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Research Article

AGR2 and AKR1B10: Potential Collaborative Markers for Nasopharyngeal Carcinoma Diagnosis and Therapy

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Abstract

Nasopharyngeal Carcinoma (NPC) is a common malignant cancer in South China. Previous studies have showed that Anterior Gradient Protein 2 (AGR2) and Aldo-Keto Reductase 1B10 (AKR1B10) progressively increase in NPC primary and metastatic tissues, and AGR2 and AKR1B10 may have the potential to reflect the development of NPC. In this study, we showed that AGR2 and AKR1B10 expressions in NPC tissues were associated with the prognosis of NPC patients, the patients with highly expressed AGR2 and AKR1B10 had a poor prognosis. Consistently, the serum levels of AGR2 and AKR1B10 were markedly higher in NPC patients compared with healthy control, and these concentrations decreased after treatment. Additionally, there was a positive linear correlation between AGR2 and AKR1B10 levels in NPC patients. Furthermore, AGR2 and AKR1B10 showed a positive relationship with Tumor Node Metastasis (TNM) grade and metastasis. Overall, there may be a tight relationship between AGR2 and AKR1B10 expression, and both of them can be used as serum biomarkers to achieve early diagnosis and provide effective therapeutic targets for NPC patients.

Keywords: AGR2; AKR1B10; Serum biomarker; Therapeutic target; Nasopharyngeal carcinoma

Abbreviations: NPC: Nasopharyngeal Carcinoma; AGR2: Anterior Gradient Protein 2; AKR1B10: Aldo-Keto Reductase 1B10; EBV: Epstein-Barr Virus; MMP: Matrix Metalloproteinase; UPR: Unfolded Protein Response; ER: Endoplasmic Reticulum; OS: Overall Survival; ECM: Extracellular Matrix; EGFR: Epidermal Growth Factor Receptor; IGF-1: Insulin-Like Growth Factor-1; DAG: Diacylglycerol; PKC: Protein Kinase C; CTL: Cytotoxic T Lymphocyte; PDI: Protein-Disulfide Isomerase

Introduction

Nasopharyngeal Carcinoma (NPC) is a commonly encountered type of malignant cancer, which arises from epithelial cells of the nasopharynx and shows a remarkably high prevalence in east Africa and Asia [1], especially South China, such as Guangdong, Guangxi and Hunan [2,3]. Increasing evidence has suggested that the etiology of NPC is not only involved with Epstein-Barr Virus (EBV) infection [4], chemical carcinogens and environmental exposures, but also tightly correlated with genetic lesions [5,6]. Furthermore, the unusual incidence patterns of NPC indicate that NPC high-risk genes play an aberrant role in Chinese and Chinese-related populations, resulting in the inheritance and familial aggregation of NPC [7]. The metastasis and invasion have been identified as significant symptoms of NPC, which contributes to unfavorable curative effect and low survival rate [8]. To improve prognosis and survival rate, novel therapeutic targets of NPC are urgently to be identified.

The previous studies have found that Anterior Gradient Protein 2 (AGR2) and Aldo-Keto Reductase 1B10 (AKR1B10) are associated with NPC development and metastasis [9,10]. AGR2 is characterized as a member of the Protein-Disulfide Isomerase (PDI) family, with the ability to modulate Unfolded Protein Response (UPR) proteins synthesis and Endoplasmic Reticulum (ER) stress-associated pathways [11]. Cancer-secreted AGR2 is a potential diagnostic and prognostic biomarker, it has a significant correlation with low Overall Survival (OS) in multiple types of cancers, including prostate cancer [11,12], breast cancer [13], ovarian cancer [14], lung adenocarcinoma cancer [15], Gastric Cancer (GC) [16], Colorectal Cancer

(CRC) [17] and Non-Small Cell Lung Cancer (NSCLC) [18]. Aldo-keto reductases (AKRs) are a group of monomeric cytoplasmic proteins, including AKR1B1 and AKR1B10 [19]. AKR1B10, a detoxification enzyme, is normally expressed in the gastrointestinal tract and catalyses cofactor-dependent oxidation-reduction reactions [19]. Of particular, AKR1B10 is secreted through lysosome-mediated non-classical pathway, then contributing to the increase of AKR1B10 in the serum. Functionally, AKR1B10 is positively associated with tumor size and lymph node metastasis [20], further leading to reduced survival in various malignancies, including Hepatocellular Carcinoma (HCC) [21], Oral Squamous Cell Carcinoma (OSCC) [22], lung adenocarcinoma [23] and GC [24]. Taken together, AGR2 and AKR1B10 are highly expressed in multiple human solid cancers and reported to promote the aggressive characteristics. Thus, they might play an indispensable role in enhancing NPC malignancy. To profoundly explore whether the serum levels of AGR2 and AKR1B10 can be considered as diagnostic biomarkers and therapeutic targets of NPC, our study would detect the expression of AGR2 and AKR1B10 in tissue and serum samples from NPC patients and control group. Further, we would combine with the analysis of clinical data to assess the feasibility of AGR2 and AKR1B10 as serum markers to achieve early diagnosis and provide effective therapeutic targets for NPC patients.

Materials and Methods

Reagents and antibodies

Antibody against AGR2 was purchased from Bior Byt Company (San Francisco California, United States). Antibody against AKR1B10 was purchased from Abcam Company (Shanghai, China). Elisa Kit against AGR2 was purchased from Mlbio Company (Shanghai, China). Elisa Kit against AKR1B10 was purchased from Light of Life Company (Changsha, Hunan).

Human serum samples

106 NPC patients were enrolled in this study, and the patients were obtained from Hunan Cancer Hospital & the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University (Changsha, China) from July 2019 to March 2020. The patients were pathologically diagnosed as NPC. Of them, 50 pairs of NPC tissue samples and matched normal tissues were obtained by biopsy. Blood samples were obtained by venepuncture from 106 NPC patients, 106 corresponding NPC patients after treatment and 40 healthy donors. Then samples were clotted at 4-8 **℃** and centrifuged at 3000rpm for 10min. The collected serum was saved in 200**μ**L EP tubes and stored at -80 **℃** until used. The 106 NPC patients contain 83 males and 23 females, their features were collected, including age, gender, EBVCA-IgG, EBVEA-IgA and TNM stages. The median age of the 106 patients was 48.5 years (range 36-78). The tumor histology and stages were classified according to the World Health Organization (WHO) classification and the TNM staging system of the Union for International Cancer Control (UICC), respectively. 40 healthy relatives of the NPC patients consisted of 22 males and 18 females. The median age of the 40 healthy relatives was 42 years (range 25-68), with no known diseases. All procedures were consistent with the National Institutes of Health Guide and approved by institutional board with patients' written consent. This study was evaluated and approved by the Ethics Committee of the Affiliated Cancer Hospital of Xiangya Medical School, Central South University.

Immunohistochemistry staining and intensity analysis

Immunohistochemistry for protein expression was performed using SP Rabbit & Mouse HRP Kit (CW2069, CWBIO, Beijing, China) according to the manufacturer's instructions. The paraffin sections were deparaffinized, rehydrated and washed in PBS. Antigen retrieval was performed in sodium citrate buffer in a microwave oven. After being blocked with 3% hydrogen peroxide, the tissue sections were permeabilized with 0.1% Triton X-100 and next immersed in 5% BSA. The primary antibodies (1:100) were added in an Immuno Histo Chemistry (IHC) Antibody Diluent, and incubation overnight. Subsequently, the sections were rinsed with PBS and incubated with a Horseradish Peroxidase (HRP)-conjugated Goat anti Rabbit IgG polyclonal antibody. After being rinsed with PBS, the section was incubated with SABC. The signal was developed with the peroxidase substrate DAB which appears as a brown reaction product or with AEC which appears as a red reaction product. All sections were counterstained with haematoxylin-eosin and were imaged under a microscope. The nucleus/cytoplasm was stained to yellow or brownish yellow (when the color was deep, it was brown), cells were considered to be positive for the target genes. Average IOD was the ratio of the accumulated optical density and the area of the sample under the visual field (for visual field of a 400X light microscope).

Haematoxylin-eosin staining

Tumor tissues were fixed with 4% paraformaldehyde for 24-72h, then dehydrated and treated with xylene until becoming transparent, embedded by paraffin, and sliced in 8-um-thick sections by using a microtome. The sections were placed on gummed paper, dewaxed and stained with haematoxylin-eosin. The prepared sections were placed under the microscope to observe for pathological changes in tumor tissues.

Enzyme-linked immunosorbent assay

Enzyme-Linked Immunosorbent Assay (ELISA) was performed following the manufacture suggested protocol. First, standard and unknown concentration samples were added into the microplate. Then, the substance to be tested and the biotin labeled antibody were incubated at the same time. After washing, HRP labeled with avidin was added. After incubation and washing, the unbound enzyme conjugates were removed, and substrate A and B were added to act simultaneously with the enzyme conjugates. Finally, the color depth is proportional to the concentration of the substance to be measured in the sample.

Statistical analysis

SPSS 21.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) were used for statistical

analysis. The relationship of AGR2 with AKR1B10 concentrations was analyzed using Spearman. The differences between groups were statistically examined using Mann-Whitney U test. Using median values as the cut-off value for the marker, the receiver operating characteristic (ROC) analysis was done. Differences between groups were considered as *, $P< 0.05$; **, $P< 0.01$; or ***, $P<0.001$.

Results

Highly expressed AGR2 and AKR1B10 in human NPC biopsy tissues

AGR2 and AKR1B10 are overexpressed in multiple human cancers, and are associated with cancer metastasis, therapeutic resistance [25], pro-inflammatory phenotype acquisition [26], Extracellular Matrix (ECM) remodeling [27], angiogenesis generation [28], autophagy regulation [29], proliferation and apoptosis [30,31]. In order to verify the clinical significance of AGR2 and AKR1B10 in NPC patients, we collected 50 pairs of NPC tissue samples. IHC data showed that the positive expression rate of AGR2 and AKR1B10 in NPC tissues was remarkably higher than those in matched normal tissues (Figure 1A). Then, the IOD/Area values of AGR2 and AKR1B10 were calculated, suggesting that there was a significant liner correlation between AGR2 and AKR1B10 expression in NPC patients ($P<0.01$, r=0.98) (Figure 1B). These results strongly demonstrated that highly expressed AGR2 and AKR1B10 might play a prominent role in accelerating NPC development.

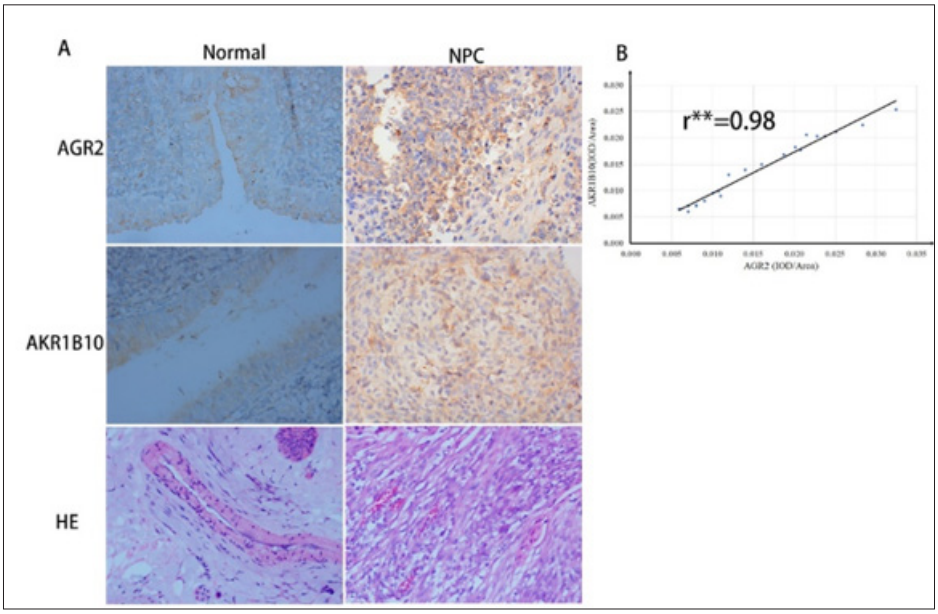

Figure 1: The highly expressed AGR2 and AKR1B10 in human NPC biopsy tissues

A. Immunohistochemistry staining detected AGR2 and AKR1B10 expressions in primary NPC tissues and matched normal tissues,

B. AGR2 and AKR1B10 expressions were quantitatively analyzed. The correlation between AGR2 and AKR1B10 IOD/Areas values was analyzed using spearman ($P<0.01$, r=0.98). These data were representative of 3 separate experiments in triplicate. Data were presented as means ±S.D. of three independent experiments and were statistically analyzed using Student's t test. **, $P< 0.01$.

The concentrations of AGR2 and AKR1B10 in NPC patients

AGR2 and AKR1B10 oncoproteins are secreted by cancer cells, and the serum levels of AGR2 and AKR1B10 are not only used to predict cancer progression and prognosis, but also evaluate therapeutic efficiency [32]. To evaluate the values of AGR2 and AKR1B10 in NPC patient treatment, we collected serum samples from NPC patients at pre-therapy and post-therapy, and healthy population served as control. ELISA assay was performed to detect AGR2 and AKR1B10 concentrations in the serum samples. The results showed that the median concentration of AGR2 is 1.46ng/mL in the NPC group, 0.78ng/mL in the NPC after treatment group and 0.55ng/mL in the healthy control. The median concentration of AKR1B10 is 156.87pg/mL in the pre-therapy NPC group, 118.65pg/mL in the post-therapy group and 77.37pg/mL in the healthy control (Table 1). These indicated that serum AGR2 and AKR1B10 concentrations were higher in NPC patients than those in the healthy control ($P< 0.01$; $P<0.001$). Meanwhile, the AGR2 and AKR1B10 concentrations significant decreased after treatment ($P< 0.05$; $P< 0.01$). However, there was no significant difference between post-therapy NPC and healthy control (Figure 2A & 2B).

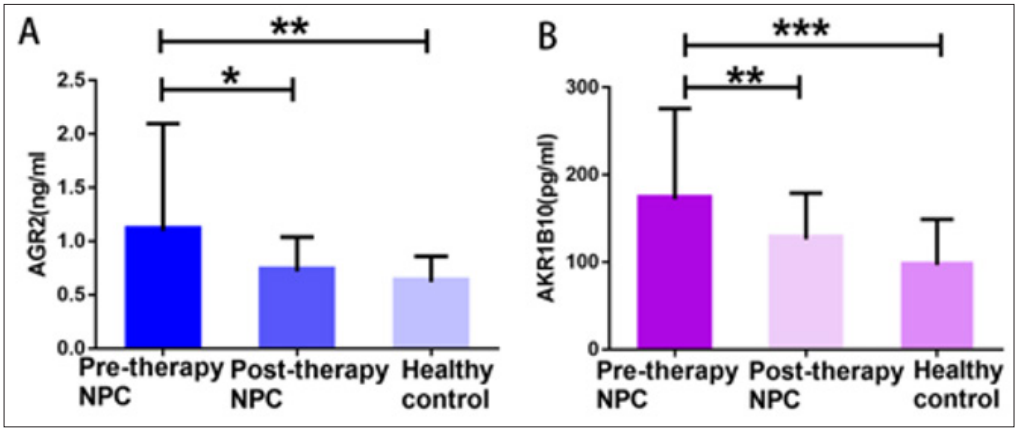

Figure 2: The concentrations of AGR2 and AKR1B10 in the serum samples from the NPC patients at pre-treatment and post-treatment

A. ELISA assay was used to detect serum AGR2 in NPC patients at pre-treatment and post-treatment and healthy population,

B. AKR1B10 was detected in the serum samples from at pre-treatment and post-treatment and healthy population. These data were representative of 3 separate experiments in triplicate. Data were presented as means ±S.D. of three independent experiments and were statistically analyzed using Student's t test. *, P<0.05; **, P< 0.01; or ***, P<0.001.

Table 1: Serum concentrations of AGR2 and AKR1B10 in NPC patients.

Proteins	Pre-Therapy NPC (n=106)	Post-Therapy NPC (n=106)	Healthy Control (n=40)	P Value
AGR2(ng/ml)	0.77(0.53, 1.22)			
		0.67(0.45, 0.95)		<0.05
			0.55(0.41, 0.77)	<0.01
AKR1B10(pg/ml)	140.45(93.10, 234.88)			
		122.93(88.26,148.56)		<0.01
			83.02(62.15, 113.87)	<0.001

The association of AGR2 and AKR1B10 with clinical characteristics

The association of AGR2 and AKR1B10 levels with and NPC patients' clinical characteristics was further analyzed. The clinicopathological traits of NPC patients were summarized in (Table 2), containing age, gender, EBVCA-IgG, EBVEA-IgA, and TNM stages. The results showed that AGR2 and AKR1B10 serum levels displayed a remarkable correlation with TNM grade (P<0.05) and metastasis (P<0.05). But there were no significant relationships of AGR2 and AKR1B10 concentrations with age, gender, EBVCA-IgG or EBVEA-IgA (Table 2). These data strongly suggested that highly expressed AGR2 and AKR1B10 may be used markers to predict NPC metastasis.

Table 2: The relevance of AGR2 and AKR1B10 levels with clinical features.

Features	Cases	AGR2 (ng/ml)	P	AKR1B10 (pg/ml)	P
Gender					
Male	83	0.75(0.49, 1.15)	>0.05	138.93(94.11, 228.92)	>0.05
Female	23	0.85(0.58, 1.26)		147.98(90.09, 256.40)	
Age					
<40	24	0.85(0.53, 1.25)	>0.05	146.23(86.71, 253.49)	>0.05
≥40	82	0.75(0.52, 1.17)		139.69(95.07, 229.69)	
TNM stage					
I+II	14	0.69±0.35	<0.05	101.41 (96.31, 241.38)	<0.05

III+IV	92	0.79(0.57, 1.30)		156.16 (89.31, 228.06)	
Metastasis					
Yes	16	1.62(0.79, 3.44)	<0.001	255.97±88.77	<0.001
No	90	0.71(0.46, 1.09)		135.99(88.95, 215.72)	
EBVCA-IgG					
+	39	0.69(0.46, 1.10)	>0.05	127.00(89.05, 216.94)	>0.05
-	67	0.85(0.53, 1.26)		145.36(94.11, 244.78)	
EBVEA-IgA					
+	36	0.70(0.49, 1.10)	>0.05	132.03(89.05, 216.54)	>0.05
-	70	0.85(0.53, 1.26)		144.91(94.11, 245.58)	

Combination of AGR2 with AKR1B10 as potential therapeutic for NPC patients

The above results showed that AGR2 and AKR1B10 concentrations significantly increased in pre-therapy NPC patients, while all of them decreased in post-therapy patients at the same time points. To further explore whether there was a linear correlation between AGR2 and AKR1B10 concentrations, we analyzed the levels of AGR2 and AKR1B10 in the same NPC patients. Data showed that there was a positive linear correlation between AGR2 and AKR1B10 concentrations in NPC patients before treatment (P<0.01, r=1.00) (Figure 3). However, there was no significance in the corresponding NPC patients after treatment. Next, we further want to determine whether the clinical performance of AGR2 and AKR1B10 could be served as evaluation criteria of therapeutic effect for NPC patients by performing ROC statistics. Based on the AGR2 and AKR1B10 protein levels in serum samples, we found that AGR2 cut-off value of 0.83ng/mL had a sensitivity of 48.1% and a specificity of 90%, and AGR2 median value of 0.77ng/mL had a sensitivity of 50% and a specificity of 70% (Figure 4A). AKR1B10 cut-off value of 93.57pg/mL had a sensitivity of 75.5% and a specificity of 70%, and AKR1B10 median value of 140.45pg/mL had a sensitivity of 50% and a specificity of 90% (Figure 4B). Meanwhile, combined ROC curve analysis for AGR2 and AKR1B10 was used to verify the clinical value of AGR2 and AKR1B10 (Figure 4C).

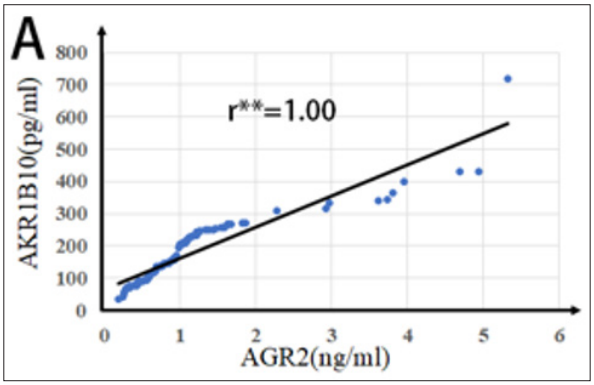

Figure 3: The relativity of AGR2 with AKR1B10 expressions in serum samples from NPC patients. AGR2 and AKR1B10 concentrations were detected in serum samples from NPC patients suing ELISA assay. The relationship of AGR2 with AKR1B10 concentrations was analyzed using Spearman (P<0.01, r=1.00). **, P< 0.01.

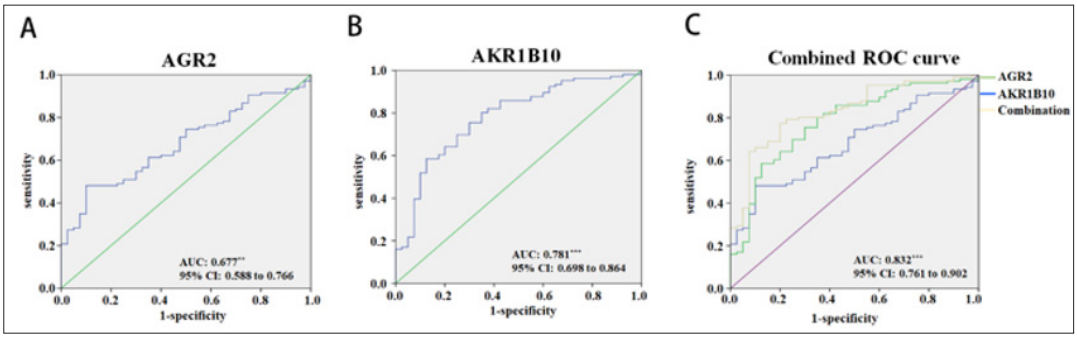

Figure 4: Combination of AGR2 and AKR1B10 as potential blood-based diagnostic and therapeutic markers for NPC patients

A. ROC curve analysis of AGR2 for NPC diagnostic and therapeutic marker

B. ROC curve analysis of AGR2 for NPC diagnostic and therapeutic marker

C. Combined ROC curve of AGR2 and AKR1B10 for NPC diagnostic and therapeutic markers. **, P<0.01; ***, P<0.001. ROC: Receiver Operating Characteristic, AUC: Area Under the Curve, 95% CI: 95% Confidence Interval.

Discussion

In clinical, most of confirmed NPC patients are in advanced stage due to the delayed diagnosis, they are often characterized by distant metastasis, poor prognosis and low OS rate [33]. Distant metastasis is still the major reason for NPC treatment failure [34], and early discovery, early intervention and treatment are essential for improving the therapeutic efficiency for NPC patients. Currently, increasing number of early detection methods are emerging, and our research also aims to look for sensitive and accurate biomarkers for early diagnosis. AGR2 and AKR1B10 have been identified as novel serum protein biomarkers to monitor progression due to their potential roles in promoting invasion and metastasis of numerous malignant cancers [20,35,36]. Of note, AGR2 and AKR1B10 proteins are significantly higher in NPC tissues compared with the matched normal nasopharyngeal tissues [22,37]. Consistently, some clinical studies have also found the increased AGR2 and AKR1B10 concentrations in serum or plasma samples of NPC patients compared with healthy control, implying the diagnostic significance of AGR2 and AKR1B10 for NPC [37]. In this study, we demonstrated that AGR2 and AKR1B10 expressions were remarkably up regulated in human primary NPC tissues compared to the matched normal nasopharyngeal tissues, and there was a positive liner correlation between AGR2 and AKR1B10 IOD/Areas values. Thus, there may be a tight correlation between AGR2 and AKR1B10 expression, and both of them can cooperatively serve as diagnostic biomarkers for NPC.

Growing number of experimental studies have indicated that silencing of AKR1B10 would restrain cancer metastasis and induce cancer cells apoptosis [19]. And the inhibitors of AKR1B10 can be utilized as adjuvant drugs to overcome chemoresistance in numerous cancers [38]. For instance, AKR1B10 inhibitor epalrestat facilitates sorafenib (an anti-cancer drug)-induced apoptosis and autophagy through inhibiting the activation of mTOR pathway in HCC [39]. In agreement with this, silencing AGR2 can inhibit cell proliferation, reduce invasion and dissemination, induce cancer cells death and improve anti-cancer therapy [30,40,41]. Moreover, the previous studies have found that AGR2 can serve as a cancer-associated antigen for facilitating anti-cancer immunotherapy through triggering AGR2 peptide-specific Cytotoxic T Lymphocyte (CTL) response and providing a potent candidate for antibody targeting, which offer a favorable avenue to improve NPC therapeutic efficiency [42,43]. Based on these works, we speculated that serum AGR2 and AKR1B10 may be potential markers for NPC therapy. To validate the above speculation, we detected AGR2 and AKR1B10 in the serum samples from the patients at pre-therapy and post-therapy. The results showed that AGR2 and AKR1B10 concentrations were increased in the NPC patients when compared with the healthy group, while they decreased in the post-treatment patients. There also was a positive relationship between AGR2 and AKR1B10 levels in NPC patients before treatment. Further, the clinical analysis showed that serum AGR2 and AKR1B10 were closely related to the TNM stages and metastasis of NPC patients. ROC curve analysis was used to evaluate the clinical performance of AGR2 and AKR1B10 as therapeutic markers for NPC patients. AGR2 and AKR1B10 could be considered as collaborative serum markers for NPC patients. Collectively, AGR2 and AKR1B10 might not only be potential diagnostic and prognostic biomarkers, but also characterized as promising therapeutic targets for NPC patients. Besides, it is worth noting that there may is a closely relationship between AGR2 and AKR1B10 expression, but the regulatory mechanisms remain to be further investigated.

Author contributions

Yuan Tan drafted the manuscript. Renhua Fan, Liguang Lin, Fang Liu and Meihua Zhou carried out the assays and collected the samples. Yuan Tan and Lei Wang conducted the study design. Lei Wang revised the manuscript. All authors reviewed and approved the final manuscript.

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