The incidence of mTOR marker in tracheal adenoid cystic carcinoma by immunohistochemical staining

Abstract

Introduction: There is an association between the activation of mammalian target of rapamycin (mTOR) signaling and aggressive tumor growth in multiple forms of cancer, including adenoid cystic carcinoma (ACC). ACCs are uncommon yet a malignant form of neoplasms that arises within the secretory glands. Therefore, the aim of this study was to investigate the increase of mTOR in the ACC tumors in order to survey the possibility of treating these tumors with mTOR inhibitors.

Material and methods: Samples from known cases of the lung and tracheal ACC were retrieved from the archives of the pathology department of Masih Daneshvari hospital, and immunohistochemical (IHC) staining for mTOR was performed on them. After preparation of the blocks with specific antibodies, tumor cells with cytoplasmic and/or nuclear expression of mTOR were considered as positive cells by applying a specific scoring method introduced in this study.

Results: The paraffin blocks of 26 patients were surveyed and the IHC marker of mTOR was positive in the tumors of 10 patients (38.5%). Out of 10 mTOR positive cases, 5 were females and 5 were males. The primary site of the surveyed tumors was the trachea and bronchus in 12 cases (46%), salivary glands in 7 individuals (27%), and lung tissue in 7 cases (27%), and there was no significant correlation between the primary site of the ACC tumors and the existence of the mTOR markers in them (P = 0.67). From all cases, 13 patients (50%) had cribriform and tubular cells without solid components, 9 cases (34.6%) had cribriform and tubular with less than 30% of solid components, and 4 cases (15.4%) had cribriform and tubular cells with more than 30% of solid components. There was no significant difference between the morphologies and the existence of mTOR markers in them (P = 0.741).

Conclusions: As the incidence of mTOR markers is seen in patients with tracheal ACC, evaluation and scoring of mTOR in these persons can be helpful as further studies can distinguish the use of it in the treatment of the disease.

Key words: immunohistochemistry, adenoid cystic carcinoma, mTOR

Adv Respir Med. 2020; 88: 305–312

Introduction

The mammalian target of rapamycin (mTOR) family is expressed in most mammalian cells to control growth and metabolism [1–5]. The mTOR1 and mTOR2 complexes are involved in normal cell growth and developmental process and are crucial for its viability by regulating the...
kinases of the AGC family, protein synthesis and autophagy [6]; accordingly, their dysregulation is implicated in the pathophysiology of several diseases, including cancer, type 2 diabetes, and neurodegeneration [7].

There is a correlation between the activation of mTOR signaling and aggressive tumor growth in many cancers [8]. As a matter of fact, after the hyperactivation of mTOR signaling, the tumors grow at an increased rate and they are less likely to disappear because of an increased amount of protein synthesis and inhibition of autophagy [9, 10]. Also, the activated mTOR pathway helps proliferation, migration, and survival of tumor cells, which increases the tumor invasiveness [11]. In addition, this pathway reduces the tumor cells sensitivity to chemotherapy and hormonal treatment [12]. Hence, inhibition of its activity increases the chemotherapy effectiveness and improves the outcome of the treatment of these tumors [13]. The natural inhibitor of mTOR is rapamycin. Temsirolimus and everolimus are other important inhibitors whose effectiveness in the treatment of different cancers has been proven [13, 14].

Adenoid cystic carcinoma (ACC) as an aggressive neoplasm of salivary glands is the second most common primary malignancy of tracheal neoplasm, in whose pathophysiology the mTOR pathway may be involved [15]. Although the incidence of this tumor is low, about 90% of them are malignant [16]. The treatment of the patients with ACC includes surgical resection of the tumors with their margins and reconstruction, chemotherapy, and radiotherapy [17], and as mentioned, if the marker of the mTOR pathway is high in these tumors, using the mTOR inhibitors may increase the chemotherapy effectiveness. The aim of this study was to investigate the incidence of mTOR in the ACC tumors in order to survey the possibility of treating them with mTOR inhibitors.

**Material and methods**

The samples of the Pathology department of Masih Daneshvari hospital were searched for ACC tumors of the trachea with diagnosis confirmed between 2010 and 2014, with the approval of the institutional review board. Then, paraffin blocks of known cases of the ACC of the lung and trachea (primary and metastatic carcinoma) were retrieved from the archives of the department and reviewed by two different pathologists without knowledge of the initial diagnosis.

**Immunohistochemistry**

Formalin-fixed paraffin blocks of known cases of the ACC of the lung and trachea were used in our study. In brief, following dewaxing, washing and rehydration of the slides through xylene and graded alcohols, microwave heating in citrate buffer were used for antigen retrieval. Endogenous peroxidase was blocked in ChemMate peroxidase-blocking solution (Dako). Phospho-mTOR (Ser2448) — Cell Signalling #2976, monoclonal rabbit, dilution 1:50 was the main antibody and EnVision™ Dual Link System (Dako) was used as a detection system [18].

A positive immunohistochemical reaction was indicated by brown cytoplasmic and/or nuclear staining. Cases were scored on the basis of the visually estimated percentage of tumor cells with positive cytoplasmic and/or nuclear staining.

**Scoring**

After the staining, tumor cells with cytoplasmic and/or nuclear expression of mTOR were considered as positive cells according to the scoring method presented below:

1. In the regions with the highest density of positive cells for each slide, the percentage of positive cells in 5 separate fields which had ≥1000 adjacent cells were counted and scored as: percentage of positive cells less than 1%: 0, between 1% and 25%: 1, 26% to 50%: 2, 51% to 75%: 3, 76% to 100: 4 (Table 1).
2. The color intensity of the stained blocks was scored based on color intensity as follows: lack of brown 0, mild: 1, moderate: 2, severe: 3 (Table 2).
3. The two obtained points for each tissue were multiplied to calculate the final scores (maximum score: 12) and the final scores were categorized into: 0-1: negative for mTOR, 2-3: weakly positive for mTOR, 4-6: positive.

| The percentage of positive cells | Score |
|----------------------------------|-------|
| < 1%                             | 0     |
| 1–25%                            | 1     |
| 26–50%                           | 2     |
| 51–75%                           | 3     |
| 76–100%                          | 4     |
for mTOR, 7–12: strongly positive for mTOR (Table 3).

4. For statistical analysis, the samples were divided into two groups: positive for mTOR marker (final score > 3) and negative for mTOR marker (final score ≤ 3).

**Statistical analysis**

The data was analyzed using SPSS V22 software and an ANOVA test followed by a Chi-square test, a T-test and a Fisher’s exact test.

| Color intensity | Score |
|-----------------|-------|
| Lack of brown   | 0     |
| Mild            | 1     |
| Moderate        | 2     |
| Severe          | 3     |

| Final score | The existence of mTOR |
|-------------|-----------------------|
| 0, 1        | Negative for mTOR     |
| 2, 3        | Weakly positive for mTOR (+) |
| 4–6         | Positive for mTOR (++) |
| 7–12        | Strongly positive for mTOR (+++) |

**Table 4. Demographic information, tumor location, mTOR existence and morphology of tumor cells**

| Variable                      | Number of ACC | Percentage | P value* |
|-------------------------------|---------------|------------|----------|
| Gender                        |               |            |          |
| Male                          | 13            | 50         | 1        |
| Female                        | 13            | 50         |          |
| Age                           |               |            |          |
| < 50 years old                | 11            | 42.3       | 0.48     |
| > 50 years old                | 15            | 57.7       |          |
| The primary site of the tumors|               |            |          |
| Trachea and bronchus          | 12            | 46         | 0.67     |
| Salivary glands               | 7             | 27         |          |
| Lung                          | 7             | 27         |          |
| Type of tumor                 |               |            |          |
| Primary                       | 20            | 77         | 0.457    |
| Metastatic                    | 6             | 23         |          |
| mTOR existence                |               |            |          |
| Positive                      | 10            | 38.5       | N/A      |
| Negative                      | 16            | 61.5       |          |
| Morphology                    |               |            |          |
| Morphology**                  | 13            | 50         | 0.741    |
| Morphology***                 | 9             | 34.6       |          |
| Morphology****                | 4             | 15.4       |          |

*P value for comparing variables based on the existence of mTOR marker; **cribriform and tubular cells without solid components; ***cribriform and tubular with less than 30% of solid components; ****cribriform and tubular cells with more than 30% of solid components

**Results**

In the study, a total number of 32 patients with adenoid cystic carcinoma were reviewed by pathologists. Six patients were excluded due to the prior history of radiotherapy. Finally, the paraffin blocks of 26 subjects were surveyed (Table 4) and the immunohistochemistry (IHC) marker of the mTOR expression was positive in the tumors of 10 patients (38.5%). From 10 mTOR positive cases in immunohistochemistry surveys, 5 were females and 5 were males. Also, negative cases for mTOR were seen equally among men and women. Therefore, there was no difference between men and women in the existence of mTOR markers in the ACC tumors (Table 4).

The mean age of patients was 53.12 ± 15.3 and 57.7% of them (n = 15) had a mean age of 50 years or older (Table 4). There was no significant correlation between the existence of mTOR markers in ACC tumors and the age of the patients (P = 0.48).

The primary site of the surveyed tumors was the trachea and bronchus in 12 cases (46%), salivary glands in 7 cases (27%), and lung tissue in 7 cases (27%) (Table 4). The frequency of mTOR positive patients based on the primary site of the tumors are summarized in Table 5. The percentage of mTOR positive cases in tracheal and bronchial tumors was 41.7%, in salivary gland tumors was 28.6%, and in lung tumors was 42.8%. In the study, there was no significant correlation between the primary site of the ACC tumors and the existence of mTOR markers (P = 0.67).
Among these tumors, 20 tumors (77%) were the primary tumors and only 6 of them (23%) were metastases from other sites (4 cases with metastasis from salivary glands and 2 cases with metastasis from the trachea) (Table 4). From the mTOR positive cases, 8 of them (40%) were primary tumors and only 2 of them (33.3%) were metastatic tumors (Table 6). There was no significant difference between metastatic or primary tumors in the existence of mTOR markers (P = 0.457).

In terms of the morphology of tumor cells, 13 cases (50%) had cribriform and tubular cells without solid components (morphology 1), 9 cases (34.6%) had cribriform and tubular with less than 30% of solid components (morphology 2), and 4 cases (15.4%) had cribriform and tubular cells with more than 30% of solid components (morphology 3). There was no significant difference between different morphologies in the existence of mTOR markers (P = 0.741). Different morphologies of the ACC tumor cells are shown in Figure 1.

Also, in the study, we investigated the site of the staining (Figure 2) in the tumor cells (cytoplasmic, nuclear, or both cytoplasmic and nuclear) based on the three types of morphology - the results are summarized in Table 7. Out of 10 patients who were considered positive (3 < final score), 4 had nuclear staining, 4 had cytoplasmic staining and 2 had both nuclear and cytoplasmic staining. Also, out of the 16 patients who were considered negative (3 > final score), 8 had no staining, 7 had cytoplasmic staining, 1 had both nuclear and cytoplasmic staining, but none had nuclear staining.

Discussion

This study was conducted to investigate the incidence of mTOR in the paraffin blocks of the patients with ACC tumors using immunohistochemistry technique. According to our surveys, the IHC marker of mTOR expression was positive in the tumors of 10 cases (38.5%) out of the 26 surveyed. In a similar study by Wang Li et al., the mTOR marker in non-small cell lung cancer in 43.5% of 78 patients was positive [19]. Also, in the study by Meiling Wen et al. about the importance of mTOR expression in patients with colon cancer, out of 106 subjects with this cancer, 80 patients (75.5%) were mTOR positive [20]. It seems that the differences can be related to the different sample sizes, which suggests the need for more studies in this regard with larger sample sizes.

In addition, out of 10 mTOR positive patients in this study, 5 were females and 5 were males, and there was no difference between men and women in the expression of mTOR. The mean age of the patients in the study was 53.12 years and there was no significant correlation between the existence of mTOR markers and the age of the patients (P = 0.48). These results are consistent with the outcomes of the study by Wang et al. on the expression of mTOR in 210 patients with lung cancer and the results of the study of Meiling Wen et al.; in both studies, it has been shown that there is no significant relationship between the expression of mTOR and age and sex [19, 20].

In our study, 20 patients with ACC (77%) had primary tumors and the mTOR marker was

| IHC survey | Trachea and bronchus | Percentage | Salivary glands | Percentage | Lung | Percentage | P value |
|------------|----------------------|------------|----------------|------------|------|------------|---------|
| Positive   | 5                    | 41.7       | 2              | 28.6       | 3    | 42.8       | 0.67    |
| Negative   | 7                    | 58.3       | 5              | 71.4       | 4    | 57.2       |         |
| Total      | 12                   | 100        | 7              | 100        | 7    | 100        |         |

IHC — immunohistochemical
positive in 8 of them (40%), 6 patients had a metastatic tumor, and the mTOR marker was positive in 2 cases (33.3%). Hence, there was no significant difference between metastatic or primary tumors in the existence of mTOR markers ($P = 0.457$), although more studies with larger sample sizes are required.

The ACC tumors were divided into three groups according to the morphology: cribriform and tubular without solid components, cribriform and tubular with less than 30% of solid components, and cribriform and tubular with more than 30% of solid components [21]. Of the 26 patients, 13 cases were in the first group with 6 (42.8%) mTOR positive patients, 9 in the second group with 3 (37.5%) mTOR positive cases and 4 in the third group with 1 (25%) mTOR positive case. Although this difference is not statistically significant in our study, it seems that there is a reverse correlation between the percentage of solid components and the existence of mTOR markers, and according to the direct correlation between the high percentage of the solid component and the grade of cancer, it can be deduced that more invasive ACCs with higher grades have lower incidence of mTOR markers. This is consistent with the results of the study by Wang Li et al. that has shown that low-grade and moderate-grade neuroendocrine tumors have a higher incidence of mTOR markers than high-grade tumors [19].

Although the incidence of tracheal ACC tumors is low, about 90% of them are malignant [22], and their diagnosis is delayed due to the slow growth rate, and their atypical symptoms and patients are misdiagnosed with asthma or bronchitis [23]. The everolimus and temsirolimus are approved as anticancer drugs which targeted the inhibition of the mTOR pathway in the clinic [24].

The limitation to the study that is worth mentioning was the restricted number of cases that was due to the rare incidence of the disease. Also,
Table 7. The site of the staining of tumor cells and the color intensity distinguishing between positive and negative cases

| Variable                          | ACC            | Morphology 1 | Morphology 2 | Morphology 3 | Total |
|-----------------------------------|----------------|--------------|--------------|--------------|-------|
| The site of the staining in the positive cases | Negative       | –            | 2            | –            | 2     |
|                                   | Cytoplasmic    | 2            | 1            | 1            | 4     |
|                                   | Nuclear        | 2            | –            | –            | 2     |
|                                   | both cytoplasmic and nuclear | 2 | – | – | 2 |
| Total                             | 6              | 3            | 1            |             | 10    |
| The site of the staining in the negative cases | Negative       | 4            | 3            | 1            | 8     |
|                                   | Cytoplasmic    | 4            | 1            | 2            | 7     |
|                                   | Nuclear        | –            | –            | –            | –     |
|                                   | both cytoplasmic and nuclear | – | 1 | – | 1 |
| Total                             | 8              | 5            | 3            |             | 16    |
| Color intensity in the positive cases | Lack of brown | –            | –            | –            | –     |
|                                   | Mild           | –            | –            | –            | –     |
|                                   | Moderate       | 5            | 4            | 1            | 10    |
|                                   | Severe         | –            | –            | –            | –     |
| Total                             | 5              | 4            | 1            |             | 10    |
| Color intensity in the positive cases | Lack of brown | 4            | 3            | 1            | 8     |
|                                   | Mild           | 6            | –            | 1            | 7     |
|                                   | Moderate       | –            | 1            | –            | 1     |
|                                   | Severe         | –            | –            | –            | –     |
| Total                             | 10             | 4            | 2            |             | 16    |

ACC — adenoid cystic carcinoma
Table 8. The percentage of positive cells in slides distinguishing between positive and negative cases and final scores for all cases

| Variable | Morphology 1 | Morphology 2 | Morphology 3 | Total |
|----------|--------------|--------------|--------------|-------|
| The percentage of positive cells in the slide for positive cases | < 1% | – | – | – |
| 1–25% | – | – | – | – |
| 26–50% | 2 | 2 | 1 | 5 |
| 51–75% | 1 | 2 | – | 3 |
| 76–100% | 2 | – | – | 2 |
| Total | 5 | 4 | 1 | 10 |
| The percentage of positive cells in the slide for negative cases | < 1% | 4 | 3 | 1 |
| 1–25% | 4 | 1 | 1 | 6 |
| 26–50% | 2 | – | – | 2 |
| 51–75% | – | – | – | – |
| 76–100% | – | – | – | – |
| Total | 10 | 4 | 2 | 16 |
| Final scores for all cases | 0, 1 (–) | 7 | 4 | 2 |
| 2, 3 (+) | 1 | 1 | 1 | 3 |
| 4–6 (+++) | 3 | 4 | 1 | 8 |
| 7–12 (++++) | 2 | – | – | 2 |
| Total | 13 | 9 | 4 | 26 |

the study only surveyed patients of one ethnicity. Future research with larger and multietnic data pool can better assess these findings.

Conclusion

For the first time in Iran, the IHC staining was used to investigate the mTOR pathway in the ACC tumors. The site of the staining in the tumor cells (nuclear, cytoplasmic, or both), the intensity of coloring, and the percentage of positive tumor cells were used to present a unique scoring system to be applied in the investigation of mTOR markers in ACC tumors. The mTOR can be used as a target for the treatment of the cancer, and in this study, the presence of mTOR marker in some of the ACC patients has been shown. Thus, further evaluation of using mTOR as suggestive indicator for targeted therapy of ACCs and the effectivity of mTOR inhibitors in the treatment for these tumors are recommended.

Conflict of interest

None declared.

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