Research Paper

Clinicopathological Significance of ATRX Expression in Nasopharyngeal Carcinoma Patients: A Retrospective Study

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Received: 2021.05.30; Accepted: 2021.09.22; Published: 2021.10.03

Abstract

Background: Nasopharyngeal carcinoma (NPC) is the most common head and neck squamous cell carcinoma in south China. Radiation technology improves the local control rates in early NPC. However, the distant metastases are still the main cause of treatment failure. Thus, to find biomarkers for prognosis will help to enhance the survival of NPC. ATRX is a chromatin remodeling protein localized in the nucleus. Deletion or mutation of ATRX gene has been demonstrated in a variety of malignancies. However, the significance of ATRX expression in the prognosis of NPC remains unclear.

Methods: Tumor tissues from 227 NPC patients diagnosed in the Second Xiangya Hospital of Central South University from 2011 to 2016 were selected. Immunohistochemistry was used to detect the ATRX expression level of the tumor tissue. Chi-square test was used to analyze the relationship between ATRX expression and clinical characteristics such as age, sex, T stage, N stage and clinical stage. Kaplan-Meier method was used for survival analysis, and log-rank was used to compare the difference in survival rate.

Results: There were 53 patients with negative ATRX expression, accounting for 24.2% of the total group. ATRX expression was not significantly associated with age, sex, N stage, clinical stage, and progression-free survival (PFS) (P>0.05). However, patients with negative ATRX expression had earlier T staging (P=0.045) and a higher 5-year overall survival (84.9% vs 66.9%, P=0.022).

Conclusions: Loss of ATRX expression may contribute to better prognosis in patients with NPC.

Key words: Nasopharyngeal Carcinoma; ATRX; Staging; Survival; Prognosis

Introduction

Nasopharyngeal Carcinoma (NPC) is the most common head and neck squamous cell carcinoma in south China and southeastern Asia [1, 2]. Due to its radiosensitive behavior, radiation (RT) and chemoradiotherapy (CRT) has become the cornerstone of the treatment for early or locoregionally advanced diseases [3, 4]. However, about 25-30% of the patients will suffer from disease relapse or metastasis, and the prognosis of advanced patients is poor. The biological behavior of NPC varied dramatically in individual patients. Therefore, seeking for biomarkers for survival prediction, patient stratification and treatment adaptation is urgently needed [5].

Alpha thalassemia/mental retardation X-linked (ATRX) is a protein containing an ATPase/helicase domain, which belongs to the switch/sucrose nonfermentable (SWI/SNF) family of chromatin remodeling proteins [6]. It is found to be involved in cell cycle-dependent phosphorylation, which regulates its nuclear matrix and chromatin association and suggests its involvement in gene regulation at interphase and chromosomal segregation in mitosis [7]. It has been demonstrated that ATRX is frequently mutated in a variety of mesenchymal tumors.
including gliomas [8], neuroendocrine neoplasms [9], and sarcomas [10]. ATRX mutation leads to decreased ATRX protein expression, and results in tumor genome instability, higher tumor mutation burden, and thus leading to increased sensitivity to chemotherapy, radiation therapy and immunotherapy agents [11, 12]. However, the data on the incidence of ATRX expression and clinical significance in epithelial carcinomas is limited. It is reported that ATRX loss predicts good prognosis in cervical carcinoma and hepatocarcinoma [13, 14]. And ATRX mutation results in increased immune checkpoint inhibitor (ICI) sensitivity in NSCLC [12]. However, the significance of ATRX expression in the prognosis of NPC remains unknown.

In the present study, we evaluated the ATRX expression in NPC patients via immunohistochemistry and analyzed the correlation between ATRX expression and clinicopathological characteristics and patient’s prognosis, and demonstrated that ATRX deficiency is correlated with earlier T stage and longer overall survival in NPC patients.

**Materials and Methods**

**Patients enrollment and clinical data**

Patients who were diagnosed with NPC at the Second Xiangya Hospital of Central South University (Hunan, China) between 2011 and 2016 were retrospectively reviewed. And patients without complete clinical data or unavailable paraffin blocks were excluded. Finally, a total of 227 patients were enrolled in this study. Two pathologists (HH Wang and SQ Fan) independently reviewed all H&E staining and IHC staining slides. And all the clinical and pathological characteristics were obtained from electronic medical records system of the hospital. Approval for using the patient material in this study was obtained from the Ethics Committee of the Second Xiangya Hospital of Central South University. All procedures were conducted in accordance with the declaration of Helsinki. Tissue samples were obtained from the Department of Pathology of the Second Xiangya Hospital of Central South University.

**Immunohistochemical staining and semi-quantitative scores**

Immunohistochemical staining for ATRX was performed with 4-μm-thick sections from paraffin blocks using the MaxVision HRP polymer anti-rat IHC kit. Each section was deparaffinized and rehydrated, and high-temperature antigen retrieval was achieved for all antibodies by heating the samples in 0.01 M citrate buffer in a domestic microwave oven at full power (1000 W) for 15 mins. Besides internal positive control, positive control slides were included in every experiment. Methanol containing 3% H2O2 was applied for fifteen minutes to inactivate endogenous peroxidase. The slides were incubated with a rabbit monoclonal antibody against human, a 1:200 dilution of the primary antibody to ATRX (Sigma, Sigma–Aldrich, St. Louis, MO, USA) at 4 °C overnight. Then, all the slides were rinsed with PBS three times for 5 mins each. After the incubation with secondary antibody, the visualization signal was conducted with 3,3′-diaminobenzidine tetrachloride. Subsequently, all slides were counterstained with hematoxylin. The specificity of the antibody was determined with matched IgG isotype antibody as a negative control. Tissue samples were evaluated independently by HHW and SQF who were blinded to the clinicopathological data, at 200× magnification light microscopy. Nucleus staining of the tumor cells was assessed. Representative microscopic photos are shown in Figure 1. According to the proportion of nucleus staining of the tumor cells, the ATRX expression was classified as negative and positive. ATRX staining loss of tumor nucleus was defined as ATRX negative while ATRX positive was defined as that ATRX is expressed in at least 1% of nuclei in the tumor cells. The concordance rate between the two pathologist is 97%.

**Figure 1.** Immunohistochemical study of ATRX expression in NPC tissues. The intensity of nucleus staining was graded as negative (A) or positive (B) (A-B, ×200).
Statistical analysis

Statistical analysis was performed using SPSS software V.22 (IBM, Armonk, USA). The χ² test was used to evaluate the association between ATRX expression and clinicopathological parameters including Age, Gender, T stage, N stage and clinical stage according to AJCC 8th staging system. Progression-free survival (PFS) and overall survival (OS) curves were plotted using Kaplan-Meier method, and the log-rank test was used to compare the differences. P<0.05 was considered as statistically significant.

Results

Patient characteristics

The clinicopathological characteristics of the patients are summarized in Table 1. A total of 227 patients were enrolled, among which 138 (60.8%) patients were less than 50 years old, with a median age of 48 years. 156 (68.7%) patients were men, with a male-to-female ratio of 2.2:1. All patients were diagnosed with NPC and treated with chemoradiation therapy. 26 (11.5%) were T1 stage, 88 (38.8%) were T2 stage, 68 (30.0%) were T3 stage and 45 (19.8%) were T4 stage. 15 (6.6%) were N0 stage, 37 (16.3%) were N1 stage, 144 (63.4%) were N2 stage and 31 (13.7%) were N3 stage. According to the 8th AJCC staging system, 24 (10.6%) were stage II, 132 (58.1%) were stage III and 71 (31.3%) were stage IV. A total of 103 (45.4%) patients received concurrent chemoradiation, and 124 (54.6%) received sequential chemoradiation. Among all the patients, 145 (63.9%) received cisplatin-based chemotherapy, 68 (30.0%) received nedaplatin-based chemotherapy, while 14 (6.1%) received other agents.

Correlations between ATRX expression and clinicopathological factors

NPC patients with ATRX expression, defined as ATRX positive staining in at least 1% of nuclei in the tumor cells, consist of 76.7% (174/227) of the cohort. And 23.3% (53/227) of the cohort had ATRX loss. The association between ATRX expression and the clinicopathological factors are shown in Table 2. ATRX loss was significantly correlated with earlier T stage (p=0.045). Statistical significance was not found in sex, age, ECOG, smoking history, hemoglobin level, treatment mode, chemotherapy agent type, N stage and AJCC stages.

Impact of ATRX expression on the prognosis of NPC patients

As shown in Figure 2, NPC patients with ATRX loss had significantly longer overall survival than those with ATRX expression (84.9% vs 66.9%, P=0.022). No prognostic significance between ATRX expression and progression free survival (PFS) was observed (73.1% vs 65.9%, p=0.124).

Table 1. Baseline characteristics of patients with NPC

| Clinicopathological characteristics | Number (%) |
|-----------------------------------|------------|
| Age <=50                          | 138 (60.8%)|
| Age >50                           | 89 (39.2%) |
| Gender                           |            |
| Male                             | 156 (68.7%)|
| Female                           | 71 (31.3%) |
| ECOG                             |            |
| 0                                | 186 (81.9%)|
| 1                                | 41 (18.1%) |
| Smoking history                  |            |
| No                               | 93 (40.9%) |
| Yes                              | 134 (59.1%)|
| HGB (M/F) (g/L)                  |            |
| <120/110                          | 9 (4.0%)   |
| ≥120/110                         | 218 (96.0%)|
| Treatment mode                   |            |
| Concurrent chemoradiation        | 103 (45.4%)|
| Sequential chemoradiation        | 124 (54.6%)|
| Chemotherapy agent type          |            |
| Cisplatin                        | 145 (63.9%)|
| Nedaplatin                       | 68 (30.0%) |
| Others                           | 14 (6.1%)  |
| Total platin dose (mg/m²)         |            |
| ≥300                              | 164 (72.2%)|
| <300                              | 63 (27.8%) |
| ECOG                             |            |
| 0                                | 185 (81.5%)|
| 1                                | 42 (18.5%) |
| Smoking history                  |            |
| No                               | 93 (41.0%) |
| Yes                              | 134 (59.0%)|
| HGB (M/F) (g/L)                  |            |
| <120/110                          | 15 (6.6%)  |
| ≥120/110                         | 212 (93.4%)|
| Treatment mode                   |            |
| Concurrent chemoradiation        | 103 (45.4%)|
| Sequential chemoradiation        | 124 (54.6%)|
| Chemotherapy agent type          |            |
| Cisplatin                        | 145 (63.9%)|
| Nedaplatin                       | 68 (30.0%) |
| Others                           | 14 (6.1%)  |
| T stages                         |            |
| T1                                | 26 (11.5%) |
| T2                                | 88 (38.8%) |
| T3                                | 68 (30.0%) |
| T4                                | 45 (19.8%) |
| N stages                         |            |
| N0                                | 15 (6.6%)  |
| N1                                | 37 (16.3%) |
| N2                                | 144 (63.4%)|
| N3                                | 31 (13.7%) |
| AJCC stages                      |            |
| II                                | 24 (10.6%) |
| III                               | 132 (58.1%)|
| IV                                | 71 (31.3%) |
Figure 2 Kaplan-Meier survival curves of patients with NPC stratified by ATRX expression. (A) Progression-free survival and (B) overall survival according to ATRX expression in NPC patients. ATRX negative (n=53) and ATRX positive (n=174).

Table 2. Correlation between ATRX expression and clinicopathological factors in NPC

| Clinicopathological factors | N  | ATRX expression | P value |
|-----------------------------|----|-----------------|---------|
|                             |    | negative | positive |         |
| Gender                      |    |          |          |         |
| Male                        | 156| 35 (66.0%)| 121 (69.5%)| 0.630   |
| Female                      | 71 | 18 (34.0%)| 53 (30.5%) |         |
| Age                         |    |          |          |         |
| ≤50                         | 138| 28 (52.8%)| 110 (63.2%)| 0.175   |
| >50                         | 89 | 25 (47.2%)| 64 (36.4%) |         |
| ECOG                        |    |          |          |         |
| 0                           | 186| 46 (84.9%)| 140 (81.4%)| 0.559   |
| 1                           | 41 | 9 (15.1%) | 52 (18.6%) |         |
| Smoking history             |    |          |          |         |
| No                          | 93 | 24 (43.4%)| 69 (40.1%) | 0.671   |
| Yes                         | 134| 31 (56.6%)| 103 (59.9%)|         |
| HGB (M/F) (g/L)             |    |          |          |         |
| <120/110                    | 9  | 1 (1.9%)  | 8 (4.7%)  | 0.370   |
| ≥120/110                    | 218| 54 (98.1%)| 164 (95.3%)|         |
| Treatment mode              |    |          |          |         |
| Concurrent chemoradiation   | 103| 26 (47.2%)| 77 (44.8%) | 0.759   |
| Sequential chemoradiation   | 124| 29 (52.8%)| 95 (55.2%) |         |
| Chemotherapy agent type     |    |          |          |         |
| Cisplatin                   | 145| 32 (58.5%)| 113 (65.7%)| 0.291   |
| Nedaplatin                  | 68 | 21 (37.7%)| 47 (27.3%) |         |
| Others                      | 14 | 2 (3.8%)  | 12 (7.0%)  |         |
| T stages                    |    |          |          |         |
| T1-2                        | 114| 33 (62.3%)| 81 (46.6%) | 0.045   |
| T3-4                        | 113| 20 (37.7%)| 93 (53.4%) |         |
| N stages                    |    |          |          |         |
| N0-N1                       | 52 | 11 (20.8%)| 41 (23.6%) | 0.670   |
| N2-N3                       | 175| 42 (79.2%)| 133 (76.4%)|         |
| AJCC stages                 |    |          |          |         |
| II-III                      | 156| 39 (73.6%)| 117 (67.2%)| 0.383   |
| IV                          | 71 | 14 (26.4%)| 57 (32.8%) |         |

Discussion

In the present study, we investigated the expression of ATRX in NPC and its correlation with clinicopathological characteristics and prognosis of NPC patients. ATRX protein expression was absent in 24.2% of NPC patients, and ATRX loss is correlated with earlier T stage and longer OS in NPC patients. ATRX is a member of the SWI/SNF chromatin remodeling superfamily which is involved in remodeling and stabilizing genome [15]. It is reported that SWI/SNF genes are found to be mutated in almost 25% of malignancies, which have prognostic significance [16]. Moreover, expression changes of specific SWI/SNF chromatin remodeling proteins are also implicated as prognostic biomarkers [16]. However, the consequences of mutations or expression alterations on prognoses are highly context-specific [16]. ATRX mutation is mostly seen in mesenchymal malignances, including glioma, sarcoma, and neuroendocrine tumors, while the report on the incidence and prognostic significance on epithelial malignancies is rare. Loss of ATRX results in impaired genome damage repair system, which leads to genetic instability, microsatellite instability (MSI), and more aggressive phenotype [17]. At the same time, it also sensitizes cancer cells to treatment agents, including DNA-damaging agents, radiotherapy [11, 18], and immune checkpoint inhibitors [12, 19]. The prognostic significance of ATRX mutation on cancer patients varies between researches. It is reported that ATRX mutation is a poor prognostic factor in pancreatic neuroendocrine tumors (pNETs) [20], hepatic angiosarcomas [21], and leiomyosarcoma [22, 23], while it is found to be a good prognostic factor in glioma [24], and cervical cancer [13]. Such complexities without universal good prognosis may suggest that mutations affecting chromatin remodeling genes or expression altering chromatin remodeling proteins do not directly determine the aggressiveness or prognosis of malignancies [16]. Besides, this discrepancy may be resulted from patient selection and treatment modality. In the present study, we firstly reported the incidence of ATRX deficiency in NPC patients, and found that ATRX deficiency predicts good prognosis.
in NPC patients. However, the underlying mechanism is not fully understood, and it may be related with the following aspects. Firstly, since the ATRX protein plays significant epigenetic roles in maintaining telomere length via depositing histones at heterochromatin and telomeric DNA, tumors with ATRX mutations or loss exhibit altered telomeres and telomere dysfunction [25, 26]. Moreover, ATRX mutations increase tumor mutational burden (TMB), PD-L1 expression, as well as manifested interferon gamma (IFN-γ) signaling [12, 19]. Given that radiotherapy or chemoradiotherapy is the primary treatment for NPC, and the potential role of ATRX mutation in glioma patients treated with RT has been reported. Collectively, the mechanism of ATRX mutation conferring survival advantages in NPC is worth further investigation.

Mutations in ATRX are thought to result in loss of ATRX protein which mediates loss of function (LOF), and therefore in the present study, immunohistochemistry (IHC) is used as a surrogate to detect mutations. In previous research in glioma, pancreatic neuroendocrine tumors, and neuroblastoma have demonstrated that ATRX mutations are associated with absence of ATRX protein. However, in another research, Chami et al. [27] reported that using IHC as a surrogate may miss ATRX mutations in neuroblastoma, which indicated detecting ATRX mutation by IHC protein expression as a surrogate still needs further validation. Furthermore, the consistency between ATRX protein absence and ATRX mutation in NPC is worth further investigation.

Although this study identified the prognostic significance of ATRX loss in NPCs, there are several limitations. First, confirmation of ATRX mutations by other techniques, such as sequencing from fresh tissue was not included. Additionally, this study is a retrospective study, and due to a limited sample size, there is a need for validation in larger cohorts with clinical, molecular alterations, conjointly with protein expression/activation.

In conclusion, we evaluated the clinico-pathological significance of ATRX expression in 227 patients with NPC. ATRX loss was significantly associated with good prognosis in patients.

**Abbreviations**

NPC: nasopharyngeal carcinoma; ATRX: alpha thalassemia/mental retardation X-linked; SWI/SNF: switch/sucrose nonfermentable; ICI: immune checkpoint inhibitor; PFS: progression-free survival; OS: overall survival; MSI: microsatellite instability; pNETS: pancreatic neuroendocrine tumors; TMB: tumor mutational burden; IFN-γ: interferon gamma; LOF: loss of function; IHC: immunohistochemistry.

**Acknowledgements**

This work was supported by grants from the National Natural Science Foundation of China (81802476, 81802620, 82172656), the Natural Science Foundation of Hunan Province (2020JJ5807, 2021JJ40882), and the Scientific Research Project of Hunan Provincial Health Commission (20201019).

**Author Contributions**

T.H., X.L.L., C.H.H. conceived and designed the study. H.H.W. and S.Q.F. contributed to data acquisition. Data analysis and manuscript drafting were performed by S.S.W. and Y.C.X. Figures and tables were created by Y.H.F. and Y.Q.F. All authors were involved in the writing or review of the manuscript and approved the final version.

**Competing Interests**

The authors have declared that no competing interest exists.

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