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

Qi Tang [a], Yao Wu [a], Yuan Tan [b], Faqing Tang [a,b,*]

[a] *The First Hospital of Hunan University of Chinese Medicine & Hunan Cancer Hospital, Changsha, 410007, China*
[b] *Department of Clinical Laboratory and Hunan Key Laboratory of Oncotarget gene, The affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha 410013, China.*

KEYWORDS

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ABSTRACT

Nasopharyngeal carcinoma is a prevalent malignant tumor, which lacks specific diagnostic markers and effective therapeutic evaluation indexes. AGR2 (Pregradient protein 2) and AKR1B10 (aldoketone reductase 1B10) are oncoproteins secreted by cancer cells into peripheral blood. Prior researches have suggested that the upregulation of AGR2 and AKR1B10 occurs in both primary and metastatic NPC tissues, indicating their potential as biomarkers for NPC development. In the present study, NPC patients had considerably greater serum levels of AGR2 and AKR1B10 than did healthy persons, and these levels decreased following treatment. Furthermore, the serum concentrations of AGR2 and AKR1B10 in patients with NPC were positively correlated with the grade of TNM (tumor lymph node metastasis) and the occurrence of meta stasis. Consistently, AGR2 and AKR1B10 positive rates in NPC tissues were notably greater than those in nearby normal tissues. Additionally, there was an affirmative linear connection between AGR2 and AKR1B10 in NPC patients. In conclusion, there appears to be a close relationship between the expression of AGR2 and AKR1B10, both serving as synergistic biomarkers for early diagnosis and treatment evaluation indicators for patients with nasopharyngeal carcinoma.

INTRODUCTION

The nasopharyngeal epithelium is the source of nasopharyngeal carcinoma (NPC), a malignant tumor that is most common in East Africa and Asia[1], particularly in southern China[2, 3]. The development of NPC is associated with EBV (Epstein-Barr virus) infection[4], environmental factors, hereditary predisposition, and contact with chemical carcinogens[5, 6]. Furthermore, high-risk pathogenic genes for NPC are predominantly found in China, leading to familial inheritance and clustering of the disease[7]. Metastasis and invasion of NPC often result in poor treatment outcomes and prognosis[8]. Hence, it is imperative to discern novel indicators for NPC in order to enhance the survival rate of patients.

Previous researches have established the correlation between the expression levels of AGR2 and AKR1B10 and the metastasis and progression of nasopharyngeal carcinoma[9, 10]. AGR2, a part of the PDI (protein disulfide isomerase) group, helps regulate the production of UPR (unfolded protein response) proteins, and is involved in endoplasmic reticulum (ER) stress-related pathways[11]. The secretion of AGR2 by tumor cells serves as a valuable biomarker for the diagnosis and prognosis of tumor. It has been significantly associated with OS (overall survival) in various sorts of cancer, containing prostate cancer[11, 12], breast cancer[13], ovarian cancer[14], lung adenocarcinoma cancer[15], GC (gastric cancer)[16], CRC (colorectal cancer)[17] and NSCLC(non-small cell lung cancer)[18]. AKRs (Aldo-keto reductases) constitute a category

* Corresponding author. E-mail address: tanfq@hnca.org.cn

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of monomeric cytoplasmic proteins, which includes AKR1B1 and AKR1B10[19].

AKR1B10 is an enzyme involved in detoxification, typically found in the gastrointestinal tract. It plays a role in catalyzing cofactor-dependent redox reactions[19]. Specifically, AKR1B10 gets released into the bloodstream in a not-so-traditional way involving lysosomes, leading to elevated serum levels of AKR1B10[20].

In terms of functionality, AKR1B10 exhibits a positive correlation with the size of tumors and the occurrence of lymph node metastasis, ultimately leading to diminished survival rates across various malignant neoplasms. Including HCC (hepatocellular carcinoma)[21], OSCC (oral squamous cell carcinoma)[22], lung adenocarcinoma[23] and GC[24]. In summary, AGR2 and AKR1B10 exhibit heightened expression levels across various human solid cancers, thereby amplifying their invasive characteristics. Hence, they could potentially play a crucial role in promoting the aggressiveness of NPC tumors.

In order to explore the potential utility of AGR2 and AKR1B10 as diagnostic biomarkers and gauges for treatment assessment in NPC, this research endeavor aims to evaluate the expression levels of AGR2 and AKR1B10 in both tissue specimens and serum samples obtained from individuals afflicted with NPC as well as those who are in good health. Through the integration of these findings with clinically relevant data, our objective is to assess the feasibility of utilizing AGR2 and AKR1B10 as co-markers for early diagnosis and efficacy evaluation in patients suffering from NPC.

MATERIALS AND METHODS

Reagents and Antibodies

The AGR2 antibody was acquired from BiorByt Company (San Francisco, California, United States), meanwhile, the AKR1B10 antibody was sourced from Abcam Company (Shanghai, China). The AGR2 Elisa Kit was gained from Mlbio Company (Shanghai, China), and the AKR1B10 Elisa Kit was acquired from Light of Life Company (Changsha, Hunan).

Human Serum Samples

This study encompassed a total of 106 cases of NPC patients, who were selectively recruited from Hunan Cancer Hospital & the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University (Changsha, China) during the period spanning July 2019 to March 2020. All patients have been diagnosed with NPC through pathological examination. Out of the total, 50 pairs of NPC tissue samples and their corresponding normal tissues were procured via biopsy. Blood samples were collected through venipuncture from 106 individuals diagnosed with NPC, as well as from 106 corresponding NPC patients post-treatment, and 40 healthy donors. Blood samples were obtained through venipuncture from 106 individuals diagnosed with NPC, as well as from 106 corresponding NPC patients post-treatment, and 40 healthy donors. The samples were then coagulated at 4–8℃ and spun around at 3000 rpm for 10 minutes. The harvested serum was carefully stored in 200μL EP tubes and kept at −80℃ until it was ready for use. Demographic characteristics of the 106 NPC patients were recorded, including gender, age, TNM stages, EBVEA-IgA, and EBVCA-IgG. Among the patients diagnosed with NPC, there were 83 males and 23 females, with a median age of 48.5 years (ranging from 36 to 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. The tumor histology and stages were categorized in accordance with the classification of the World Health Organization (WHO) and the TNM staging system of the Union for International Cancer Control (UICC), respectively. In addition, 22 males and 18 females constituted the total healthy participants, which median age was 42 years (ranging from 25 to 68).

All procedures were conducted in accordance with the National Institutes of Health Guide and approved by the institutional board. Written consent was obtained from all patients prior to their participation in the study. This study underwent evaluation and received approval from the Ethics Committee of the Affiliated Cancer Hospital of Xiangya Medical School, Central South University.

Immunohistochemistry Staining and Intensity Analysis

Immunohistochemistry to analyze protein expression was conducted utilizing the HRP assay kit (CW2069, CwbiO, Beijing, China) in accordance with the guidelines provided by the manufacturer.

This includes antigen extraction, incubation with primary and secondary antibodies, and finally observing experimental results under a microscope. The average iodine is calculated as the ratio of cumulative optical density to the area of the sample within the field of view (using a 400x optical microscope).

Hematoxylin-Eosin (HE) Staining

After fixation and dehydration, the tumor tissues were embedded in paraffin, sliced, deparaffinized, and stained with hematoxylin-eosin. The pathological morphology of the tumor was observed under a microscope.

Enzyme-Linked Immunosorbent Assay

The Enzyme-linked immunosorbent assay (ELISA) was carried out in accordance with provided instructions. The samples undergoing testing were incubated with biotin-labeled antibodies. Subsequently, any unbound enzyme conjugate was removed through washing, and the color development was initiated by adding substrate A and B liquid to the enzyme conjugate. It is noteworthy that the intensity of color produced was directly related to the concentration of the substance being measured within the sample.

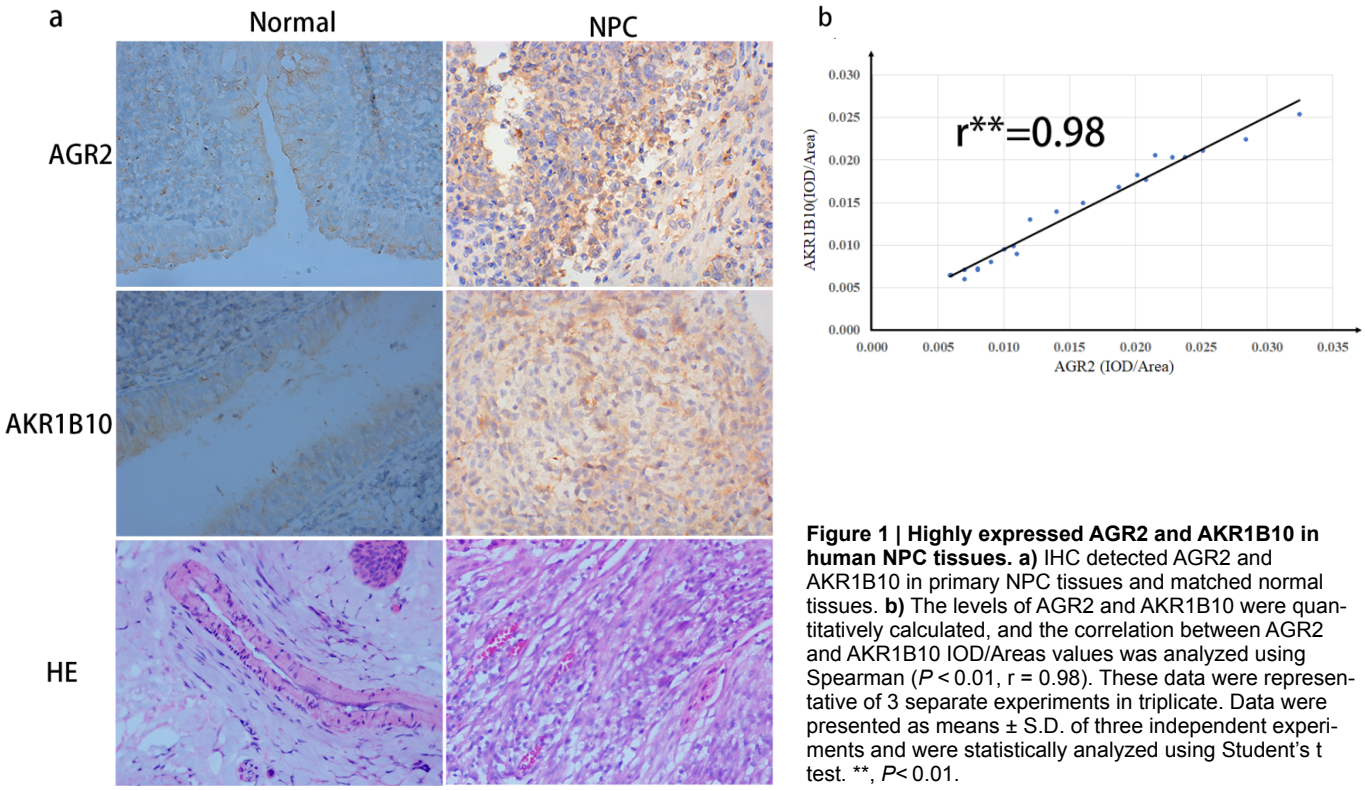

Figure 1 | Highly expressed AGR2 and AKR1B10 in human NPC tissues. a) IHC detected AGR2 and AKR1B10 in primary NPC tissues and matched normal tissues. **b)** The levels of AGR2 and AKR1B10 were quantitatively calculated, and the correlation between AGR2 and AKR1B10 IOD/Areas values was analyzed using 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. **, $P < 0.01$.

Statistical Analysis

Statistical analysis was conducted using SPSS 21.0. The difference between the groups was evaluated using the Mann-Whitney U test. Receiver operating characteristic (ROC) analysis utilized the median as the cut-off value for labeling. Significance levels were denoted as *, $P < 0.05$; **, $P < 0.01$; or ***, $P<0.001$ to indicate differences between groups.

RESULTS

Highly Expressed AGR2 and AKR1B10 in Human NPC Tissues

Each of AGR2 and AKR1B10 is a secretory oncoprotein, and can be secreted into peripheral blood by cancer cells. The serum levels of AGR2 and AKR1B10 are not only utilized for predicting cancer progression and prognosis, but also for evaluating therapeutic effectiveness. These biomarkers play a crucial role in providing valuable information for the management of cancer patients. They are involved in therapeutic resistance[25], pro-inflammatory phenotype acquisition[26], extracellular matrix (ECM) remodeling[27], angiogenesis generation[28], autophagy regulation[29], proliferation and apoptosis[30, 31]. To delve into the clinical significance of AGR2 and AKR1B10 in patients with nasopharyngeal carcinoma, we procured 50 pairs of nasopharyngeal carcinoma tissue samples and conducted immunohistochemistry to detect the expression levels of AGR2 and AKR1B10. The findings revealed that the rates of positive expression of AGR2 and AKR1B10 in NPC tissues were significantly higher than those in healthy individuals (**Figure 1a)**. Furthermore, the IOD/Area values of AGR2 and AKR1B10 indicated a significant linear correlation between their expressions in NPC patient tissues ($P < 0.01$, r = 0.98) (**Figure 1b**). These findings suggest that elevated levels of AGR2 and AKR1B10 may play a crucial role in promoting the progression of nasopharyngeal carcinoma, and could potentially serve as biomarkers for assessing therapeutic efficacy in patients with this condition.

AGR2 and AKR1B10 in NPC Patient Serum

The proteins AGR2 and AKR1B10 are excreted by neoplastic cells, and their levels in serum can be utilized to assess the prognosis and therapeutic efficacy of cancer[32]. To assess the role of AGR2 and AKR1B10 in the diagnosis and treatment of NPC, we have gathered serum samples from 106 pre- and post-treatment patients with NPC. ELISA was used to determine the expression levels of AGR2 and AKR1B10 in serum, the results revealed that the median AGR2 level was 1.46 ng/mL in the NPC group before treatment, 0.78 ng/mL in the NPC group after treatment, and 0.55 ng/mL in the healthy group. The median levels of serum AKR1B10 in the pre-treatment group, post-treatment group, and healthy individuals were 156.87 pg/mL, 118.65 pg/mL, and 77.37 pg/mL, respectively (**Table 1**). Serum AGR2 and AKR1B10 levels were significantly higher in patients with NPC compared to healthy individuals ($P<0.01$; $P<0.001$), and these concentrations decreased significantly after treatment ($P<0.05$; $P <$

Table 1 | Serum concentrations of AGR2 and AKR1B10 in NPC patients and healthy population

Proteins	Pre-therapy NPC (n=106)	Post-therapy NPC (n=106)	Healthy control (n=40)	*P*
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

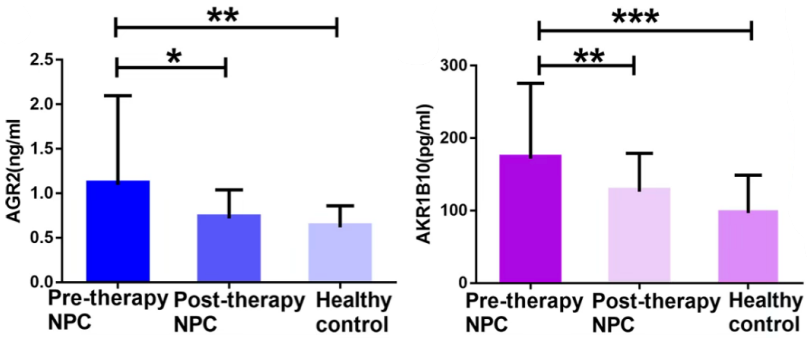

Figure 2 | Serum concentrations of AGR2 and AKR1B10 in NPC patients at pre- and post-therapy. a) Serum AGR2 was detected in the NPC patients at pre-therapy and post-therapy. The volunteer served as healthy control. **b)** Serum AKR1B10 was detected in the NPC patients at pre-therapy and post-therapy. The volunteer served as healthy control. 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.

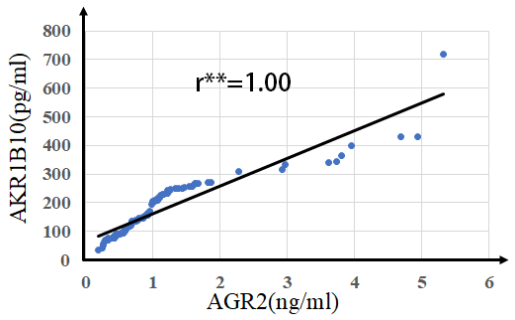

Figure 3 | Relativity of AGR2 with AKR1B10 expressions in serum samples from NPC patients. a) AGR2 and AKR1B10 concentrations were detected in the serum samples from the NPC patients using ELISA assay. The relationship of AGR2 with AKR1B10 was analyzed using Spearman (P< 0.01, r=1.00). **, P< 0.01.

0.01). However, there was no significant difference between NPC patients and healthy individuals after treatment (**Figures 2a, 2b**). In conclusion, serum levels of AGR2 and AKR1B10 can serve as predictive markers for assessing therapeutic outcomes in patients with nasopharyngeal carcinoma.

Association of AGR2 and AKR1B10 With Clinical Characteristics

To further investigate the correlation between AGR2 and AKR1B10 and the general clinical data of patients with NPC, Table 2 summarizes the clinicopathological characteristics of patients, including gender, age, TNM stages, EBVEA-IgA, and EBVCA-IgG. The results indicate that serum levels of AGR2 and AKR1B10 are significantly correlated with tumor TNM grade (P<0.05) and metastasis (P<0.05), but not with gender, age, EBVEA-IgA or EBVCA-IgG (**Table 2**). These findings suggest that elevated levels of AGR2 and AKR1B10 may indicate progression and metastasis of nasopharyngeal carcinoma, leading to a poor prognosis.

Combination of AGR2 With AKR1B10 as Potential Diagnostic Biomarkers for NPC Patients

The aforementioned studies indicate that the levels of AGR2 and AKR1B10 in NPC patients were significantly elevated prior to treatment, but decreased after treatment at the same time point. To further investigate a potential linear correlation between AGR2 and AKR1B10 levels, we examined their expression in patients with NPC. The data revealed a significant positive linear correlation between AGR2 and AKR1B10 concentration in patients with nasopharyngeal carcinoma prior to receiving treatment ($P < 0.01$, $r = 1.00$) (**Figure 3**).

The results of serum AGR2 and AKR1B10 protein levels indicated that when the cutoff value of AGR2 was 0.83 ng/mL, the sensitivity was 48.1% and the specificity was 90%. When the median AGR2 was 0.77 ng/mL, the sensitivity was 50% and the specificity was 70%. The AUC was 0.667 and 95% CI was 0.5888-0.766 (**Figure 4a**). The truncation value of AKR1B10 was 93.57 pg/mL, the sensitivity was 75.5%, and the specificity was 70%; the median value of AKR1B10 was 140.45 pg/mL, the sensitivity was 50%, and the specificity was 90%. The AUC was 0.781 and the 95% CI was 0.698 to 0.864(**Figure 4b**). At the same time, combined with the ROC curve analysis of AGR2 and AKR1B10, the clinical value of AGR2 and AKR1B10 in the diagnosis of nasopharyngeal carcinoma was verified. The AUC was 0.832 and 95% CI was 0.761 ~ 0.902. (**Figure 4c**).

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)	

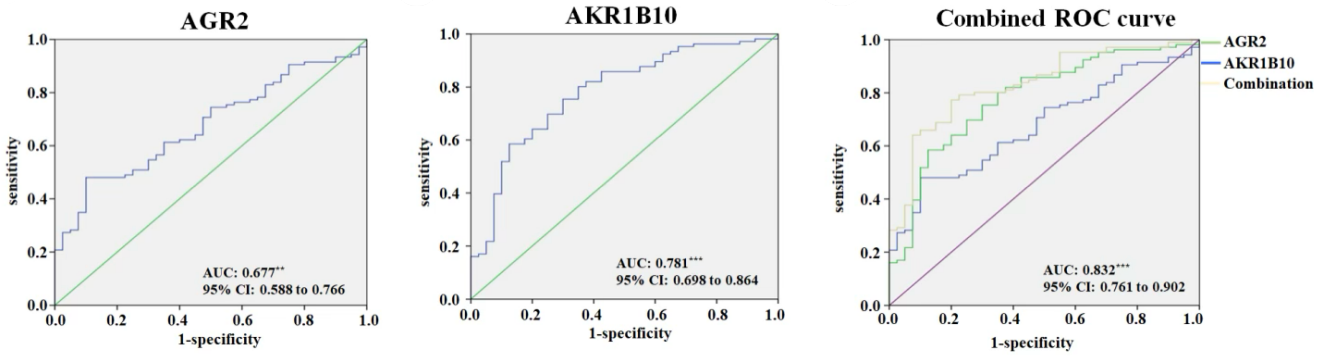

Figure 4 | Combination of AGR2 and AKR1B10 as potential blood-based diagnostic markers for NPC patients. a) ROC curve analysis of AGR2 for NPC diagnosis. **b)** ROC curve analysis of AGR2 for NPC diagnosis. **c)** Combined ROC curve of AGR2 and AKR1B10 for NPC diagnosis. **, P< 0.01; ***, P< 0.001. ROC: receiver operating characteristic, AUC: area under the curve, 95% CI: 95% confidence interval.

DISCUSSION

Clinically diagnosed nasopharyngeal carcinoma patients are often found to be in the advanced stage with distant metastasis, leading to an unfavorable prognosis and diminished likelihood of survival33. Remote metastasis is the primary factor contributing to the unfavorable prognosis and low survival rate of nasopharyngeal carcinoma patients34. Hence, the timely implementation of early screening, intervention, and treatment is imperative in enhancing the overall survival rate of these patients.

Consequently, our study aims to identify sensitive and precise biomarkers for the early diagnosis and treatment of nasopharyngeal carcinoma.

AGR2 and AKR1B10 have been found to promote invasion and metastasis of various malignancies, and these serum protein biomarkers have been identified as novel markers for monitoring tumor progression.20, 35, 36. Studies have indicated that patients with nasopharyngeal cancer exhibit higher serum levels of AGR2 and AKR1B10 compared to healthy individuals37. Similarly, our study demonstrated significantly elevated serum levels of AGR2 and AKR1B10 in patients with NPC compared to healthy individuals, which decreased significantly after treatment. This suggests that these two

proteins may serve as predictive markers for the efficacy of NPC treatment. Prognostic analysis of NPC patients revealed a positive correlation between AGR2 and AKR1B10 levels, The prognostic analysis of NPC patients has revealed a positive correlation between AGR2 and AKR1B10 levels, while ROC curve analysis results suggested their significant value as synergistic serum markers in diagnosing NPC. Furthermore, the levels of AGR2 and AKR1B10 in serum were closely associated with TNM stage and metastasis in NPC patients, indicating their important role in predicting NPC metastasis.

Research has revealed that the inhibition of AKR1B10 can effectively impede tumor metastasis and trigger apoptosis in cancer cells19. Inhibitors targeting AKR1B10 can serve as adjuvants to overcome chemotherapy resistance in various types of cancers38. For instance, the AKR1B10 inhibitor epalrestat has been shown to promote apoptosis and autophagy in cancer cells induced by sorafenib (an anticancer drug) by inhibiting the activation of the mTOR pathway in HCC39. Additionally, silencing AGR2 has been found to inhibit the proliferation, migration, and invasion of cancer cells, ultimately leading to cancer cell death30, 40, 41. Moreover, AGR2 has also been demonstrated to enhance anti-cancer drug immunotherapy by eliciting a specific cytotoxic T lymphocyte (CTL) response against AGR2 peptides, offering a promising approach to enhance treatment efficacy for nasopharyngeal carcinoma42, 43. This investigation unveils a noteworthy elevation in the expression levels of both AGR2 and AKR1B10 within human nasopharyngeal carcinoma tissues. Furthermore, there exists a positive linear correlation between the IOD/Areas values of AGR2 and AKR1B10. Therefore, it is proposed that the expression patterns of AGR2 and AKR1B10 may be closely linked and could potentially be utilized as therapeutic markers for patients with nasopharyngeal carcinoma.

CONCLUSION

In conclusion, the levels of serum AGR2 and AKR1B10 exhibit a close correlation with the onset, progression, and metastasis of nasopharyngeal carcinoma. The decrease in serum AGR2 and AKR1B10 levels in patients with nasopharyngeal carcinoma indicates the effectiveness of treatment. Therefore, AGR2 and AKR1B10 may not only serve as potential biomarkers for tumor diagnosis and prognosis but also as promising therapeutic markers for patients with nasopharyngeal carcinoma. Additionally, it is worth noting that AGR2 may be closely related to the expression of AKR1B10; however, its regulatory mechanism requires further study.

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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.

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Availability of data and material All data generated or analyzed during this study are included in this article.

Author contributions Qi Tang drafted the manuscript. Qi Tang and Yuan Tan carried out the assays and collected the samples. Faqing Tang conducted the study design and revised the manuscript. All authors reviewed and approved the final manuscript.

Competing interests The authors declare that there are no competing interests associated with the manuscript.

Consent for publication The study participants have given consent to participate as well as consent to publish the data.

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