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EVALUATION OF P21^{WAF} EXPRESSION AND CDKN1A EXON 2 MUTATION IN SALIVARY ADENOID CYSTIC CARCINOMA

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Abstract – *Objective:* *The P21^{waf} is a tumor-suppressor protein encoded by CDKN1A gene. In this study, we evaluated P21^{waf} expression and CDKN1A exon 2 mutation and their relationships with clinicopathological parameters and cancer development in salivary adenoid cystic carcinoma (ACC).*

Patient and Methods: *Forty paraffin blocks from patients with salivary ACC were collected. Immunohistochemical staining was performed using P21 antibody. Genomic DNAs were extracted from the deparaffinized sections of the embedded tissue. Exon 2 of CDKN1A gene was amplified by PCR and the PCR products were sequenced. Spearman's correlation coefficient, Fisher's exact test, and Kruskal-Wallis test were used for data analysis.*

Results: A significant inverse correlation was observed between P21 expression and histologic grade (p=0.033, r=-0.338). The correlation of tumor size with recurrence (p=0.048) and tumor stage (p=0.046) was also evidenced. No mutation was detected in the exon 2 of CDKN1A gene.

Conclusions: *Regarding the association of P21 expression and histologic grade as a major prognostic indicator of ACC, P21 may be a useful prognostic indicator in ACC. On the other hand, CDKN1A exon 2 mutation seems inapplicable as a risk factor for ACC development.*

KEYWORDS: *Adenoid Cystic Carcinoma (ACC), P21^{waf}, CDKN1A gene, Exon 2.*

INTRODUCTION

Tumors of salivary glands constitute 1% of all and 3% of head and neck neoplasms[1]. With the frequency of 36%, adenoid cystic carcinoma (ACC) comprises the most common malignancy of the submandibular gland. ACC is specifically characterized with distant metastasis and local recurrence leading to poor prognosis[2]. Histopathologically, it is composed of ductal and myoepithelial cells, which are arranged in solid, cribriform, and tubular growth patterns. Several investigations have indicated that histologic subtype and clinical stage are the most important prognostic factors predicting survival rate in ACC[3].

Despite recent progress in molecular medicine, there is insufficient information about the possible involvement of cell-cycle regulatory proteins and the relevant genes in the pathogenesis of head and neck tumors, especially those originated in salivary glands[1]. Cyclin-dependent kinases (CDKs) and their negative regulators CDK Inhibitors (CDKIs), are important modulators involved in regulating cellular proliferation. There are two distinct groups of CDKIs including INK4 and CIP/KIP inhibitory proteins[4]. The CIP/KIP family includes P21 waf1, P27 Kip1, and P57/Kip2[5].

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The P21^{waf} protein, as a cell-cycle regulator encoded by CDKN1A (P21) gene, acts at the G1-S transition[5]. Binding of wild-type P53 to the promoter region of CDKN1A gene occurs following its accumulation in response to DNA damage. As a result, P21 suppresses the cell-cycle progression via inhibiting the activity of CDK complex. The P21^{waf} level may also be regulated by other mechanisms. Furthermore, P21 is also involved in terminal differentiation and cell senescence, which may play a role in cell maturation and cell death[6,7].

CDKN1A encoding P21^{waf} protein is a tumor suppressor gene located on chromosome 6p21.2. Considering the significant role of this gene in controlling cell-cycle, CDKN1A mutation has been suggested to participate in the development of breast carcinoma, thyroid carcinoma, oral squamous cell carcinoma and cervical cancer[8-11]. Accordingly, most alterations of CDKN1A have been identified in exon 2[8,12,13]. To date, little and controversial information has been reported on CDKN1A gene mutations and p21^{waf} expression in salivary gland tumors. The present study aimed to determine the immunohistochemical expression of p21^{waf} and CDKN1A exon 2 mutation in salivary ACC.

PATIENTS AND METHODS

Study population

A total of forty cases of ACC diagnosed in the Pathology Department of Amir Alam Hospital, Tehran University of Medical Sciences, Tehran, Iran and Oral Pathology Department, Shahid Beheshti University of Medical Sciences, Tehran, Iran between 2010 and 2017 were collected. This research was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (Code No: 1394-80).

The patients' medical records and slides were selected and reviewed to obtain their demographic and clinicopathologic information, including age, sex, tumor location, tumor size, histologic grade, clinical stage, neural invasion, lymph node metastasis, and distant metastasis. The cases with incomplete data and inadequate paraffin-embedded materials were excluded from the study. The histologic grade of the tumors was determined according to WHO classification as follows: Grade I (tubular pattern), Grade II (cribriform pattern), and Grade III (more than 30% of solid pattern) [14].

Immunohistochemistry

The 4-µm-thick sections of formalin-fixed paraffin-embedded blocks were mounted on silane-coated slides (silonized S 3003; Dako, Copenhagen, Denmark). Upon deparaffinization, they were hydrated in 100% xylene and graded ethanol series treated with 3% hydrogen peroxide. The sections were immersed in 10 mM of citrate buffer (PH=6.0) and heated in a microwave oven at 750 W to retrieve the antigens. After cooling to room temperature, the slides were incubated with P21 monoclonal mouse antibody (MS-230-R7, Ready-to-use, Thermo Fisher Scientifi, Waltham, MA, USA) for 24 h. Then, they were washed in Tris-Buffered Saline (TBS) and treated with Dako Envision polymers. The sections were finally counterstained with Mayer's hematoxylin after being incubated with 3,3-diaminobenzidine (K 3468; Dako, Santa Clara, CA, USA) at room temperature for 2-5 min. The positive and negative controls were colon carcinoma and an omitted primary antibody, respectively.

Evaluation of IHC

Tumor cells with brown nuclear or cytoplasmic staining were regarded as positive. Positive cells were independently counted for 1000 cells and graded semi-quantitatively as follows: 0% positive tumor cells (-); < 10% positive tumor cells (score I); 11-50% positive tumor cells (score II); >50% positive tumor cells (score III)[15].

DNA extraction and PCR

DNA was extracted from the paraffin-embedded tissues. Exon 2 of CDKN1A gene was examined for mutation through PCR and sequencing methods. Five to eight 10-µm sections of the embedded tissues were collected from the 1.5-mL autoclaved microtubes. Then, the tissue sections were deparaffinized by washing in 1-mL xylene 3 times at 37°C for 1 h based on the modified method of Goelz et al[16]. The deparaffinized tissues were rehydrated by decreasing ethanol concentration (100% to 70 and 50%). 180 µm of Lysis buffer (50 M Tris-Hcl, 50 mMNaCL, 50 mMEDTA, 1% SDS, pH: 7.6) was added to the rehydrated tissues, which were incubated at 56°C for 24 h after adding 20 µL of proteinase K. Next, 1 µL of RNase was added to each microtube to be then incubated at 37°C for 15-30 min. Afterwards, DNA was refined by using the instruction provided by a tissue and blood kit for DNA extraction (DYNABIO Co., Cat No: KI0015).

PCR and Sequencing

For amplifying exon 2 of CDKN1A gene, we utilized PCR by using *pfu* polymerase (Master Mix, Bioneer Co., Code: K2022, Republic of Korea) and primer pairs of the following sequences:

- Forward primer: GCGCCATGTCAGAACCGGC
- Reverse primer: GAGAATCCTGGTCCCTTAC

The PCR was carried out under the following conditions: initial denaturation (94°C for 5 min); denaturation (35 cycles at 94°C for 45 s); annealing (55°C for 60 s); extension (72°C for 60 s): and final extension (72°C for 5 min).

The PCR products and DNA ladder (50-1000 bp) were separately loaded into agarose gel wells and agarose gel electrophoresis was performed. The 496-bp band was separately purified from the gel for each sample. The purified products were sequenced by using the forward and reverse primers via the Sanger method (Bioneer Co., Republic of Korea). To analyze the sequencing procedure, NCBI Reference Sequence (NG_009364.1) was applied to NCBI Basic Local Alignment Search Tool (BLAST) program.

Statistical analysis

SPSS 18 software package (SPSS Inc., Chicago, IL, USA) was utilized for storing and analyzing the data. Pearson's chi-square test, Fisher's exact test, Kruskal-Wallis test, and Spearman's correlation coefficient were employed to evaluate the relationships of P21 expression and mutation with the clinicopathologic parameters. The significant level of all tests was set as $p<0.05$.

RESULTS

In this study, the samples of 40 patients with ACC (18 males and 22 females) aged 29-80 years (average of 51.85±13.55) were investigated. The tumor size ranged from 1.5 to 11 cm (mean of 4.7±2.7). The locations of the tumors are depicted in Table 1. The tumors with histologic grades of I, II, and III represented 12 (30%), 18 (45%), and 10 (25%) cases, respectively. The clinical stages I, II, III, and IV constituted 4 (10%), 15 (37.5%), 12 (30%), and 9 (22.5%) of patients, respectively. Neural invasion, lymph node metastasis, distant metastasis, and recurrence were observed in 19 (47.5%), 3 (7.5%), 2 (5%), and 14 (35%) cases, respectively (Table 1). According to Spearman's correlation coefficient analysis, the tumor size revealed significant association with recurrence (p=0.048) and clinical stage (p=0.046).

TABLE 1. Clinicopathologic parameters of patients with adenoid cystic carcinoma.

Parameter	*No (%)*
Age (year)	
Mean (SD)	51.85 (13.55)
Range	29-80
Gender	
Female	22 (55)
Male	18 (45)
Location	
Palate	20 (50)
Parotid gland	8 (20)
Submandibular gland	7 (17.5)
Buccal mucosa	2 (5)
Floor of mouth	2 (5)
Tongue	1 (2.5)
Histologic grade	
I	12 (30)
II	18 (45)
III	10 (25)
Stage	
I	4 (10)
II	15 (37.5)
III	12 (30)
IV	9 (22.5)
Lymph node metastasis	
Yes	3 (7.5)
No	37 (92.5)
Distant metastasis	
Yes	2 (5)
No	38 (95)
Recurrence	
Yes	14 (35)
No	26 (65)
Neural invasion	
Yes	19 (47.5)
No	21 (52.5)

P21 Immunoexpression

Well-delineated nuclear staining of P21 was detected in 85% (34/40) of the tumors. Figures 1 to 3 demonstrate P21 nuclear expression in the different histologic types of ACC. Of 34 cases with positive P21 staining, 12 (30%), 14 (35%), and 8 (20%) cases showed scores of I, II, and III, respectively. Based on Spearman's correlation coefficient analysis, a significant inverse correlation was found between P21 expression and histologic grade (p=0.033, r=-0,338). However, no significant correlations were detected between P21 expression and neither age, sex, tumor size, location, stage, neural invasion, lymph node metastasis, distant metastasis, nor recurrence (Table 2).

Molecular analysis of CDKN1A gene

Out of the 40 paraffin-embedded tissue blocks, the 496-bp PCR products of 32 samples had enough concentrations for being sequenced based on the Sanger method (Bioneer Co., Republic of Korea) (Figure 4). No mutation was found in the exon 2 in the sequence of each sample via NCBI BLAST program as compared to NCBI Reference Sequence: NG_009364.1 (Figure 4).

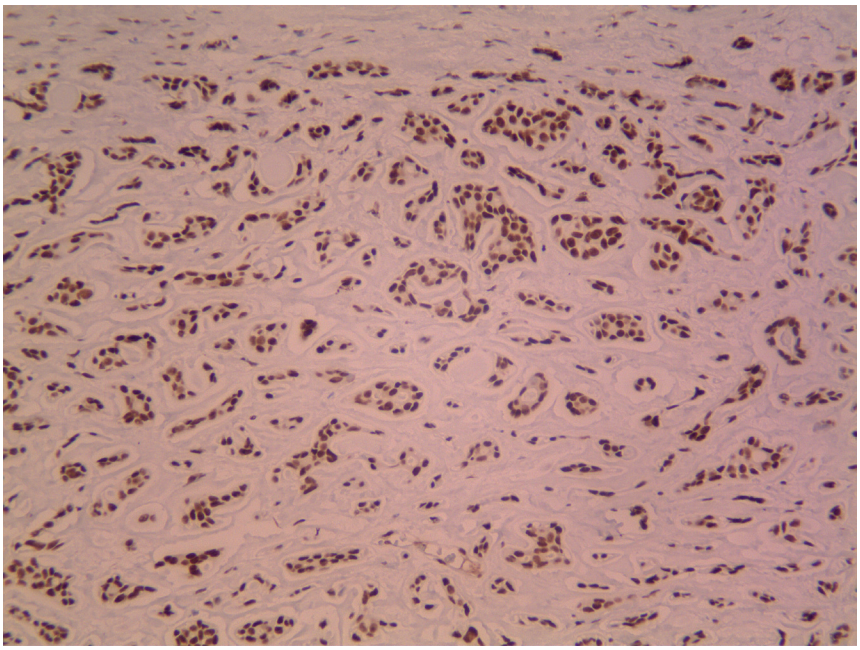

Fig. 1. P21 Immunoexpression in tubular pattern of ACC (x200).

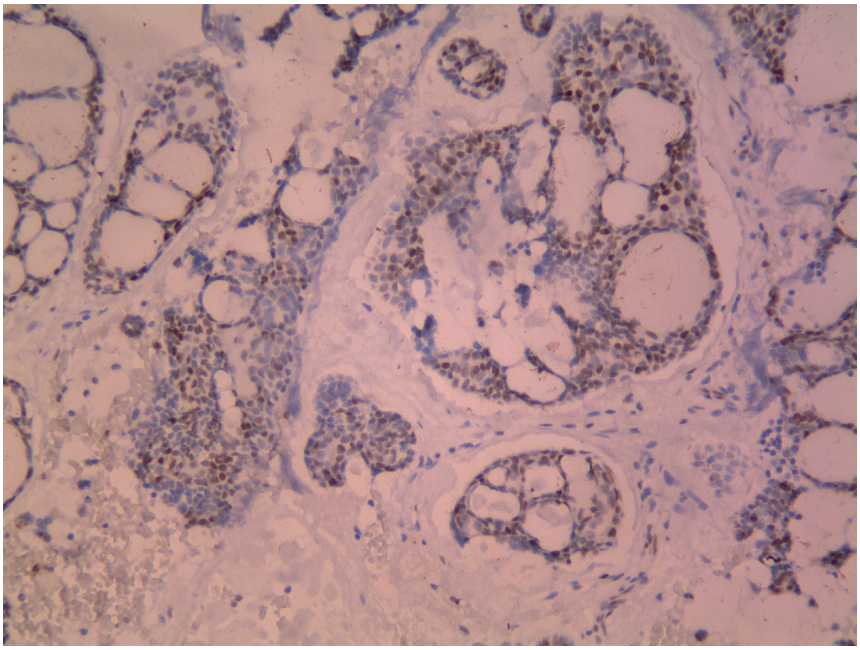

Fig. 2. P21 Immunoexpression in cribriform pattern of ACC (x200).

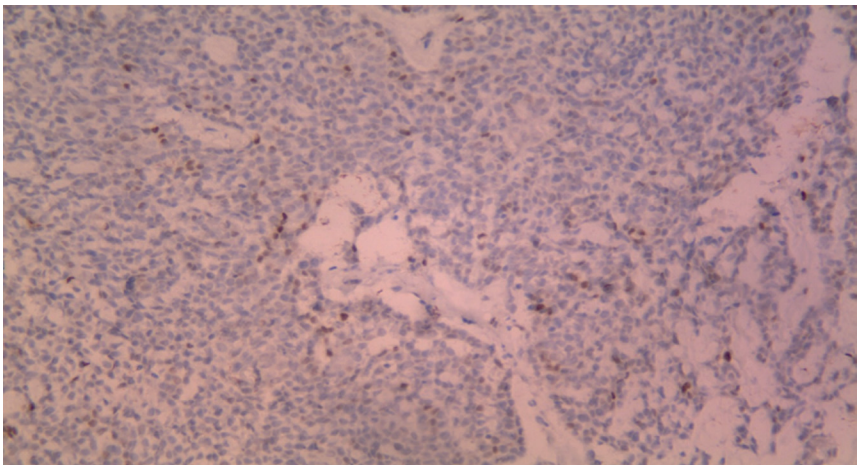

Fig. 3. P21 Immunoexpression in solid pattern of ACC (x200).

TABLE 2. Correlation of clinicopathologic variables with P21 expression in adenoid cystic carcinoma.

Variable	*P21 expression*				
	0 (n, %)	*I (n, %)*	*II (n, %)*	*III (n, %)*	*p-value*
Gender					
Male	2 (11.1%)	5 (27.8%)	7 (38.9%)	4 (22.2%)	0.891
Female	4 (18.2%)	7 (31.8%)	7 (31.8%)	4 (18.2%)	
Histologic grade					
I	1 (8.3%)	2 (16.7%)	5(41.7%)	4 (33.3%)	0.033*
II	1 (5.6%)	7 (38.9%)	8 (44.4%)	2 (11.1%)	
III	4 (40.0%)	3 (30.0%)	1 (10.0%)	2 (20.0%)	
Clinical Stage					
I	0 (0.0%)	2 (50.0%)	1 (25.0%)	1 (25.0%)	0.289
II	1 (6.7%)	4 (26.7%)	7 (46.7%)	3 (20.0%)	
III	2 (16.7%)	4 (33.3%)	4 (33.3%)	2 (16.7%)	
IV	3 (33.3%)	2 (22.2%)	2 (22.2%)	2 (22.2%)	
Neural invasion					0.828
Yes	4 (66.7%)	5 (41.7%)	6 (42.9%)	4 (50.0%)	
No	2 (33.3%)	7 (58.3%)	8 (57.1%)	4 (50.0%)	
Lymph node metastasis					
Yes	1 (16.7%)	0 (0.0%)	1 (7.1%)	1 (12.5%)	0.484
No	5 (83.3%)	12 (100.0%)	13 (92.9%)	7 (87.5%)	
Distant metastasis					
Yes	0 (0.0%)	0 (0.0%)	1 (7.1%)	1 (12.5%)	0.785
No	6(100.0%)	12 (100.0%)	13 (92.9%)	7 (87.5%)	
Recurrence					
Yes	3 (50.0%)	5 (41.7%)	4 (28.6%)	2 (25.0%)	0.700
No	3 (50.0%)	7 (58.3%)	10 (71.4%)	6 (75.0%)	

$p<0.05$ is significant*

DISCUSSION

ACC is a rare cancer with a high potential for recurrence and distant metastasis. The efficacies of current therapeutic approaches (i.e. surgery and radiation) have been very limited, particularly in patients with advanced ACC, due to lack of understanding on the molecular etiology and potential therapeutic targets[17].

P21 (WAF1/CIP1; CDKN1A) is a universal cell-cycle inhibitor, which is regulated through both P53- dependent and P53-independent pathways[18]. The altered P21waf expression has been demonstrated in human malignancies. P21 over-expression in cutaneous squamous cell carcinoma (SCC) [7], non-small-cell lung carcinoma (NSCLC)[19], head and neck cancers[20], hepato-cellular carcinoma (HCC)[21]

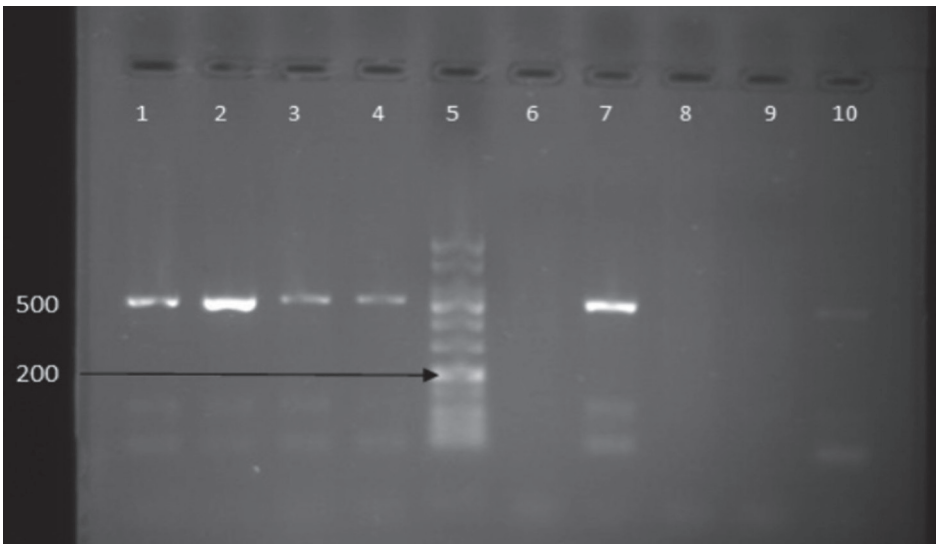

Fig. 4. 1.5% gel electrophoresis analysis of PCR products. Lane 1, 2, 3, 4, 6, 7, 8, 9, 10: PCR products of amplified DNA extraction from 9 patients' deparaffinized blocks sections. Lane 5: DNA size marker (50-1000 bp).

and glioma[22] have been reported. On the other hand, P21 low expression has been observed in colorectal carcinoma and epithelial ovarian cancer[23,24].

In the present study, 85% of the examined ACCs showed nuclear P21 immunoreactivity, which was opposed to the results of the previous studies conducted on salivary gland tumors[4, 25]. This discrepancy may be due to differences in studied tumors, staining methods, and manufactured antibodies. For instance, in the study of Affolter et al[4], 72% of the cases showed negative or low P21 expression based on routine IHC staining. However, TSA-IHC staining led to P21 expression in all the cases. The recent findings indicated that TSA-IHC staining is probably a more useful method compared with routine IHC staining for detecting P21.

Considering the complex and different functions of P21 in cancer development, the association of P21 expression and clinicopathological parameters have been evaluated in various cancers. However, these studies resulted in highly variable and controversial findings. In the present study, a statistically significant inverse correlation was observed between histologic grade and P21 expression in ACC, which was in line with the results of Matsushima et al[26]. This finding may root in the tumor suppressor function of P21, as well as its significant roles in cellular differentiation and maturation. Accordingly, we observed P21 overexpression in well-differentiated tumors. Nonetheless, no significant relationships were noted between P21 expression and other clinicopathological parameters, including clinical stage, neural invasion, metastasis, and recurrence. Our results were in accordance with previous studies[15,26-32]. In another study, however, P21 low expression has been associated with advanced tumor stage in epithelial ovarian cancer[33]. Similarly, Pérez-Sayáns et al[28] and Xie et al[34] reported that either the lack of expression or low expression of P21 has been related to lymph node metastasis and lower disease-free survival in oral and tongue SCC. On the other hand, Nemes et al[35] reported an association between P21 over expression and both advanced tumor stage and lymph node metastasis in oral SCC suggesting P21 overexpression as a poor prognostic indicator in oral SCC. In a study by Ng et al[18], P21 expression also correlated with the proliferating activity of tumors, particularly in elderly and female patients. It is hypothesized that P21 immunoreaction and function in different tumors may reflect the expression level of CDKN1A and intracellular localization of P21 [6].

Few studies have been conducted on the genetic alterations in ACC. Seethala et al[36] reported C-MYC amplification and its role in high-grade transformation in ACC. Aberrant methylation of promoter regions in CDKI genes in 34 (92%) patients of ACC was reported by Daa et al[37]. The present report was the first human study evaluating the mutational status of exon 2 of CDKN1A gene in ACC. Nevertheless, we observed no mutation in exon 2 of CDKN1A gene. Genetic alterations of CDKN1A gene have been evaluated in different cancers. Ralhan et al[10] reported polymorphism of codon 149 (A-G) in CDKN1A gene in 37% of patients with oral SCC and oral dysplastic lesions. Interestingly, oral SCC and precancerous lesions with the wild type of P53 represented higher frequency of the codon 149 polymorphism than those harboring mutated P53. Ibrahim et al[12] documented mutation of exon 2 of CDKN1A gene in 14-43% of different tumors with more predominance in Sudanese people. In another study, Facher et al[38] demonstrated the role of polymorphism in exons 2 and 3 of CDKN1A in the pathogenesis of prostate adenocarcinoma and head and neck SCC indicating P21 as a major participant in the development of these cancers. Polymorphisms of CDKN1A have also been demonstrated in Indian consumers of betel quid[39]. Akhter et al[8] stated that CDKN1A gene mutation and hyper-methylation led to complete silencing of CDKN1A gene in breast cancer indicating an interaction between genetic and epigenetic mechanisms in regulating CDKN1A gene. Allahbakhshian et al[40] who evaluated P14ARF, P27KIP1 and P21CIP1 in acute myeloid leukemia (AML) described no significant alteration in P21 mRNA expression in AML patients.

Other studies have asserted that CDKN1A mutation or deletion may rarely be identified in human tumors. Instead, P21 relocation from nucleus to cytoplasm may be the main contributor inactivating tumor-suppressor functions of P21 and inducing its oncogenic features. In accordance, the cytoplasmic localization of P21 has been associated with poor prognosis in a wide range of tumors[6]. On the other hand, Galanos et al[41] proposed that nuclear P21 can induce genomic and chromosomal instability through modulating replication stress and error-prone DNA repair in P53 mutant cells. These functions reflect the intricacy of P53/P21 pathway in cancer pathogenesis.

It is worth noting that P21/P53 interaction and expression level have been proposed to affect the efficacy of cancer treatment modalities such as chemotherapy and immunotherapy[42,43]. In this regard, Bakhshi et al[42] reported apoptosis induction through increasing level of P53, P21 and Caspase-8 mRNA in HCC cell line exposed to Vanadium complex. In the recent study, the highest rate of expression among the pro-apoptotic genes was related to P21 highlighting the role of P53-P21 signaling pathway in the Vanadium –induced apoptosis in cancer cells.

In recent years, immune checkpoint blockade therapy has presented a promising anticancer therapy increasing the therapeutic efficacy in syn-

ergy with chemotherapy[43,44]. In this vein, Iano et al[44] examined the impact of Doxorubicine (DXR) treatment in three human breast cancer cell lines senescence and their susceptibility to two different types of immune cell-mediated cytotoxicity. They observed that DXR treatment induced typical senescence in cell lines with mutant P53 and increased P21 expression in cell line with WTP53. Importantly, DXR-treated senescent cells showed increased sensitivity to activated CD4+ T cells and natural killer cells.

CONCLUSIONS

Regarding the significant association between P21 expression and histologic grade as a major prognostic indicator of ACC, P21 may serve as an applied prognostic indicator in ACC. According to our findings, CDKN1A exon 2 mutations seem to impart no role in the development of ACC. However, further investigations are recommended to evaluate p53 expression, methylation and other epigenetic changes in CDKN1A in tumorigenesis in ACC.

Ethical approval:
This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.

Conflict of interest:
The authors declare that they have no conflict of interest.

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