shown in the Human Protein Atlas Database that the expression of CAV2 was increased significantly in oral cancer tissues compared with that in normal tissues ($P<0.05$), and patients with a low expression of CAV2 had a longer survival time than those with a high expression of CAV2 ($P<0.05$). The results of qRT-PCR and immunohistochemistry manifested that the mRNA expression level of CAV2 and the percentage of CAV2-positive cells were significantly higher in oral cancer tissues than those in paracancerous tissues ($P<0.05$). The CAV2 expression was correlated with clinical stage and pathological differentiation degree ($P<0.05$). In comparison with those in patients with a low CAV2 expression, the overall survival (OS) curve, relapse-free survival (RFS) curve and survival rate declined significantly in patients with a high CAV2 expression ($P=0.001$). Besides,

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the CAV2 expression, clinical stage and pathological differentiation degree were independent influencing factors for the postoperative OS and RFS of patients. The expression of CAV2 had relatively high predictive value for the OS and RFS of patients with oral cancer within 5 years after operation, of which the area under curve was 0.827 and 0.874, and the optimal cut-off value was 27.97% and 32.84%, respectively. CAV2 is highly expressed in oral cancer. With rising CAV2 expression level, the survival time of patients is shortened and the relapse risk is elevated, suggesting a poor prognosis.

Keyword: caveolin-2, oral cancer, clinicopathological parameter

INTRODUCTION

Oral cancer is one of the common malignant tumors in clinical practice. Over 260,000 people are newly diagnosed with oral cancer every year, about 130,000 of whom die ultimately, and oral squamous cell carcinoma accounts for 90% of all cases. Numerous researchers in China and foreign countries are committed to elaborating the pathological mechanism of oral cancer, but no definite conclusions have been reached yet. Factors such as heredity, smoking, alcohol abuse, radiation, viral invasion and immune deficiency can lead to the occurrence and development of oral cancer (POPOVIĆ *et al.*, 2010; BEYNON *et al.*, 2018; LAFUENTE IBÁÑEZ DE MENDOZA *et al.*, 2020; MADATHIL *et al.*, 2020). Nevertheless, as oral cancer mostly occurs in relatively hidden parts including buccal mucosa, hard palate, tongue (dorsal and ventral parts) and upper and lower gums, most patients have been in the advanced stage when diagnosed. Despite fairly prominent progress in the diagnosis and treatment techniques of oral cancer recently, the 5-year survival rate of patients has not been remarkably increased, and the disease is extremely prone to local relapse and lymphatic metastasis, seriously bothering the clinical diagnosis, treatment and prognosis assessment by clinicians (GELAŽIUS *et al.*, 2019). Therefore, searching specific biomarkers closely related to the occurrence and development of oral cancer and establishing effective methods for monitoring the invasion and metastasis of cancer cells will facilitate the timely implementation of individualized and targeted intervention, improve the accuracy of evaluating postoperative prognosis of patients and prolong patients' survival. A number of cellular, biochemical, genetic and epigenetic markers have been proposed with the aim of better defining oral cancer (ELJABO *et al.*, 2018; CHANG *et al.*, 2020; ROI *et al.*, 2020; YANG *et al.*, 2020).

Caveolae on cell membranes refer to the spherical depressions with stable morphology on eukaryotic cell membranes, and caveolins (CAVs) are the key components on the caveola membrane. LIU *et al.* (2018) reported that the high expression of CAV2 was closely correlated with the progression of renal cancer. JIAO *et al.* (2020) showed that the expression of CAV2 was of high predictive value for the poor prognosis of patients with pancreatic cancer, while ANDO *et al.* (2007) found that CAV2 overexpression was correlated with the progression of esophageal squamous cell carcinoma. However, there are no reports about the role of CAV2 expression in oral cancer. Hence, the associations of CAV2 expression with the clinicopathological parameters of oral cancer patients were analyzed by searching a protein database and verifying clinical tissue specimens.

MATERIALS AND METHODS

Online Biological Database Analysis

The distribution of CAV2 in different tissues of normal human body was analyzed online through Human Protein Atlas Database (https://www.proteinatlas.org/). Then the expression of CAV2 in oral cancer and its influence on the survival curves of oral cancer patients were inquired.

Baseline Clinical Data

To further verify the results of the online database, the pathological tissue sections and the sections of normal tissues around the cancer (distance between paracancerous tissues and cancer tissues >2 cm) were collected from 173 patients with oral squamous cell carcinoma admitted to and treated in our hospital from June 2011 to June 2015. There were 121 males and 52 females aged 40-67 years old and (52.8 ± 13.3) years old on average. The tumor-node-metastasis (TNM) stage of the patients was determined according to the 8th edition of TNM staging system for lip and oral cavity tumors jointly published by the American Joint Committee on Cancer and Union for International Cancer Control (SINGHAVI *et al.*, 2020), including 33 cases of stage I, 41 cases of stage II, 47 cases of stage III and 52 cases of stage IV. Moreover, oral cancer was classified into 3 grades based on the pathological differentiation degree (KUKREJA *et al.*, 2020), i.e. highly differentiated (grade I, n=55), moderately differentiated (grade II, n=61) and lowly differentiated (grade II, n=67). The inclusion criteria involved patients definitely diagnosed with oral cancer by pathological examination, those who received no radiotherapy, chemotherapy and immunotherapy previously and underwent surgery for the first time, those with complete clinical and pathological data, and those subjected to standard return visits for prognosis after operation. The exclusion criteria were set as follows: 1) patients complicated with other tumors, 2) those with metastatic cancer or multiple cancers not originating in the oral cavity, 3) those with cardiac or pulmonary insufficiency, 4) those with disturbance of consciousness or lack of independent consciousness, or 5) those with incomplete medical records. All the patients and their families were informed of this study and signed the informed consent. The manipulations and objective of this study were reviewed and approved by the Ethics Committee of our hospital.

CAV2 mRNA Expression by Real-Time Quantitative PCR

Total RNAs in the oral cancer tissues and paracancerous tissues were extracted using RNAiso Plus (TaKaRa, Japan), dried and dissolved in DEPC-treated water. Then the concentration and purity of the total RNAs were measured by a spectrophotometer, and the total RNAs were reversely transcribed *as per* the instructions of reverse transcription kit (TaKaRa, Japan). Then PCR amplification was conducted with cDNA as the template using CFX96™ RT-PCR Detection System (Bio-Rad, USA). The primer sequences were designed (MONDEJAR-PARREÑO *et al.*, 2019). GAPDH: forward primer: 5'-TCAAGAAGGTGGTGAAGCAGG-3' and reverse primer: 5'-AG-CGTCAAAGGTGGAGGAGTG-3', and CAV2 messenger RNA (mRNA): forward primer: 5'-ACTGGTCCGACTGGTACGACA-3' and reverse primer: 5'-AAGAACTGCTGAGGCTTGGGT-3'. PCR conditions included pre-denaturation at 95°C for 30

s, 95°C for 5 s and 62°C for 30 s, 45 cycles in total. Afterwards, the relative expression level of CAV2 mRNA was calculated using $2^{-\Delta\Delta Ct}$ method.

CAV2 Protein Expression by Immunochemical Assay

The oral cancer tissues and paracancerous tissues were separately deparaffinized and hydrated prior to washing with phosphate-buffered saline (PBS). Then they were placed in citrate buffer (pH 6.0), heated in a microwave oven for 10 min and washed again with PBS, followed by soaking in 3% hydrogen peroxide for 10 min for quenching, so as to block endogenous peroxidase. After sealing in 2% bovine serum albumin solution for 30 min, the tissues were incubated with monoclonal antibody against CAV2 (Abcam, USA) at 4°C overnight and with universal secondary antibody (Abcam, USA) at room temperature for 30 min. Next, DAB was added in drops for color development, and the tissues were counterstained with hematoxylin, dehydrated with gradient ethanol, mounted in neutral balsam and observed under CKX31 biological microscope (Olympus, Japan). Through observation, the color developed on the cell membrane and in the cytoplasm was determined as positive reaction, and the cells presenting positive reaction were recorded as positive cells. The intensity of positive reaction was evaluated based on the extent of cell staining and the proportion of positive cells in the field of vision. The interpretation standards of results were set below (MAGNI *et al.*, 2019). At least 5 high-power fields of each pathological section were observed, in which CAV2 negative expression meant that the number of stained cells accounted for less than 25% of the total. On the contrary, the percentage of stained cells ≥25% indicated a positive expression.

All patients were followed up *via* telephone interview and outpatient visits by special physicians who were systematically trained, and the follow-up was terminated on Tuesday, June 30th, 2020. Overall survival (OS) referred to the duration from the day of surgery to the death of patients or the end of follow-up.

Statistical Analysis

SPSS 20.0 software was used for statistical analysis. The categorical data were expressed by percentage and analyzed *via* χ^2 test. Kaplan-Meier survival curves were plotted to analyze the relation between CAV2 expression and the survival of oral cancer patients, the influencing factors for prognosis were analyzed using Cox's proportional hazards regression model, and receiver operating characteristic (ROC) curves were plotted to evaluate the diagnostic value of CAV2 protein expression for prognosis. $P<0.05$ indicated that the difference was statistically significant.

RESULTS

Distribution of CAV2 in Different Tissues of Normal Human Body, in Oral Cancer and Its Relationship with Survival

It was shown in the Human Protein Atlas Database that CAV2 was widely distributed in tissues and organs such as nasopharynx, bronchus, esophagus, oral mucosa, placenta, uterus and adipose, suggesting that it is of significance to study the difference of CAV2 expression in oral cancer (Figure 1).

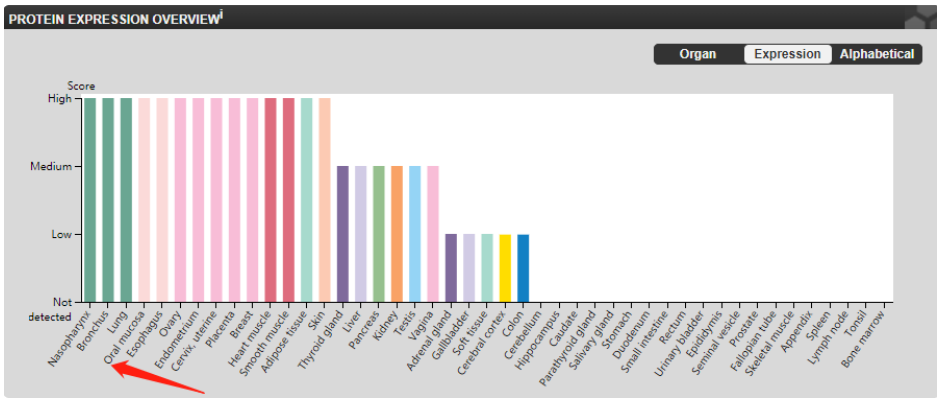

Figure 1. Distribution of CAV2 in different tissues of normal human body.

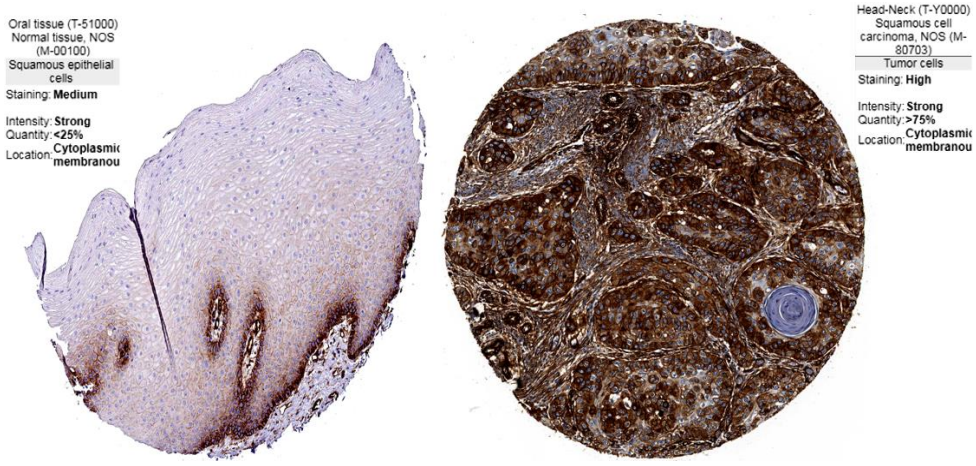

Figure 2. Expression of CAV2 in oral mucosa and oral cancer.

The expression of CAV2 in the case of oral cancer was further investigated through the Human Protein Atlas Database. The results manifested that the expression of CAV2 was increased significantly in oral cancer tissues compared with that in normal tissues ($P<0.05$) (Figure 2), and patients with a low expression of CAV2 had a longer survival time than those with a high expression of CAV2 ($P<0.05$) (Figure 3).

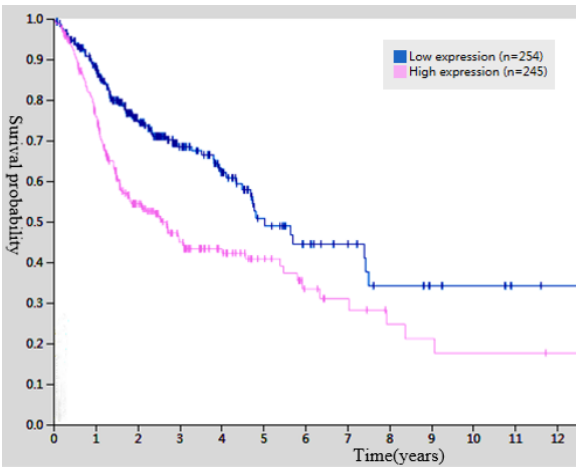

Figure 3. Relationship between expression level of CAV2 and survival time of oral cancer patients.

CAV2 Expressions in Oral Cancer and Paracancerous Tissues

It was indicated in the results of qRT-PCR that the mRNA expression level of CAV2 was significantly higher in oral cancer tissues than that in paracancerous tissues ($P<0.05$). Besides, the results of immunohistochemistry revealed that the percentage of CAV2-positive cells was significantly elevated in oral cancer tissues in comparison with that in paracancerous tissues ($P<0.05$) (Figure 4).

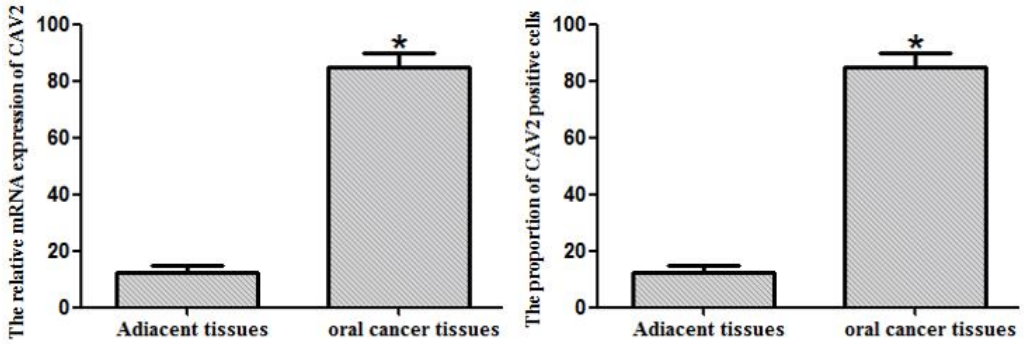

Figure 4. CAV2 mRNA expressions in oral cancer and paracancerous tissues. $P<0.05$ *vs.* paracancerous tissues.

Correlations of CAV2 Expression with Clinicopathological Parameters of Oral Cancer Patients

According to the statistical analysis of basic clinical data, the CAV2 expression was correlated with the clinical stage and pathological differentiation degree ($P<0.05$), but it had no relations to the age, gender, smoking history, drinking history, lymphatic metastasis and tumor site ($P>0.05$) (Table 1).

Table 1. Correlations of CAV2 expression with clinicopathological parameters of oral cancer patients

Category	n	CAV2		χ^2	P
		+	-		
Age					
≤50 years old	102	82	20	0.203	0.652
≥50 years old	71	59	12		
Gender					
Male	121	102	19	2.086	0.149
Female	52	39	13		
Drinking history					
Yes	116	99	17	3.447	0.063
No	57	42	15		
Smoking history					
Yes	105	85	20	0.0537	0.817
No	68	56	12		
Stage					
Stage I	33	18	15	24.660	$P<0.001$
Stage II	41	32	9		
Stage III	47	41	6		
Stage IV	52	50	2		
Pathological differentiation degree					
Grade I	55	37	18	12.549	0.002
Grade II	61	51	10		
Grade III	57	53	4		
Lymphatic metastasis					
Yes	64	48	16	2.849	0.0914
No	109	93	16		
Tumor site					
Tongue	56	49	7	7.331	0.062
Cheek	55	48	7		
Gingiva	32	22	8		
Lip	30	22	10		

Associations of CAV2 Expression Level with 5-Year OS and RFS of Oral Cancer Patients

After 5 years of follow-up of the patients, the OS curve and relapse-free survival (RFS) curve were analyzed using Kaplan-Meier method. The results demonstrated that the survival curves and survival rate of patients with a high expression of CAV2 declined significantly compared with those of patients with a low expression of CAV2 (P=0.001) (Figure 5).

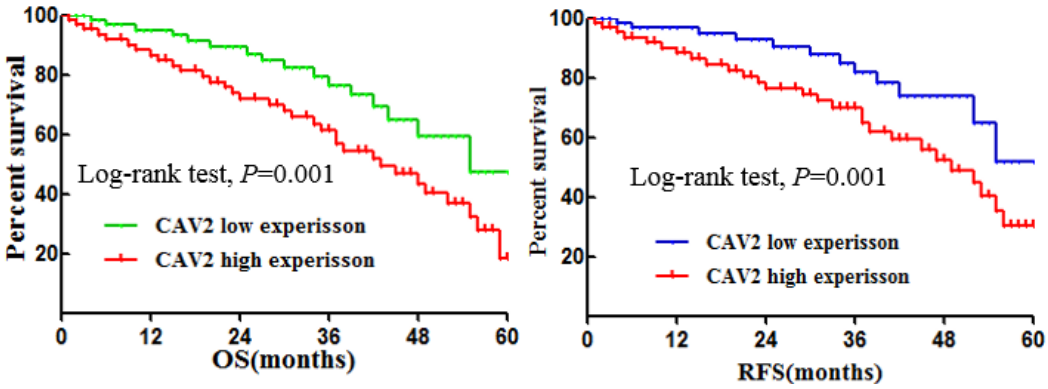

Figure 5. Associations of CAV2 expression level with 5-year OS and RFS of oral cancer patients.

Cox Univariate and Multivariate Survival Analysis Results

The Cox regression analysis of survival showed that CAV2 expression, clinical stage and pathological differentiation degree were independent influencing factors for the postoperative OS and RFS of patients. A higher expression of CAV2 indicated a poorer prognosis of patients and a higher risk of relapse (Table 2 and 3).

Table 2. Relationship between CAV2 expression and OS studied by Cox regression analysis

Index	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age	0.995 (0.945~1.015)	0.605	0.993 (0.948~1.013)	0.589
TNM stage	2.518 (1.371~4.027)	0.006	2.029 (1.592~2.624)	0.002
Pathological grade	2.167 (1.846~3.071)	0.042	1.634 (1.173~2.257)	0.031
Lymphatic metastasis	0.425 (0.325~0.697)	0.723	0.473 (0.307~0.567)	0.128
CAV2 expression	0.530 (0.337~0.829)	0.003	0.619 (0.236~0.991)	0.027

Age ≥50 years old = 1, age <50 years old = 0, TNM stage: stage III + IV = 1, stage I + II = 0, pathological grade: grade III = 1, grade I/II = 0, lymphatic metastasis: Yes = 1, No = 0, and CAV2 expression: positive = 1, negative = 0.

Table 3. Relationship between CAV2 expression and RFS explored by Cox regression analysis

Index	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age	0.984 (0.905~1.003)	0.063	0.983 (0.912~1.007)	0.067
TNM stage	2.186 (1.715~4.274)	0.001	2.029 (1.785~2.849)	0.001
Pathological grade	2.379 (1.728~3.153)	0.033	1.628 (1.473~2.572)	0.026
Lymphatic metastasis	0.374 (0.227~0.563)	0.497	0.533 (0.045~0.827)	0.087
CAV2 expression	0.512 (0.316~0.806)	0.001	0.597 (0.338~0.986)	0.004

Age ≥50 years old = 1, age <50 years old = 0, TNM stage: stage III + IV = 1, stage I + II = 0, pathological grade: grade III = 1, grade I/II = 0, lymphatic metastasis: Yes = 1, No = 0, and CAV2 expression: positive = 1, negative = 0.

Predictive Values of CAV2 Expression for OS and RFS

As shown in the ROC curves, the expression of CAV2 had relatively high predictive value for the OS and RFS of patients with oral cancer within 5 years after operation, of which the area under curve was 0.827 and 0.874, and the optimal cut-off value was 27.97% and 32.84%, respectively (Figure 6, Table 4).

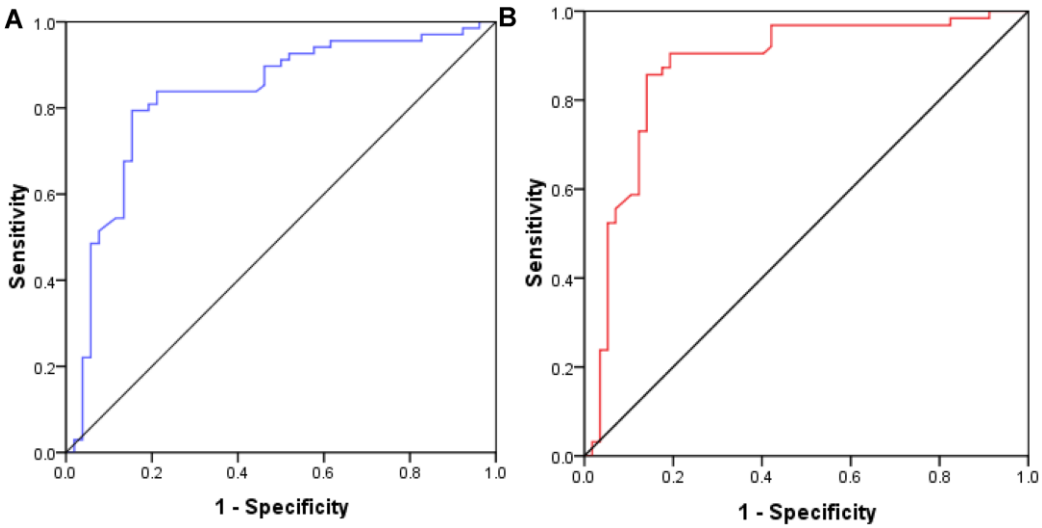

Figure 6. Predictive values of CAV2 expression for (A) OS and (B) RFS of oral cancer patients within 5 years after operation.

Table 4. Predictive values of CAV2 expression for OS and RFS of oral cancer patients within 5 years after operation

Index	Area under ROC curve	95% CI	Sensitivity	Specificity
OS	0.827	6.27~10.37	0.822	0.853
RFS	0.874	5.98~14.61	0.835	0.878

DISCUSSION

Population genetic study provides valuable information about genetic structure of plants, the stratification versus gene flow among the species populations, genetic divergence of the populations, etc. (ESFANDANI- BOZCHALOYI, 2017a; 2017b; 2017c; 2017d). These information have different applications, and from pure understanding of biology of the species to conservation of endangered species, choosing of proper parents for hybridization and breeding and phylogeography and mechanism of invasion (FREELAND *et al*., 2011).

Pistachio has important socio-economic and ecological impacts in the arid and semi-arid agricultural regions of Iran (KAFKAS *et al*., 2006). In addition, Iran hosts a wide genetic diversity of *Pistacia* spp. and more than 300 pistachio genotypes have been collected across the country. Iran therefore possesses valuable germplasm for pistachio improvement and conservation programs. Assessing genetic diversity and relationships among cultivars of Iranian pistachio, using discriminative and robust markers, is therefore important.

This study was aimed at evaluating the genetic diversity of Iranian pistachio in order to aid the conservation of its germplasm. The obtained information about the genetic variation between and within different populations will prepare the ground for the formulation of appropriate conservation strategies. The present analysis revealed that Iranian-cultivated pistachio germplasm is highly variable, presumably due to specific local genetic backgrounds, breeding pressure and/or limited interchange of genetic material. The unique nature of the Iranian pistachio germplasm revealed by our results, supports the case for the implementation of more intense characterization, conservation and breeding strategies. Also, the ISSR markers used were useful for determination of genetic diversity among pistachio cultivars in Iran.

The results of this molecular assay in fingerprinting of the 11 pistachio genotypes are presented in table 3. In ISSR, according to the reported results of (KAFKAS *et al*., 2006), first six primers were used and after initial screening three out of them primers eventually selected for the final analysis. A total of 28 bands were amplified by the three primers, an average of 9.3 bands per primer of which 13 (46/42%) were polymorphic. The total number of amplified fragments was between seven to 12 and the number of polymorphic fragments ranged from three to five.

During the ISSR screening in this study, good amplification products were obtained from primers based on GA, CA and GAA repeats. But primers based on CT, GT and CAA repeats produced few large separate bands which finally were eliminated for the final analysis. KAFKAS *et al*., 2006) using 20 primers obtained a total of 156 bands, an average of 7.7 bands per primer, of which 73(46.2%) were polymorphic which is similar to our results in this study.

MIRZAEI *et al*. (2005) reported 80% polymorphism among 22 Iranian cultivars and wild pistachio species. The difference in polymorphism reported in the current study and that of Mirzaei et al. (2005) could be attributed to differences in the tested genotypes and the selected

primers. Katsiotis et al. (2003) obtained 82.41% polymorphism and of a total of 22.11, there were 18.2 polymorphic bands. In a study reported by Golan-Goldhirsh et al. (2004) in assessing polymorphisms among 28 Mediterranean *Pistacia* accessions, twenty seven selected primers produced 259 total bands (average 9.59) and 86.1 of them were polymorphic.

KHADIVI (2018) revealed high level of polymorphism among the studied genotypes. The seven SSR primer pairs generated a total of 18 alleles that 13 of them were polymorphic among the genotypes. The range of the polymorphic alleles was 1 (for Ptms9, Ptms40, Ptms41, and Ptms42) to 5 (for Ptms7 locus) with an average of 2.57. The amplified allele sizes ranged from 120 to 250bp. Pair-wise genetic similarity coefficients varied from 0.20 to 0.75.

The present study indicated that a higher genetic diversity was found in the older genotypes. This fact confirms our speculation that pistachio cultivations have increasingly led to the reduction of their genetic variation due to deployment of improved cultivars and to the availability of private or public grafted seedling nurseries for pistachio, as well as the changing livelihood conditions. Recently, the method of pistachio cultivation is changing leading towards an increased reduction of crop diversity deployed on farm. In the past, pistachio diversity was maintained high in the field through a number of cultivation practices, s. a. use of male varieties derived from seed, use of wild *Pistacia* species to boost pollination and hence the fruit setting, use of natural populations of wild *Pistacia* (*P. atlantica*) as a rootstock due to their well-known resistance to stony and calcareous soils. In order to utilize different genotypes of pistachio efficiently, it is important to know the genetic variation and genetic relationships that exist between them. The SSR primer pairs used in the present study revealed moderate levels of polymorphism in Iranian pistachios.

AHMAD *et al*. (2003, 2005) reported moderate diversity among the Syrian, Turkish, and American pistachios. Protection against the loss of genetic diversity is required urgently (HAMRICK and GODT, 1996). In traditional areas of pistachio cultivation, contact between the cultivated clones and wild species is quite common and has existed for hundreds or even thousands of years (ZOHARY, 1996). As a result, interspecific hybridization of *P. vera* with other *Pistacia* species led to the development of various hybrids with different backgrounds (MAGGS, 1973; BARAZANI *et al*., 2003).

In conclusion, although Iran is one of the two major centers of *Pistacia* diversity and the main pistachio producer in the world, the Iranian pistachio industry has a very narrow genetic base. The present results demonstrated that the study of genetic diversity among some *Pistacia* genotypes and cultivars using ISSR markers provided information that is relevant for the conservation of pistachio germplasm. The current results showed that Iranian genotypes have a moderate genetic variation and therefore are very important for genetic conservation and the planning of future breeding programs. The present results may be used for the conservation, core collection and future breeding of the pistachio.

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EKSPRESIJA KAVEOLINA-2 KOD BOLESNIKA SA ORALNIM KARCINOMOM I KORELACIJA SA KLINIČKOPATOLOŠKIM PARAMETRIMA

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Izvod

Cilj nam je bio da analiziramo ekspresiju kaveolina-2 (CAV2) kod pacijenata sa rakom usne šupljine i njegove korelacije sa kliničkopatološkim parametrima. Ekspresija CAV2 u raku usne šupljine i njegov uticaj na krive preživljavanja pacijenata sa rakom usne šupljine ispitivani su putem baze podataka Atlas humanog proteina. Uzorci tkiva karcinoma i normalni uzorci parakoncentralnog tkiva (≥2 cm udaljeni od tkiva karcinoma) prikupljeni su od 173 pacijenta sa rakom usne šupljine potvrđenim patologijom. Štaviše, izvedena je qRT-PCR i imunohistohemija kako bi se otkrila mRNK i ekspresija proteina CAV2 u tkivima karcinoma usne duplje i odgovarajućim parakoncentroznim tkivima, i analizirane su kliničkopatološke karakteristike i uslovi preživljavanja pacijenata sa rakom usne šupljine. U bazi podataka Atlasa humanog proteina pokazano je da je ekspresija CAV2 značajno povećana u tkivima karcinoma usne šupljine u poređenju sa onom u normalnim tkivima (P <0,05), a pacijenti sa niskom ekspresijom CAV2 imali su duže vreme preživljavanja od onih sa visokom ekspresijom CAV2 (P <0,05). Rezultati qRT-PCR i imunohistohemije pokazali su da su nivo ekspresije mRNK CAV2 i procenat CAV2 pozitivnih ćelija bili značajno veći u tkivima karcinoma usne šupljine od onih u parakanceroznim tkivima (P <0,05). Ekspresija CAV2 korelirala je sa kliničkom fazom i stepenom patološke diferencijacije (P <0,05). U poređenju sa onima kod pacijenata sa niskom ekspresijom CAV2, kriva ukupnog preživljavanja (OS), kriva preživljavanja bez relapsa (RFS) i stopa preživljavanja značajno su opali kod pacijenata sa visokom ekspresijom CAV2 (P = 0,001). Pored toga, ekspresija CAV2, klinička faza i stepen patološke diferencijacije bili su nezavisni faktori uticaja na OS i RFS pacijenata. Ekspresija CAV2 imala je relativno visoku prediktivnu vrednost za OS i RFS pacijenata sa rakom usne šupljine u roku od 5 godina nakon operacije, od čega je površina pod krivom bila 0,827 i 0,874, a optimalna granična vrednost bila je 27,97% i 32,84%, redom. CAV2 je visoko izražen kod karcinoma usne šupljine. Sa porastom nivoa ekspresije CAV2, vreme preživljavanja pacijenata se skraćuje, a rizik od relapsa je povišen, što ukazuje na lošu prognozu.

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