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Aldo-keto reductase 1C3 mediates chemotherapy resistance in esophageal adenocarcinoma via ROS detoxification

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CONTENTS

I. **ABSTRACT**

Background:

The incidence and mortality of esophageal adenocarcinoma (EAC) are very high, and the prognosis is poor in Europe. During the past years, Aldo-keto reductase 1C 3 (AKR1C3) as an oxidoreductase has attracted much attention in the field of cancer research. Especially in the treatment of tumors, AKR1C3 seems to show its protective effect on tumor therapy in many cancers. However, whether its role in the treatment of EAC is still unknown.

Methods:

Public datasets were downloaded and applied. Western blot (WB) was used to check the protein level. Immunofluorescence was also used to measure the expression and localization of AKR1C3. Cell lines in SKGT-4 ($SKGT\text{-}4^{AKR1C3\text{-}KD}$ /$SKGT\text{-}4^{AKR1C3\text{-}shNT}$) and OACP4C ($OACP4C^{AKR1C3\text{-}KD}$ /$OACP4C^{AKR1C3\text{-}shNT}$), and cell lines in OE33 ($OE33^{AKR1C3\text{-}OE}$ /$OE33^{AKR1C3\text{-}VEC}$) and FLO-1 ($FLO\text{-}1^{AKR1C3\text{-}OE}$ /$FLO\text{-}1^{AKR1C3\text{-}VEC}$) were established. Basic cell experiments such as cell proliferation/colony formation/wound healing assay were carried out in this study on those genetically modified EAC cell lines. MTT assay was used to quantify the cells' viability after anti-cancer drugs treatment. Then cisplatin-induced cell apoptosis was checked by FACS using Annexin V/DAPI. Reactive oxygen species (ROS) of EAC cells were checked by FACS using H2DCFDA. Chromatin-immunoprecipitation (ChIP) was carried out to validate the transcriptional regulation of AKR1C3. $SKGT\text{-}4^{NRF2\text{-}KD}$ and $SKGT\text{-}4^{NRF2\text{-}shNT}$ cell lines were established. Then, PCR and WB were used to check its level in $SKGT\text{-}4^{NRF2\text{-}KD}$ and $SKGT\text{-}4^{shNT}$ cell lines. GSH quantification of genetically modified EAC cells was also checked. Moreover, mitochondrial stress test was performed in evaluating oxygen consumption rate (OCR) of EAC cells.

Results:

AKR1C3 mRNA level in EAC tissues is higher than in the normal squamous esophageal epithelium in the data of GSE26886 and GSE92396 from public database. However, only about half of the 12 patients' AKR1C3 protein level had elevated in tumor tissue than in matched normal tissue. Although public databases revealed the trend that higher AKR1C3 means poorer survival probabilities, no difference in survival can be observed

between the AKR1C3^{high} and AKR1C3^{low} group. Then, we detected its protein level in OE19, OE33, OACP4C, FLO-1, JHesoAD1 and SKGT-4 cell lines. It high-expressed in OE19, OACP4C and SKGT-4 cell lines, and it low-expressed in OE33, JHesoAD1 and FLO-1 cell lines. Immunofluorescence showed the same results with WB. Furthermore, immunofluorescence results also found that AKR1C3 was mainly expressed in the cytoplasm.

OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines were more capable of proliferating/ colony-forming/migrating than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cell lines. Opposite results were observed in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines. Moreover, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cells had less apoptosis induced by anti-cancer drugs, while SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells showed the opposite results. Furthermore, we found that higher ROS in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells and lower ROS in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cells compared with their control cell lines. The nuclear factor erythroid 2-related factor 2 (NRF2) controls redox homeostasis, and it binds with AKR1C3's promoter and mediates its expression. And their expression was decreased in SKGT-4$^{NRF2\text{-}KD}$ cells than in the SKGT-4$^{NRF2\text{-}shNT}$ cells. Moreover, the rescue experiment showed that NAC and BSO could make AKR1C3' effect disappear whether in SKGT-4/OACP4C $^{AKR1C3\text{-}KD/shNT}$ cells or OE33/ FLO-1$^{AKR1C3\text{-}OE/VEC}$ cells. Furthermore, the phosphorylation of AKT was decreased in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C $^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. Conversely, it was increased in OE33 $^{AKR1C3\text{-}OE}$ and FLO-1 $^{AKR1C3\text{-}OE}$ cell lines than OE33 $^{AKR1C3\text{-}VEC}$ and FLO-1 $^{AKR1C3\text{-}VEC}$ cells lines. AKT inhibitor could inhibit the phosphorylation of AKT but had no effect on AKR1C3 expression. And inhibitor of AKT could diminish the protective effect of the decrease for apoptotic cells caused by the elevated of AKR1C3 in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines. Moreover, GSH levels was decreased, and the inhibitor of AKT had the same effect with AKR1C3-KD for its regulation. We found that the OCR is increased in OE33 $^{AKR1C3\text{-}OE}$ than in OE33$^{AKR1C3\text{-}VEC}$, while SKGT-4$^{AKR1C3\text{-}sh1}$ and SKGT-4$^{AKR1C3\text{-}sh2}$ showed the opposite results than SKGT-4$^{AKR1C3\text{-}shNT}$. Lipid metabolism substances such as fatty acids, steroids were enriched in AKR1C3^{high} group from public databases

Conclusions:

AKR1C3 regulates chemotherapy of EAC and may contribute to chemotherapy resistance of tumor cells. The possible mechanism is that the elevated AKR1C3 enhanced its own antioxidant capacity by reducing the ROS levels through AKT/GSH signaling. Tumor cells were exposure in chemotherapy drugs, an abundance of ROS will be produced in tumor cells, while the cells with higher AKR1C3 expression in the cells will show a stronger antioxidant capacity against ROS and thus be more likely to survive. So, this study provides rationale for the design of remedy that targeting AKR1C3 might enhance the anti-tumor effect and prolong survival time of EAC patients.

Key words: esophageal adenocarcinoma; Aldo-keto reductase 1C 3; chemotherapy; resistance; AKT signaling; ROS regulation; apoptosis; redox-homeostasis

II. INTRODUCTION

2.1.Esophageal cancer

2.1.1. Introduction

Esophageal cancer (EC) is a common cancer of the digestive tract, and its incidence and mortality vary greatly by region [1]. In the early stage of esophageal cancer, the clinical symptoms of patients are often not obvious. When the patient is swallowing rough and hard food, there may be discomfort, such as food discomfort, foreign body sensation and different degrees of pain. Sometimes the symptoms are obvious and sometimes they are not [2]. So many early esophageal cancer patients do not pay attention to it, and it is easy to develop into the middle and late stage. However, for these advanced patients the survival rate is significantly reduced, the 5 year overall survival (OS) ranges from 15% to 25% [3]. So, it is of great significance to pay attention to early screening of esophageal cancer, not only because of its high incidence, but also because EC such as EAC and esophageal squamous cell carcinoma (ESCC) can be prevented and controlled by early screening. These two main histopathological subtypes account over 95% of esophageal cancers [4]. During the past several decades, the incidence of ESCC has been declining while EAC has strikingly increased in Germany, however, the exact cause of esophageal cancer is still unknown [5]. The incidence of ESCC has been declining while EAC has strikingly increased in Germany, however, the exact cause of esophageal cancer is still unknown [6]. The incidence of EAC is much higher than ESCC in Germany, while ESCC occurs more in China [7, 8].

For esophageal cancer patients, in addition to surgery, chemoradiotherapy actually has a great effect on the prognosis of the disease. However, due to various reasons, some advanced patients have no effect on these treatments at all [9, 10]. So, this is indeed a troublesome problem, and the treatment of esophageal cancer is indeed an important challenge, especially now that its increasing incidence. Therefore, esophageal cancer is still a major malignant tumor which threatening the health of people all over the world.

2.1.2. Management of EAC

Although EAC and ESCC are both belong to esophageal cancer, they also have some common features, but they are completely different esophageal cancers. The clinical

characteristics of EAC and ESCC are completely different and its responses to clinical treatments is also different [11, 12]. Therefore, it is best to study EAC and ESCC separately and treat them as heterogeneous diseases. Therefore, for the treatment of EAC, we should treat EAC according to the treatment principles of adenocarcinoma. At the same time, we know that the occurrence and development of EAC is also very important for its treatment. There are many factors related to the occurrence of EAC, such as gastroesophageal reflux disease (GERD) and BE are considered as risk factors. GERD is that gastroesophageal reflux of the stomach and bile acids flow backward into the esophagus and this chronic irritation of the esophagus could increase the likelihood of developing BE. The simple understanding is that because of this chronic injury, GERD is more likely to develop into BE, and then BE is more likely to develop into EAC. Meanwhile, it is well accepted that BE is considered as the main precursor of EAC [13].

In Western countries, the EAC incidence is rising rapidly, the early diagnosis rate is low, and the median survival rate is low [14, 15]. EAC seriously endangers human health. Effective therapy for EAC still remains a big challenge [13]. For some early-stage EAC patients, endoscopic therapy may be the most common approaches. However, for late stage of EAC patients, neoadjuvant treatment carried out before surgery. Chemotherapy or chemoradiotherapy (CRT) is still an optional therapy for the patients in the final stages with metastatic cancer. Several pivotal trials such as the FLOT trial and the CROSS trial have been established for locally advanced esophageal cancers.

ECF and ECX were carried out in the FLOT4 trial, which also shows improved OS than perioperative chemotherapy with ECF/ECX [9, 16]. The CROSS trial involved 368 patients was first published in 2012 and emphasized the advantage of a neoadjuvant CRT regimen [10]. Subsequently, the CROSS regimen was quickly adopted by many centers around the world and thought to be a clinical guideline for those patients [17]. Recently, Paireder et al. reported that modification of CROSS with higher radiotherapy doses is safe and effective [18]. Unfortunately, even with the above improvements in both detection and treatment, the OS remains low [14, 19].

At the same time, for resectable EAC patients, there are also some upcoming randomized trials that will assess the advantages of neoadjuvant chemotherapy. Furthermore, a phase III trial for locally advanced esophageal cancer [20] and Neo-AEGIS trial for EAC and

GE junction adenocarcinoma [21]. These trails will probably give us more insight into the treatment and research of esophageal adenocarcinoma.

2.1.3. Chemotherapy resistance in EAC

Chemotherapy resistance is very common for tumor treatment, which is also an important reason for the poor prognosis of tumor patients. Although there are now many new therapeutic strategies for cancer treatment, such as combined chemotherapy and/or radiotherapy to prolong the EAC patients' OS. The high resistance rate to anti-cancer drugs is still very common and it also makes the treatment of cancer very complicated [22, 23]. Chemotherapy resistance can easily be observed in many different cancers during treatments [24, 25]. There are many potential mechanisms of chemoresistance in EAC, both intrinsic and extrinsic mechanisms. The PI3K/AKT, and TGF-β/JNK signaling pathways, and redox-homeostasis are the intracellular regulatory mechanisms that could cause chemoresistance. As for the external mechanism, the tumor microenvironment (ETM) contains a large number of cells, such as macrophages, cancer-associated fibroblasts (CAF), and immune cells, which have a very important impact on tumor cells. ETM is also widely recognized for chemoresistance [26-29].

PI3K/AKT pathway participates and makes up several key cellular pathways, including survival, proliferation, migration, invasion, apoptosis, and angiogenesis in human cancers [30-33]. AKT signaling pathway participate in regulating chemotherapy resistance of cancers [34-36]. Indeed, research on AKT-related pathways has become the main research content of many research centers who render AKT a valuable therapeutic target [37, 38].

It was reported that AKT is higher in the EAC than that in the normal and it confers EAC cells' resistance to epirubicin [39, 40]. Furthermore, Myers et al. reported that targeting PI3K/AKT signal pathway could sensitize resistant EAC cells to standard neoadjuvant chemotherapy [41]. AKT signaling pathways also show their properties in controlling cancer stem cells (CSCs) [42]. There may be many CSCs cells in EAC, and these cells have high differentiation potential, so EAC contains more CSCs will be more resistant to chemotherapy drugs [43].

2.2.Aldo-Keto-Reductase 1C3 (AKR1C3)

2.2.1. AKRs family

AKRs protein superfamily contains more than 190 annotated proteins which are divided into 16 families such as AKR1-16 [44]. AKRs possess some features in the oxidoreduction of a diverse range of natural and foreign substrates [45]. Moreover, an increased number of studies showed its detoxification effect in glycolysis-derived cytotoxic compounds and lipid peroxidation [46]. AKRs enzymes are oxidoreductases that reduce aldehydes and ketones to corresponding alcohols, making them involved in the detoxification processes of living cells [47] **(Figure 2.1)**. It is well known that the accumulation of many aldehydes and ketones in the body is harmful to the human body. However, once they are converted into non-toxic alcohols, they will be metabolized by the liver to achieve detoxification effect. AKRs proteins are usually 34 to 37 kDa monomers. Each enzyme has an (α/β)8-barrel motif and is characterized by the same protein fold [48, 49].

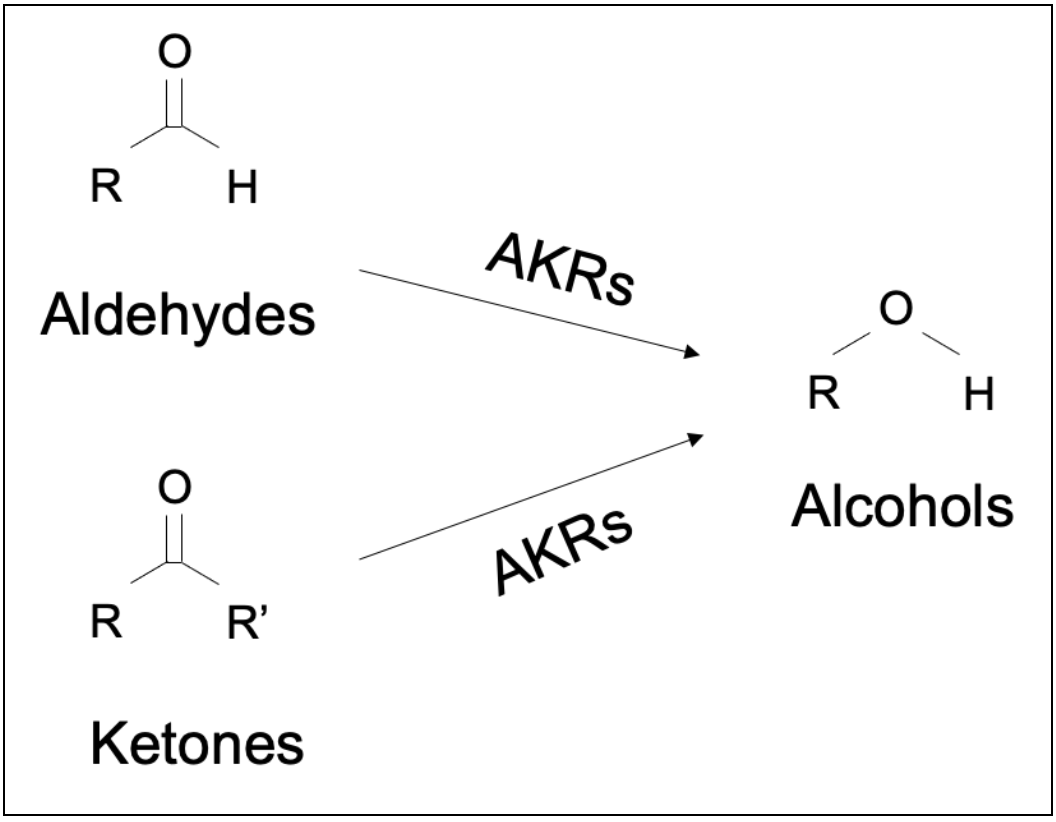


Figure 2.1. Diagrammatic drawing of the detoxification of AKRs.

The Aldo-keto reductase 1 (AKR1) family is the largest group in the AKRs superfamily. It contains the aldehyde reductases, aldose reductases, steroid 5β-reductases, and hydroxysteroid dehydrogenases [44]. Meanwhile, AKR1C includes AKR1C1-4 **(Table 2.1)**. AKR1C1-4 has been confirmed that they could participate in oxidoreduction reactions. Their substrates main include steroids and prostaglandins and they also metabolize polycyclic aromatic hydrocarbons, pharmacologic drugs, carcinogens, and other toxicants [50]. All these four members were found in the liver and have also been validated for their different extrahepatic distribution. AKR1C1-2 are commonly expressed in many organizations; AKR1C3 is mainly expressed in the prostate and breast; AKR1C4 is mainly expressed in the liver and mediates the bile acids synthesis [51, 52]. AKR1C1-4 have different ratios of NADPH-dependent reductase activities [53]. AKR1C3, as a critical number of AKR1Cs subfamily, could catalyze the conversion of PGD2 and PGH2 to PGF2 [54] **(Figure 2.2)**.

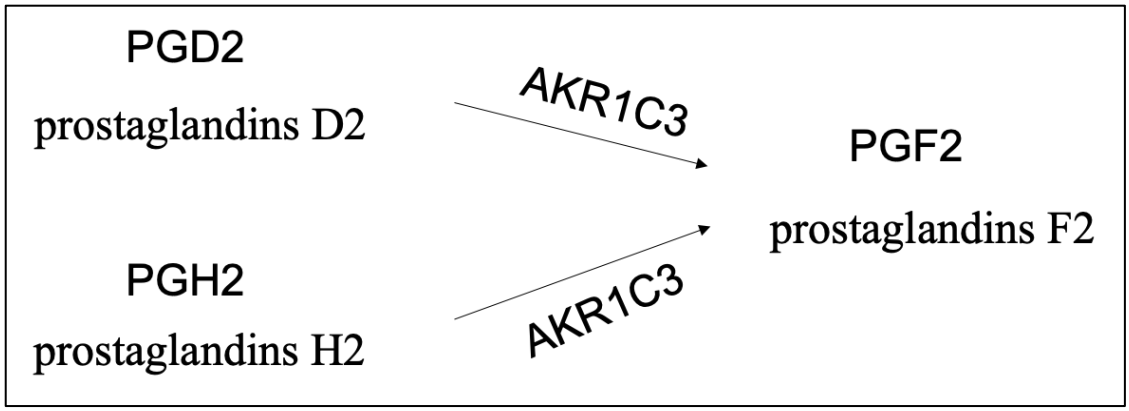


Figure 2.2. Diagrammatic drawing of catalyze the conversion of AKR1C3.

Table 2.1 Human AKR1C1-4

Name/Gene ID	**Chromosomal Localization**
AKR1C1 ID:1645	NC_000010.11 (4963415..4983283)
AKR1C2 ID:1646	NC_000010.11 (4987775..5018000)
AKR1C3 ID:8644	NC_000010.11 (5048781..5107686)
AKR1C4 ID:1109	NC_000010.11 (5196837..5218949)

2.2.2. AKR1C3 in different cancer types

Over recent years, the enzyme AKR1C3 has entered the research field of researchers. The enzyme of AKR1C3 is closely linked with many diseases, and a great of studies on this gene has been performed. It is upregulated and identified as a prognostic marker in many cancers, mainly including hepatocellular carcinoma (HCC) [55], breast cancer [56], colon cancer [57], prostate cancer [58-60], lung cancer [61], acute myeloid leukemia (AML), renal cell carcinoma (RCC) [62], cervical cancer and oropharyngeal squamous cell carcinoma (OPSCC) [63, 64]. The elevated of this gene contribute to the progression of carcinomas. Zhou et al. reported AKR1C3 promotes proliferation and metastasis of hepatocellular carcinoma cells by mediating the STAT3 [55]. AKR1C3 is upregulated in ductal carcinoma in breast [65], in DOX-resistant cells [66] and in non-small-cell lung cancer (NSCLC) tissues [61]. A study by Yoda and colleagues reported that AKR1C3 could lead to breast cancer cell survival by producing FP receptor ligands [56]. In renal cell carcinoma (RCC), Immunohistochemical staining results showed that higher AKR1C3 with higher RCC grade and stage [62]. In cervical cancer, a report by Wu et al. silenced the AKR1C3 and found that the cancer cell metastasis was also prevented. The results from another study proved that AKR1C3 affected cervical cancer cells' invasion [64]. Huebbers et al. also showed this gene indicates a poor prognosis in OPSCC [63]. Besides, AKR1C3's expression increased in more aggressive prostate cancer cells [51], and silencing of the this gene could inhibit cells' migration and EMT through activating ERK signaling [59].

2.2.3. The role of AKR1C3 in chemotherapy

As an important detoxification enzyme in vivo, AKR1C3 certainly influences the therapeutic effect of tumor and its role in drug resistance has also been reported [57, 67-69]. The overexpressed of AKR1C3 resulted in the hyposensitivity of docetaxel (DTX) to prostate cancer, AKR1C3 can effectively reduce reactive 4-hydroxy nonenal (HNE) levels, which could increase antioxidant activities of cancer cells, and silencing of AKR1C3 enhanced DTX toxicity [70, 71]. Indomethacin is known as a AKR1C3' inhibitor, was used by Liu et al. in prostate cancer cells, and that inhibiting AKR1C3 could induce cells to death in Abiraterone and Enzalutamide [71, 72].

In colon cancer, with the continuous explosion to the anti-cancer agent, the elevated AKR1C3 could be found in cisplatin-resistant cells [57]. Silencing of the AKR1C3 in these resistant cells could increase sensitivity to cisplatin [57]. Matsunaga et al. also have studied that AKR1C3 could contribute to the promotion of the chemo-resistance by detoxifying the reactive aldehydes [73].

In hepatocellular carcinoma, Zhou. et al. revealed that AKR1C3 inhibitors can inhibit tumor growth and promote apoptosis in HCC cells. Besides, an AKR1C3/NF-κB/STAT3 signaling loop has been demonstrated in hepatocellular carcinoma cells that could result in its proliferation and metastasis. Targeting AKR1C3 would prolong the OS of HCC [55].

The protective effect of AKR1C3 can also be reflected in the study of human myeloid leukemia, it makes human myeloid leukemia cells more resistant DAU [74]. This effects also was reported by Morell et al [75]. It has been proved that AKR1C3 catalyzes the conversion of doxorubicin (DOX) to doxorubicinol (DOX-ol) protecting the cells from anticancer treatment [76]. Further studies revealed that the DOX-resistant breast cancer cells elevated AKR1C3's expression. they later proved that the overexpressed AKR1C3 cells were more DOX-resistant [69, 76]. Another study also found that AKR1C3 protecting the cells from DOX [77].

2.3. Reactive oxygen species (ROS)

2.3.1. Introduction

ROS can be simply named as oxygen-containing reactive species [78, 79]. They are formed by the partial reduction of molecular oxygen which has unpaired electrons. It includes both oxygen radicals and non-radicals [80]. Although reactive oxygen intermediates (ROIs), reactive oxygen metabolites (ROMs), and oxygen radicals are the used terms to describe oxygen-containing reactive species in some literature, ROS is still widely used in biology and medicine [79].

Mitochondria and NADPH oxidases can produce ROS in cancer [81]. The elevated intracellular ROS levels were related with increased intracellular oxidant production [82]. NRF2 participates in and defends the stability of the cellular redox state **(Figure 2.3)**. NRF2 is an important gene regulating the balance of intracellular oxygen stress and has

received extensive attention. NRF2 could combine with the ARE and then regulates multiple antioxidant genes' expression, detoxification genes, metabolism, and transporters [83]. These antioxidants have a strong antioxidant capacity which could directly neutralize the intracellular ROS and protect the cells from harmful stimuli, so that cells can maintain a stable redox level [83, 84]. The Nrf2/ARE signaling pathway can target nearly 500 genes such as NADPH-quinone oxidoreductase-1 (NQO-1) and cystine transporter (SCL7A11/xCT), which is involved in counteracting ROS [85].

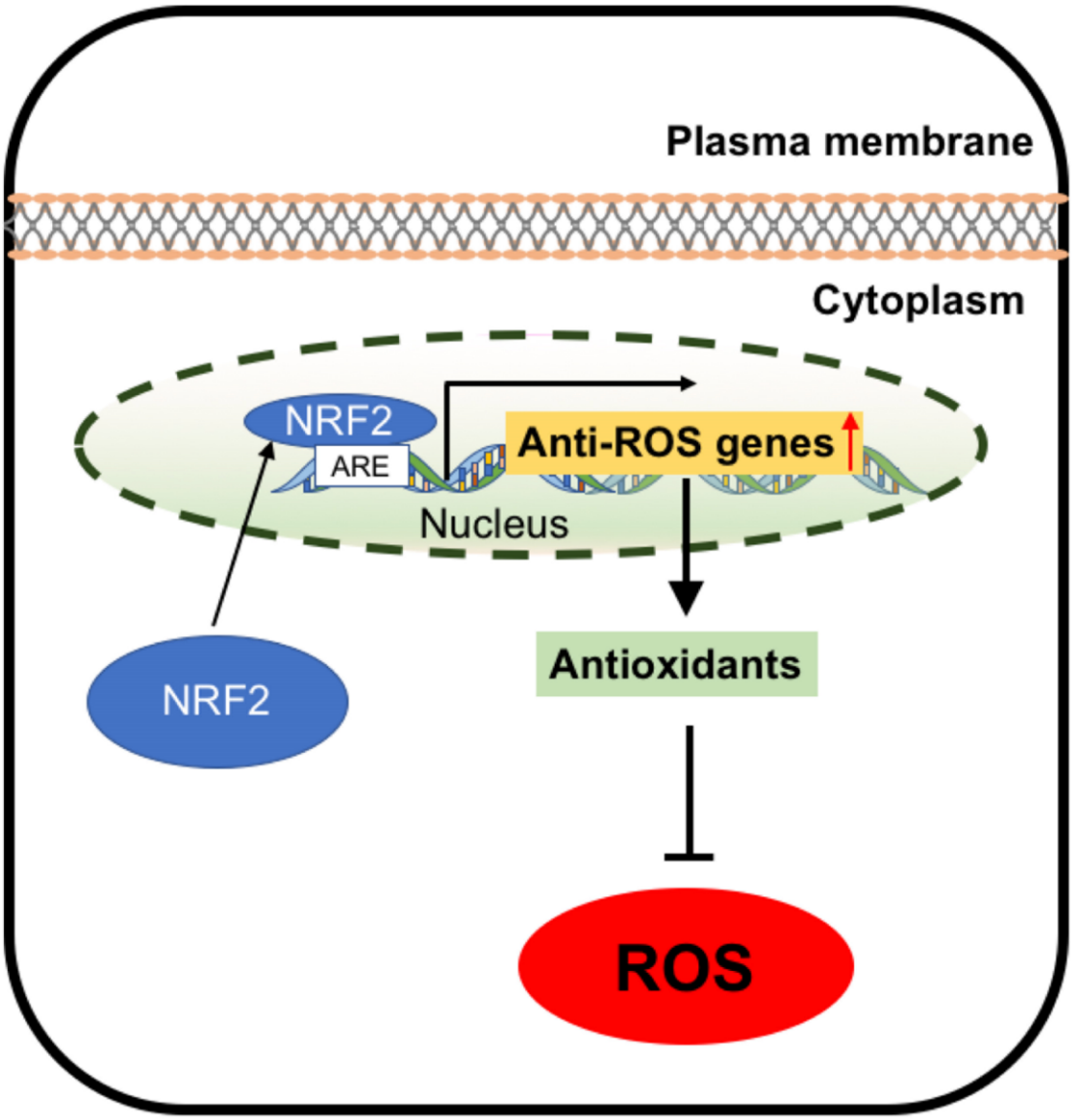


Figure 2.3. Diagrammatic drawing of NRF2-ROS signaling pathway.

2.3.2. ROS in Cancer

ROS is essential for both pathophysiology and physiology of aerobic life [81], meanwhile, excessive ROS could result in oxidative stress which result in many diseases, including inflammation [86], neurodegenerative diseases [87] and cancer [81]. Therefore, too much ROS is not acceptable in the cell. The stable redox level in the cell is very important, which affects the cell signaling pathways of cell cycle, progression, migration, and cell survival [88]. Rather, excessive ROS levels may overwhelm the antioxidant capacity of cancer cells then cause potential toxic effects to biomolecules, including proteins, lipids, RNA, and DNA leading to cell and tissue injury [81]. Moreover, excessive intracellular ROS levels may induce apoptosis [81]. Therefore, in order to better maintain a stable intracellular redox level, the antioxidant capacity of cells should also be greatly improved. A variety of antioxidant enzymes are equipped in cells improve its ability against ROS [89]. Dysregulation of redox are hallmarks of cancer and ROS has been associated with cancers which was showed to produce more ROS than their normal counterparts [90].

2.3.3. The role of ROS and GSH in chemotherapy resistance

There is increasing evidence that chemotherapeutic drugs induce numerous biological responses in cells, such as induction of cellular stress, ROS production, and oxidative DNA damage [88]. These chemotherapeutic drugs can break the redox homeostasis of tumor cells to kill the cells, resulting in the accumulation of a large amount of ROS in the body to induce apoptosis [91]. Therefore, a large amount of ROS levels in cells undoubtedly brings great challenges to the survival of cells [88]. Tumor cells also respond biologically when exposed to these chemotherapy drugs. However, higher levels of antioxidants, such as GSH and Superoxide dismutase (SOD2), are indeed found in these cancer cells that evade cell death. These antioxidants are undoubtedly a powerful mechanism of chemoresistance in tumor cells [89].

GSH pool is a potent reducing system that balances redox homeostasis [89]. Then GSH was regarded as the primary antioxidant in cells, which could eliminate excessive ROS [92]. Furthermore, cystine transporter SCL7A11/xCT mediate the generation of GSH, which plays an important role inside the cell [93] **(Figure 2.4)**. Owing to these properties of GSH, targeting GSH pool decreased drug resistance [94, 95]. Wang et al. found that ferroptosis was induced in mice with GSH depletion [96]. GSH could detoxify ROS and

then protect the cell from ferroptosis in pancreatic cancer, lung cancer, liver cancer, and colorectal cancer [92, 97, 98]. Zhang et al. have examined that GSH-mediated detoxification of cisplatin [99]. Moreover, some novel and effective treatments were carried out, Hou et al. revealed a GSH-responsive nano-prodrug system, which was constructed by covalent modification of anticancer drugs, for the combination therapy [94]. Tang et al, found that depleting GSH could inhibit tumor activity [100]. Above all, the GSH-related therapeutic strategy could be a promising treatment for cancer **(Figure 2.4)**.

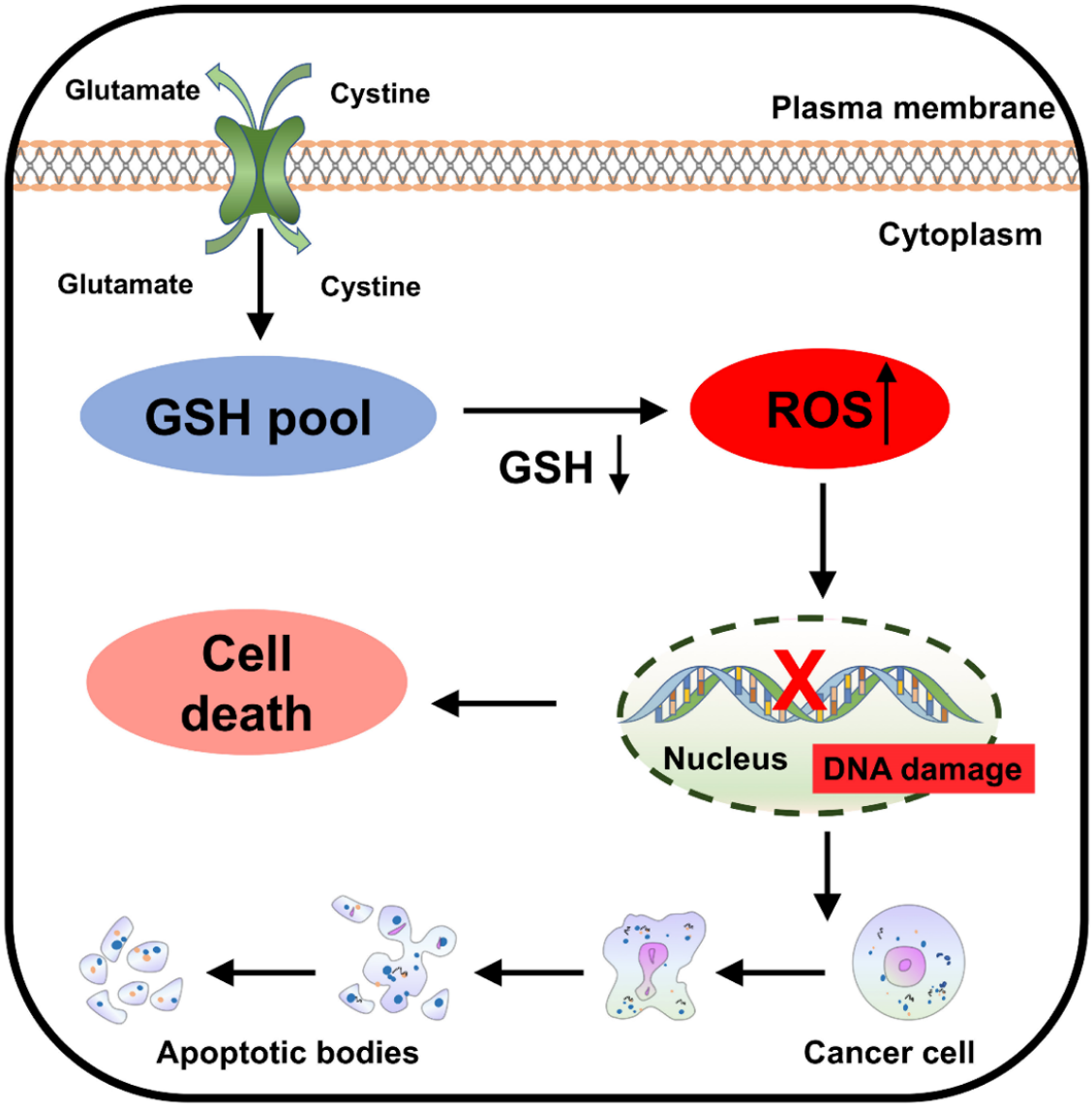


Figure 2.4. Diagrammatic drawing of overproduction ROS in cancer.
Excessive ROS induce apoptosis. GSH pool was regarded as the antioxidant pool in cancer cells, which could fight against the DNA damage and protect the cells from excessive ROS.

In short, in this study, in an attempt to evaluated AKR1C3's effect on the chemotherapy of EAC cells. We carried out *in vitro* experiments to investigate whether AKR1C3 could enhance cancer cells' antioxidant capability to reduce the damage from the anti-cancer drugs via AKT/GSH/ROS signaling.

III. **MATERIALS AND METHODS**

3.1.Materials

3.1.1. Cell lines and human specimens

Cell lines and human specimens	Source
OE19	Pathology of Cologne University
SKGT-4	
OACP4C	
FLO-1	
JHesoAD1	
OE33	Sigma Cell Line Bank (Sigma, 96070808)
12 pairs of human specimens	Universität zu Köln (BIOMASOTA, ID: 13-091)

3.1.2. Cell culture

Materials	Company, Germany (Cat#)
RPMI1640	Life technology
FBS	Capricorn Scientific GmbH (FBS12-A)
P/S	Invitrogen (15140122)
Trypsin	Invitrogen (25300054)
Doxycycline hyclate	Peprotech (2431450)
Trypan	Invitrogen (T10282)

DMSO	AppliChem (A36720100)
DPBS	PAN Biotech (P04-36500)

3.1.3. Medium

3.1.3.1. Medium for cell culture

Cell lines	Supplements	
OE33		
FLO-1		
OACP4C		
SKGT-4	RPMI1640 +	10% FBS 1% P/S
OE19		
JHesoAD1		
HEK293T		

3.1.4. Materials for PCR

3.1.4.1. RNA extraction

Name	Company, Germany (Cat#)
NucleoSpin® Tissue	MACHEREY-NAGEL (740952)
TRI reagent	Sigma (T9424)
NucleoSpin® Gel PCR Clean-up	MACHEREY-NAGEL (740609)

3.1.4.2. Quantitative real-time PCR

Name	Applied Biosystems, USA /Cat#
cDNA Kit	# 4368814
Green Master Mix	# 4385612
Clear Adhesive Film	# 4306311
96-Well Plate	# N8010560

3.1.5. Materials for WB

3.1.5.1. Reagents

Name	Company
RIPA Buffer (10X)	Cell Signaling Technology, USA
Roti®-Block (10X)	Carl Roth, Germany

Name	Thermo Scientific™, Germany/ Cat#
BCA Kit	# 23225
PMSF Protease Inhibitor	# 36978
LDS Sample Buffer	# 84788
Prestained Protein Ladder	# 26617
West Pico PLUS Chemiluminescent Substrate	# 34577

3.1.5.2. Solutions

Solutions	Formula

Running buffer	pH 8.3	Tris (25 mM) Glycine (190 mM) SDS (0.1%)/ H2O
Transfer buffer	pH 8.3	Tris (25 mM) Glycine (190 mM) Methanol (10%) H2O
TBST	pH 7.4	Tris (20 mM) NaCl (150 mM) Tween 20 (0.1%) HCl H2O
Blocking solution		Roti-Block (10X) H2O
Primary antibody solution		Roti-Block (10X) BSA (0.1%) Sodium azide (0.05%) H2O
Striping solution	pH 2.0	Glycine (20 mM)

			HCl SDS (1%)/ H2O

3.1.5.3. Antibodies

Name	Company	Cat#	Specificity/Host (Human-H, Mouse-M, Goat-G)
NRF2	Cell signaling technology	# 12721	H/R
phospho-AKT(T308)		# 13038	H/R
phospho-AKT(Ser473)		# 4058	H/R
AKT		# 9272	H/R
α-tubulin		# 3873	H/M
IgG		# 2729	-/-
h-AKR1C3	R&D Systems	# MAB7678	H/M
CXCR4	Abcam	# Ab124824	H/R
ZEB-1	Santa Cruz	# sc-25388	H/R
SNAIL1	Santa Cruz	# sc-28199	H/R
2nd antibody	Invitrogen	# 31430	M/G
2nd antibody	Invitrogen	# 31460	R/G

3.1.6. Materials for plasmids construction and lentiviral transduction of EAC cells

Name	New England Biolabs, England/ Cat#
CutSmart® Buffer	# B7204S
NEBuffer™ 2	# B7002S
T4 DNA Ligase	# M0202S
AgeI-HF	# R3552S
XhoI-HF	# R0146S
EcoRI-HF	# R3101S

Name	Addgene, USA / Cat#
pMDLg/pRRE	# 12251
pMD2.G	# 12259
pRSV-Rev	# 12253
Tet-pLKO-puro	# 21915

Name	Company,USA/ Cat#
Puromycin	PeproTech/# 5855822,
Mix & Go! Competent	Zymo Research /# T3017

Name	Sigma, Germany/ Cat#
PEI	# 408727

Hexadimethrine bromide/polybrene	# H9268
LB Broth (without agar)	# L2542
LB Broth (with agar)	# L3147

Name	Germany/ Cat#
Ampicillin Trihydrat	# SIALA1593
NucleoSpin® Plasmid	# 740588
NucleoBond® Xtra Midi	# 740412

3.1.7. Materials for flow cytometry

Name	Company
H2DCFDA	Sigma-Aldrich (4091-99-0)
APC Annexin V	Biolegend, USA (640920)
DAPI	Thermo Scientific (62247)
HBSS	Gibco Invitrogen (14175095)

3.1.8. Materials for ChIP

3.1.8.1. Reagents

Name	Company, Germany (Cat#)
FORMALD	Sigma (252549)
Dynabeads Protein G/A	Invitrogen (10004D/10002D)
Proteinase K	Invitrogen (4333793)

Protease Inhibitor	Sigma (S8820)

3.1.8.2. Primers

Primer	**Sequence (5' to 3')**
AKR1C3-ChIP-For	ACATCTTTACCCCTAGTGTTCAGT
AKR1C3-P1-Rev	AGTTCTTGAGATTTTGACTGGATGC

3.1.9. Materials for quantification of GSH

Name	Company, Germany
GSH/GSSG Kit	Abcam 205811
Trichloroacetic acid (TCA)	T0699-100ml, Sigma
NP-40	NP40S-100ml, Sigma
Flat bottom black polystyrene wells (96-well)	M0562-32EA, Sigma
$NaHCO_3$	Nr. 6885.2. Carl Roth

3.1.10. Materials for Mito Stress

Name	Company
Seahorse XF Kit	Agilent
Seahorse XF Calibrant Solution	Agilent
Seahorse XF DMEM medium	Agilent
D-glucose	Sigma

Antimycin A	Sigma
Rotenone	Biomol
Sodium pyruvate	Life Tech
L-Glutamine	Invitrogen

3.1.11. Chemicals and chemotherapy agents

3.1.11.1. Chemicals

Name	Carl Roth, Germany (Cat#)
NaCl	# 3957.2
BSA	# 8076.2
HEPES	# HN78.2
Tris	# 9127.2
Methanol	# 4627.5
2-Propanol (99%)	# 9866.5
Tween 20	# 9127.2

Name	Sigma, Germany (Cat#)
TCEP	# C4706
2-Mercaptoethanol	# M6250
Chloroform	# C7559

Name	Th. Geyer, Germany (Cat#)
Sodium azide	# 8690
Triton X 100	# 8013
HCL	# 182108
NaOH	# 1340
Ethanol (99%)	# 2212

Name	Company, Germany (Cat#)
SDS	AppliChem (A72495000)
Glycine	AppliChem (1313400914)
Nonidet® P40	AppliChem (A1694)
MTT	Biomol (cay- 21795)
EDTA	Merck (E-5134)

3.1.11.2. Chemotherapy agents

Name	Klinikum der Universität zu Köln
Cisplatin (CIS)	NeoCorp, Hexal AG
Oxaliplatin (OXA)	Accord HealthCare
5-fluorouracil (5-FU)	Accord HealthCare
Paclitaxel (PTX)	NeoTaxan, Hexal AG

3.1.11.3. Inhibitors

Name	Company, Germany
AKT inhibitor	MedChemExpress, HY-10355
NAC	Sigma-Aldrich, A9165
BSO	MedChemExpress, HY-106376A

3.1.12. Equipment

Name	Company, Germany
Class II Safety Cabinets	
CO2-incubators Heracell 150i,	Thermo Scientifi
Microcentrifuge	
Centrifuge	Megafuge 1.0R, Heraeus
Automatic pipettes	Eppendorf
Vortex	Lab dancer, VWR
Phase Contrast Microscope	Leica
IX83 Inverted Microscope	OLYMPUS
Water bath	Störk-Tronic
Fridge 4°C	Liebherr
Fridge -20°C	Bosch
Plate Reader	BMG Labtech
CellCamper® Mini, freezing box	NeoLab

Ecl Chemostar	Intas Science Imaging
Thermocycler	Tpersonal, Biometra
ThermoMixer C	Eppendorf
NanoDrop	Thermo Scientific

Name	Company, USA
PCR	Applied Biosystems
CytoFLEX	Beckman coulter
Cell Counter II	Invitrogen
Mini-PROTEAN® System Trans-Blot® Turbo™ Transfer System	Bio-Rad

Name	Company
Sonicator	Bioruptor® Pico, Diagenode, Belgium
Incubators	MCO-230AICUV-PE, Panasonic, Japan
Fridge (-80°/-150°C)	Sanyo, Japan

3.1.13. Consumable materials

Name	From Sarstedt, Germany
Tube (1/4/5/15/50mL)	-
SafeSeal tube	-
Centrifuge tube (15/50ml)	-

Cryotubes (1.8 mL)	-
Serological pipettes (5/10/ 25mL)	-
Pipette tips (1/10/20/200/1000µL)	-
6/12/24/ 96-well plates for culture	-
Dish (10 cm)	-

Name	VWR, Germany/ Cat#
Cell counting slide	# 734-2676
Syringe filters (0.2/ 0.45µm)	# 512-3180/3182
Cell strainer (40/70/100 µm)	# 734-2760

Name	Company, Germany
Filter Paper	# 88610, Thermo Scientific
PVDF membrane (0.2µM)	# 741260, MACHEREY-NAGEL

3.1.14. Software

Name	Company, USA
Office	Microsoft Corporation
OS X (10.11.6)	MacBook Air
ImageJ	ImageJ
Prism 7	GraphPad
Endnote X9	Thomson Reuter

GSEA (Version: 4.1.0)	Broad Institute
FlowJo software	Treestar Inc., Ashland

3.2.Methods

3.2.1. Cell culture and cell counting

Esophageal adenocarcinoma cells were maintained in a 10 cm dish or T-25T, T-75 flask in an incubator, with a humidified atmosphere of 5% CO_2 at 37 °C. Countess II Cell Counter (Invitrogen) was used for cell counting.

3.2.2. Proliferation assay

This assay was performed as our previously described [101].

3.2.3. Colony formation assay

This assay was performed as our previously described [101].

3.2.4. Wound healing assay

This assay was performed as our previously described [101].

3.2.5. Immunofluorescence

This assay was described previously [102]. Briefly. The primary antibody we used in this study was Anti-AKR1C3. Secondary antibody Alexa 488-conjugated anti-mouse was used. Finally, DAPI was used to stain the nuclear. An IX83 Inverted Microscope from OLYMPUS used for the pictures.

3.2.6. Quantitative Real-time PCR

This assay was described previously [101]. Primers are listed below **(Table 3.1)**. The experiments were performed in triplicate.

Table 3.1 Primers for qRT-PCR

Gene	Sequence (5’ to 3’)
GAPDH-for	GAAGGTGAAGGTCGGAGTC
GAPDH-rev	GAAGATGGTGATGGGATTTC
hAKR1C3-for	GTCATCCGTATTTCAACCGGAG

hAKR1C3-rev	CCACCCATCGTTTGTCTCGTT
hNRF2-for	TTCCCGGTCACATCGAGAG
hNRF2-rev	TCCTGTTGCATACCGTCTAAATC

3.2.7. Western blot (WB)

This assay was performed as our previously described [101].

3.2.8. Flow cytometry analysis

This assay was performed as our previously described [101].

3.2.9. Plasmids constructs and lentiviral transduction

Plasmids constructs and lentiviral transduction assay were performed previously [101]. Briefly, for the gain-function AKR1C3, an AKR1C3 overexpressing plasmid was designed and purchased from a GenScript, and pLenti-CMV-neo vector was used as control. For the loss-functional of AKR1C3, short hairpin RNA (shRNA) for AKR1C3 and NRF2 were constructed into the Tet-pLKO-puro vector via AgeI and EcoRI. shRNA target sequences were listed below **(Table 3.2)**.

Table 3.2 shRNA target sequences

Name	Sequence (5’ to 3’)
non-target control	AGGTAGTGTAATCGCCTTGTT
shAKR1C3-1	CTCACTGAAGAAAGCTCAATT
shAKR1C3-2	CCAGAGGTTCCGAGAAGTAAA
shNRF2	GCTCCTACTGTGATGTGAAAT

3.2.10. Measuring GSH level in EAC cells

GSH/GSSG Kit was used to measured GSH level of cancer cells follow the instructions of the kit. Firstly, we used the cold PBS to wash the cells and collected the cells after NP-40 treatment. Then we used the TCA to deproteinization. Finally, we put the

samples into the black 96-well plate to measure the fluorescence after neutralization. For details, please refer to our previous article[101].

3.2.11. Chromatin immunoprecipitation

This assay was described previously [101]. Diagrammatic drawing was shown below **(Figure 3.1)**. The used primers were listed below **(Table 3.3)**.

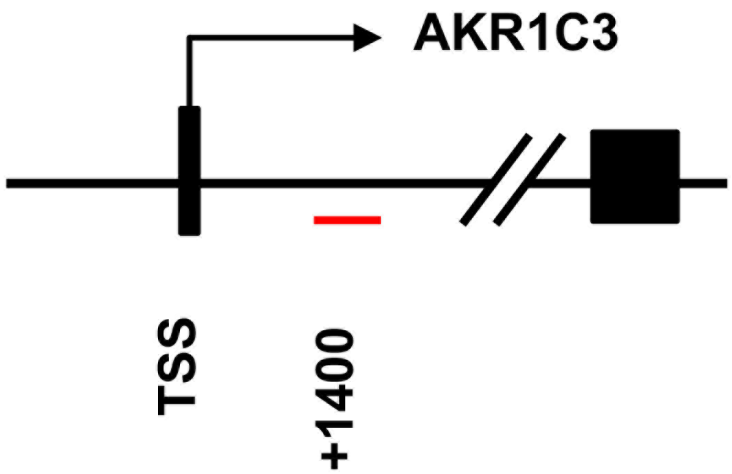


Figure 3.1. Diagrammatic drawing of the promoter region of AKR1C3.
This is a diagrammatic drawing of the transcription factor binding to the AKR1C3' promoter. The binding area is around transcription start site (TSS) +1400.

Table 3.3 Primers for ChIP.

Gene	Sequence (5' to 3')
AKR1C3-ChIP-for,	ACATCTTTACCCCTAGTGTTCAGT
AKR1C3-ChIP-rev,	AGTTCTTGAGATTTTGACTGGATGC

3.2.12. The Mito stress Assay for EAC cells

This assay was described previously [102]. Briefly, Seahorse XF Kit was performed. In brief, Human EAC cells such as SKGT-4 and OE33 were cultured with the XF-96 plates. The XF base medium minimal DMEM media was in the list above. Real-time dynamic monitoring OCR in different condition. The basal conditions of EAC cells

checked then with mitochondrial inhibitors. OCR was measured with the XF96 extracellular flux analyzer.

3.2.13. Statistical analysis

Statistical evaluation was performed by GraphPad Prism 7. OS was calculated using the Kaplan-Meier method. Data were presented as mean ± SD of triplicate samples, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: non-significant, $p > 0.05$. Statistical significance was validated by two-sided unpaired t-test. The public available data here were described in our previous study [101].

IV. **RESULTS**

4.1.Characterization of AKR1C3 in EAC

4.1.1. AKR1C3's expression in EAC patients

In the present project, AKR1C3 mRNA level in the EAC is higher than in the normal in the data of GSE26886 and GSE92396 via public database **(Figure 4.1.1 A and B)**. We also collected 12 paired tumor tissues with matched normal tissues in our cohort to demonstrate the AKR1C3' protein levels. However, only about half of the 12 patients had elevated AKR1C3 expression in tumor tissue **(Figure 4.1.1 C).** Although the trend elevated AKR1C3' expression correlates with poorer survival probabilities in EAC patients, it was not statistically significant with the *P-value* = 0.6719 **(Figure 4.1.1 D)** [101].

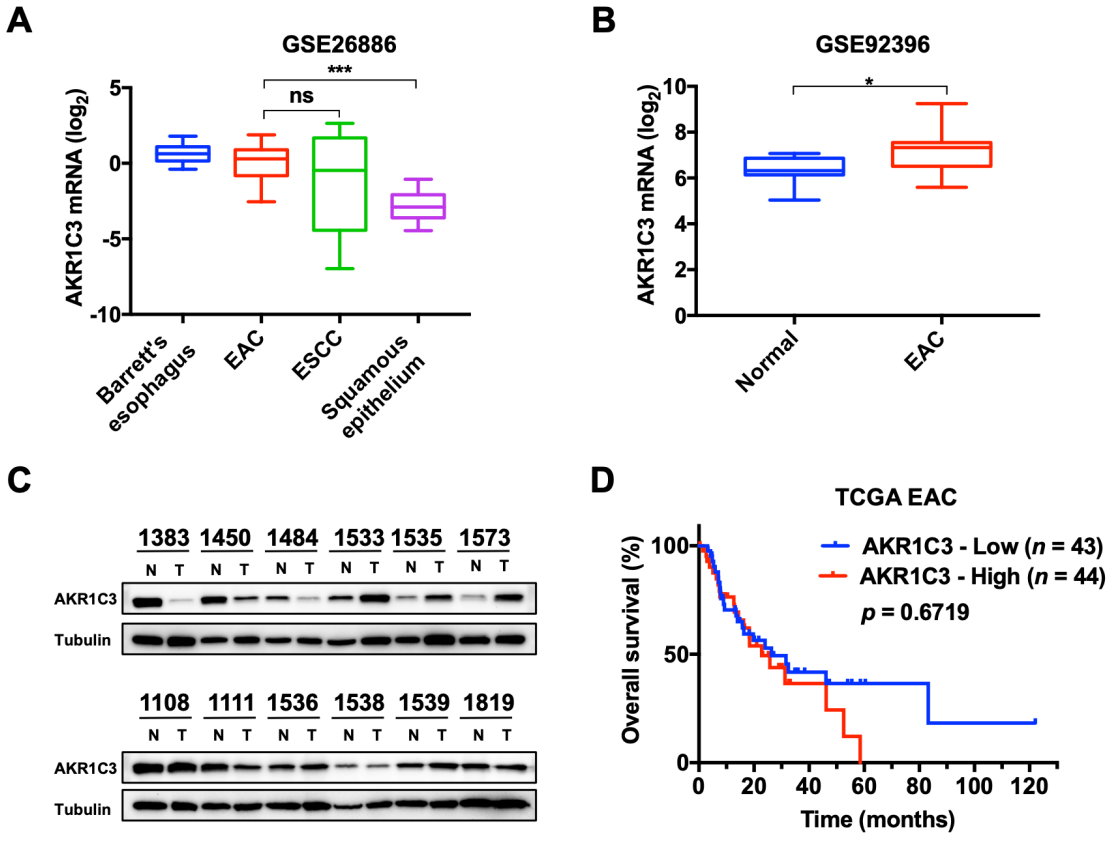


Figure 4.1.1 AKR1C3's expression in EAC.

(A and B) Public database showed AKR1C3 in EAC is higher than in the normal (C) AKR1C3 protein level was evaluated by WB (T = tumor; N = normal). (D) The EAC patients were chosen out from the data of TCGA-ESCA to check on their prognostic status.

4.1.2. AKR1C3's expression in EAC cell lines

To evaluate AKR1C3's expression in different EAC cell lines, six human EAC cell lines were used in this project such as OE19, OE33, OACP4C, FLO-1, JHesoAD1 and SKGT-4. The AKR1C3 was detected by western blot in OE19, OE33, OACP4C, FLO-1, JHesoAD1 and SKGT-4 cell lines. The result showed 3 cell lines such as OE19, OACP4C and SKGT-4 have high AKR1C3 expression. While other 3 cell lines such as OE33, JHesoAD1 and FLO-1 is different with OE19, OACP4C and SKGT-4. The protein level was relatively low in OE33, JHesoAD1 and FLO-1 **(Figure 4.1.2 A)**. Furthermore, we chose 2 cell lines such as SKGT-4 and OE 33 for the immunofluorescence. Its expression in SKGT-4 and OE33 cells were detected by WB. The result showed that its expression was consistent with the results by WB above. Moreover, its expression in SKGT-4 and OE33 cells were also checked by anti-AKR1C3 and DAPI immunofluorescence. The result showed that it was higher in SKGT-4 than in OE33, which also was consistent with the results by WB above **(Figure 4.1.2 B)**.

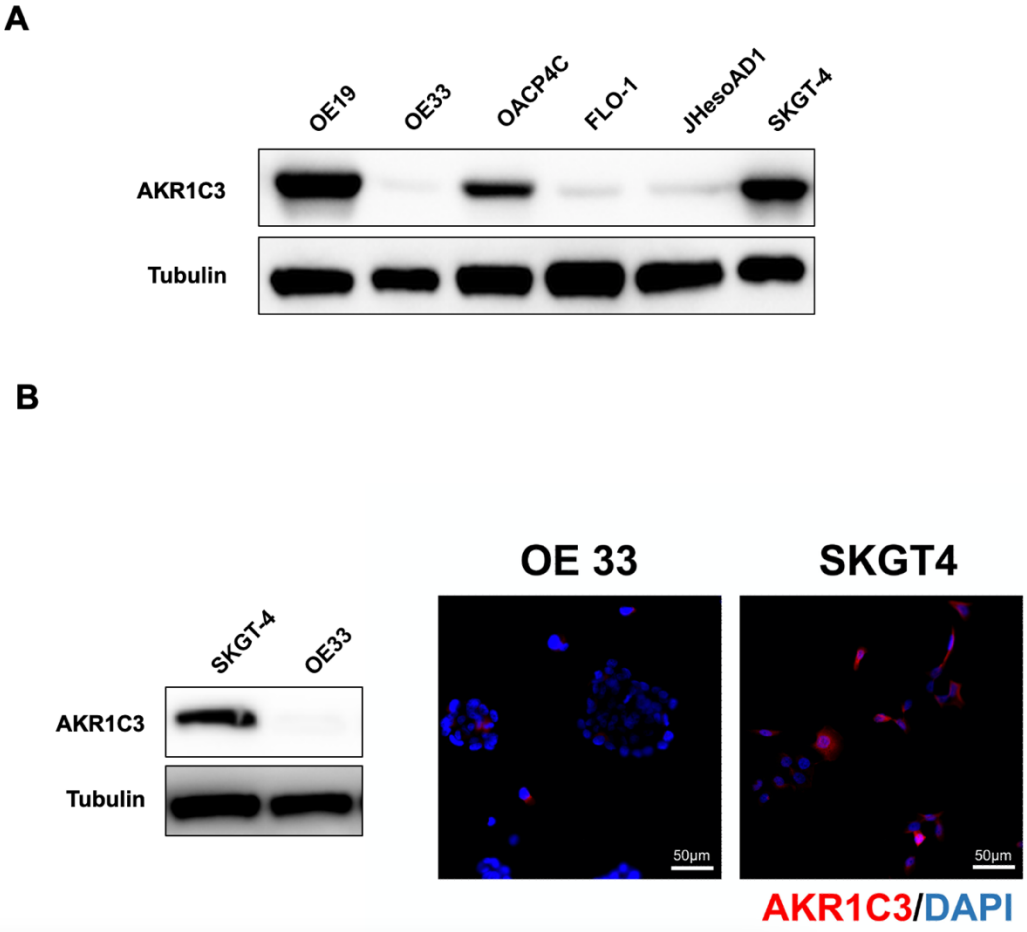


Figure 4.1.2 AKR1C3's expression in different EAC cell lines.

(A) The level of endogenous AKR1C3 in 6 human EAC cell lines, including OE19, OE33, OACP4C, FLO-1, JHesoAD1 and SKGT-4. (B) Western blot and Immunofluorescence results showed the AKR1C3's expression in SKGT-4 and OE 33.

4.1.3. Localization of AKR1C3 in EAC cells

To investigate the localization of AKR1C3, we selected SKGT-4 cell lines with strong fluorescence expression to further examine the location of its expression. SKGT-4 cell line was used with immunofluorescence. Anti-AKR1C3 (red)/DAPI (blue) was used. The immunofluorescence result showed AKR1C3 was mainly in the cytoplasm, while a small portion of AKR1C3 might be expressed in the nucleus **(Figure 4.1.3)**.

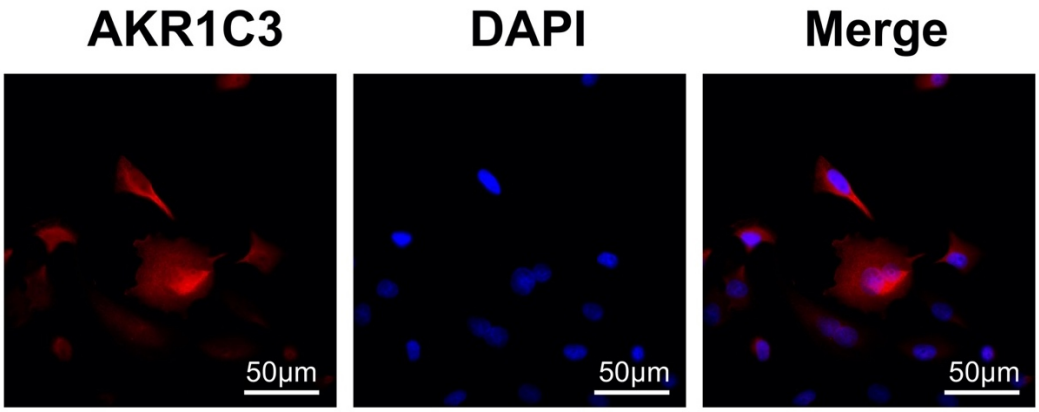


Figure 4.1.3 AKR1C3 is mainly located in cytosol.
Immunofluorescence labeled with anti-AKR1C3 and counterstained with DAPI were used in SKGT-4 cells. Red is for AKR1C3, and blue is for DAPI.

4.2.AKR1C3 regulates cell proliferation, colony formation and migration

4.2.1. AKR1C3 enhances cells' proliferation and colony formation

We chose 4 EAC cell lines such as OE33, SKGT-4, FLO-1, and OACP4C cells for further study. Next, we constructed SKGT-4 (SKGT-4$^{AKR1C3\text{-}KD/NT}$) and OACP4C (OACP4C$^{AKR1C3\text{-}KD/NT}$) cell lines for the loss-function experiment. We constructed and OE33 (OE33$^{AKR1C3\text{-}OE/VEC}$) and FLO-1 (FLO-1$^{AKR1C3\text{-}OE/VEC}$) cell lines to study the gain-functional experiment of AKR1C3 in EAC cells *in vitro* **(Figure 4.2.1 A)**. Because overall silencing efficiency in shAKR1C3-1 cells is lower than in shAKR1C3-2 cells, we choose the shAKR1C3-2 cells to do the further research.

We found that the SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells could decrease cell proliferation capability and form fewer colonies than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C $^{AKR1C3\text{-}shNT}$ cells **(Figure 4.2.1 B and D)**. Conversely, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cells had the significant opposite effect **(Figure 4.2.1 C and E)**.

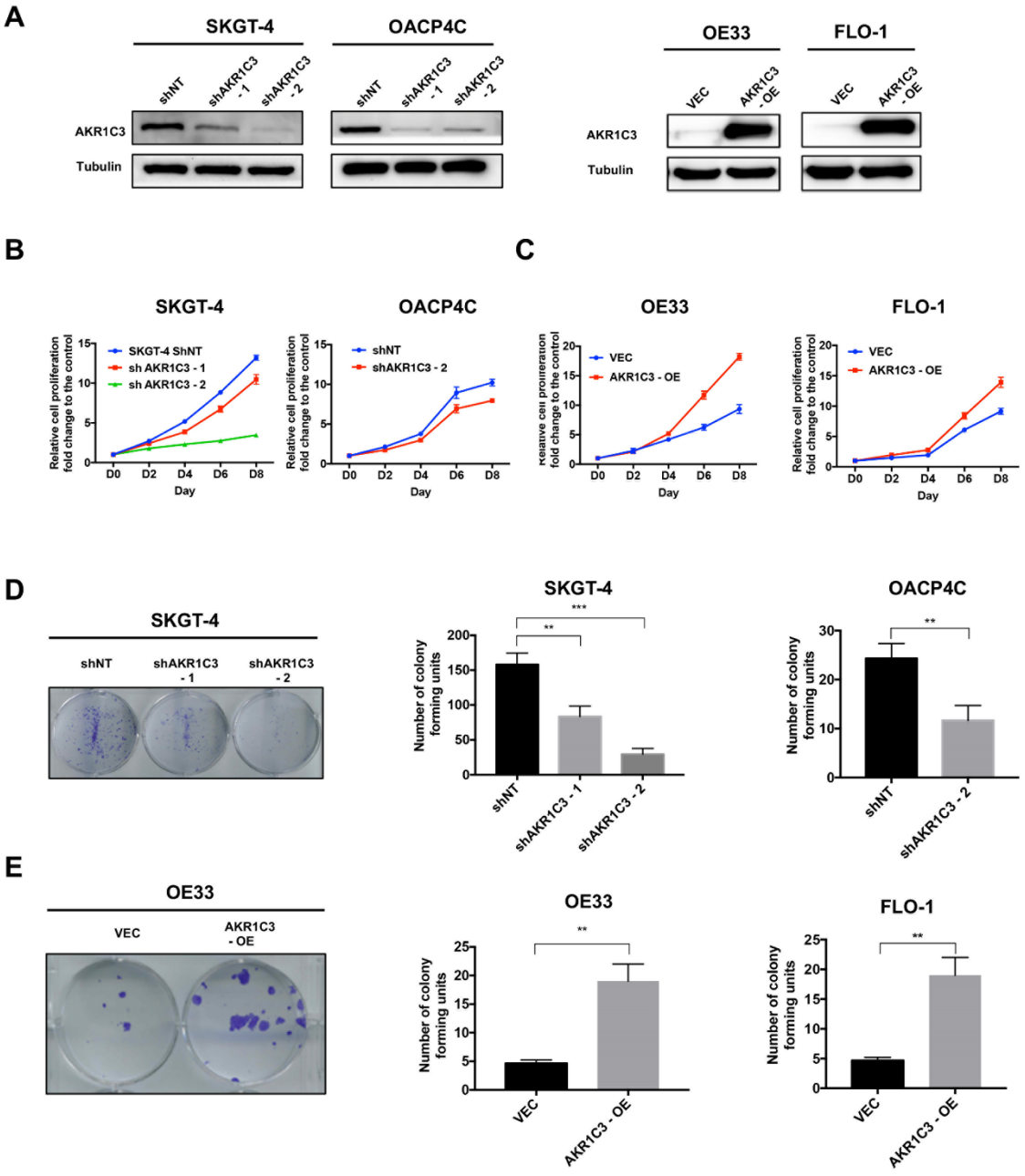


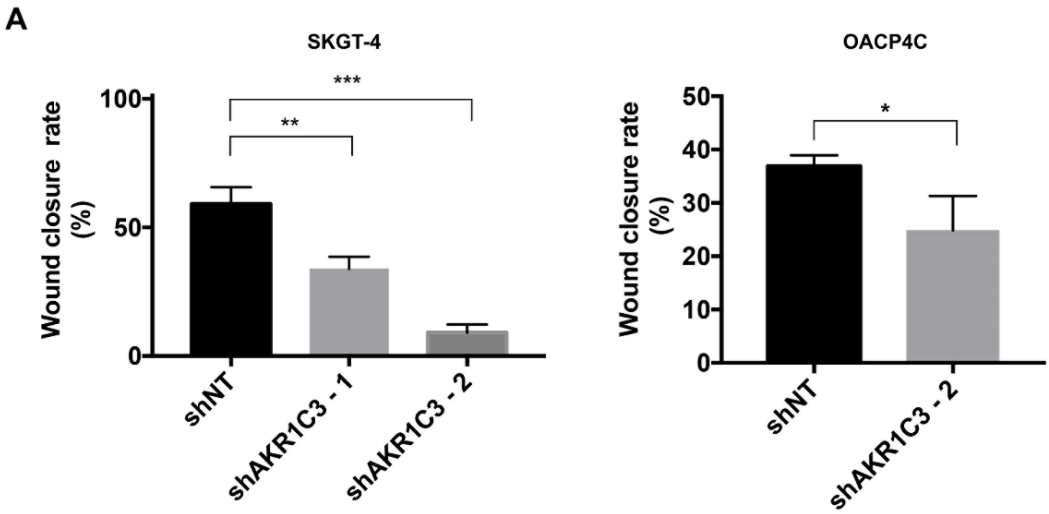
Figure 4.2.1 Proliferation and colony formation of EAC cells.

(A) Construction SKGT-4 (SKGT-4 $^{AKR1C3\text{-}KD/shNT}$) and OACP4C (OACP4C $^{AKR1C3\text{-}KD/shNT}$)), and OE33 (OE33 $^{AKR1C3\text{-}OE/VEC}$) and FLO-1 (FLO-1 $^{AKR1C3\text{-}OE/VEC}$) cell lines. (B and D) Proliferation rates and (C and E) colony forming capacity were examined.

4.2.2. AKR1C3 accelerates cells' migration

Next, we wanted to check whether AKR1C3 has an effect on the migration and metastasis of tumor cells, so we used the wound healing assay. The results showed that SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells had reduced migratory ability than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cells, whereas OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cells could obviously enhance the wound-healing rate than OE33 $^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells **(Figure 4.2.2 A and B)**.

Besides, the higher migration ability of tumor cells means that the tumor cells have a greater ability to metastasize, which has also been confirmed to be related to the epithelial-mesenchymal transition (EMT) of the tumor. So, CXCR4, ZEB-1 and SNAIL1 were checked. The results showed that SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells showed down-regulated of CXCR4, ZEB-1, and SNAIL1**(Figure 4.2.2 C)**. However, these markers not significantly increase in OE33$^{AKR1C3\text{-}OE}$ cells but in FLO-1$^{AKR1C3\text{-}OE}$ cells **(Figure 4.2.2 D)**. CXCR4 is not obviously decreased in the SKGT-4$^{AKR1C3\text{-}KD}$ cells. Considering these results together, we suggest that the elevated AKR1C3 might promote the migration of EAC cells via inducing EMT [101].



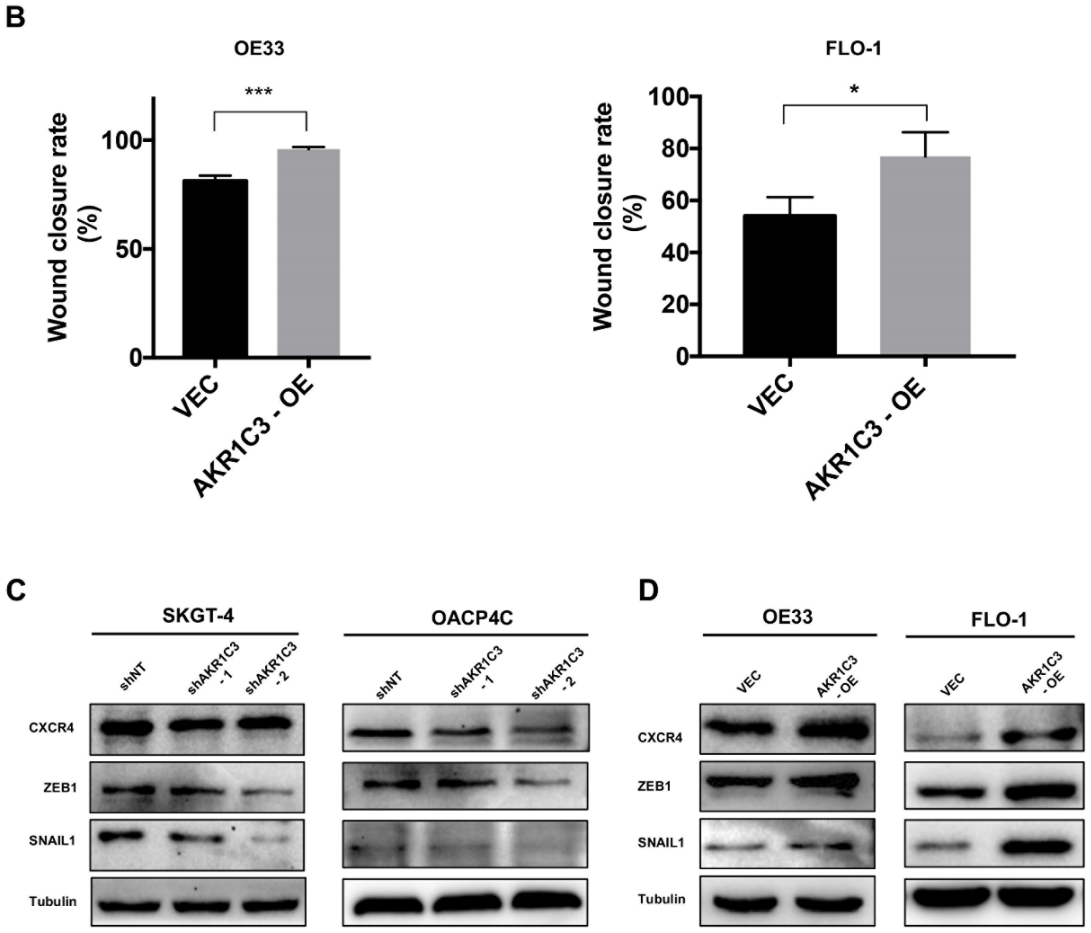


Figure 4.2.2. Analysis of EAC cells migration by *in vitro* wound healing assay.
(A and B) Migration capacity were examined. (C and D) CXCR4, ZEB1 and SNAIL1 were examined by WB.

4.3.AKR1C3 mediates chemotherapy resistance in EAC cells

4.3.1. AKR1C3 mediates chemo-resistance of cisplatin, oxaliplatin, 5-FU and paclitaxel

Four chemotherapeutic agents such as CIS, OXA, 5-FU, and PTX were applied. The MTT results showed that SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines had more apoptosis than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cells upon chemotherapeutic treatment **(Figure. 4.3.1 A and B)**, whereas OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines had less apoptosis than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells **(Figure. 4.3.1 C and D)**. Considering these results together, AKR1C3 could protect the EAC cells from CIS, OXA, 5-FU, and PTX **(Figure. 4.3.1)** [101].

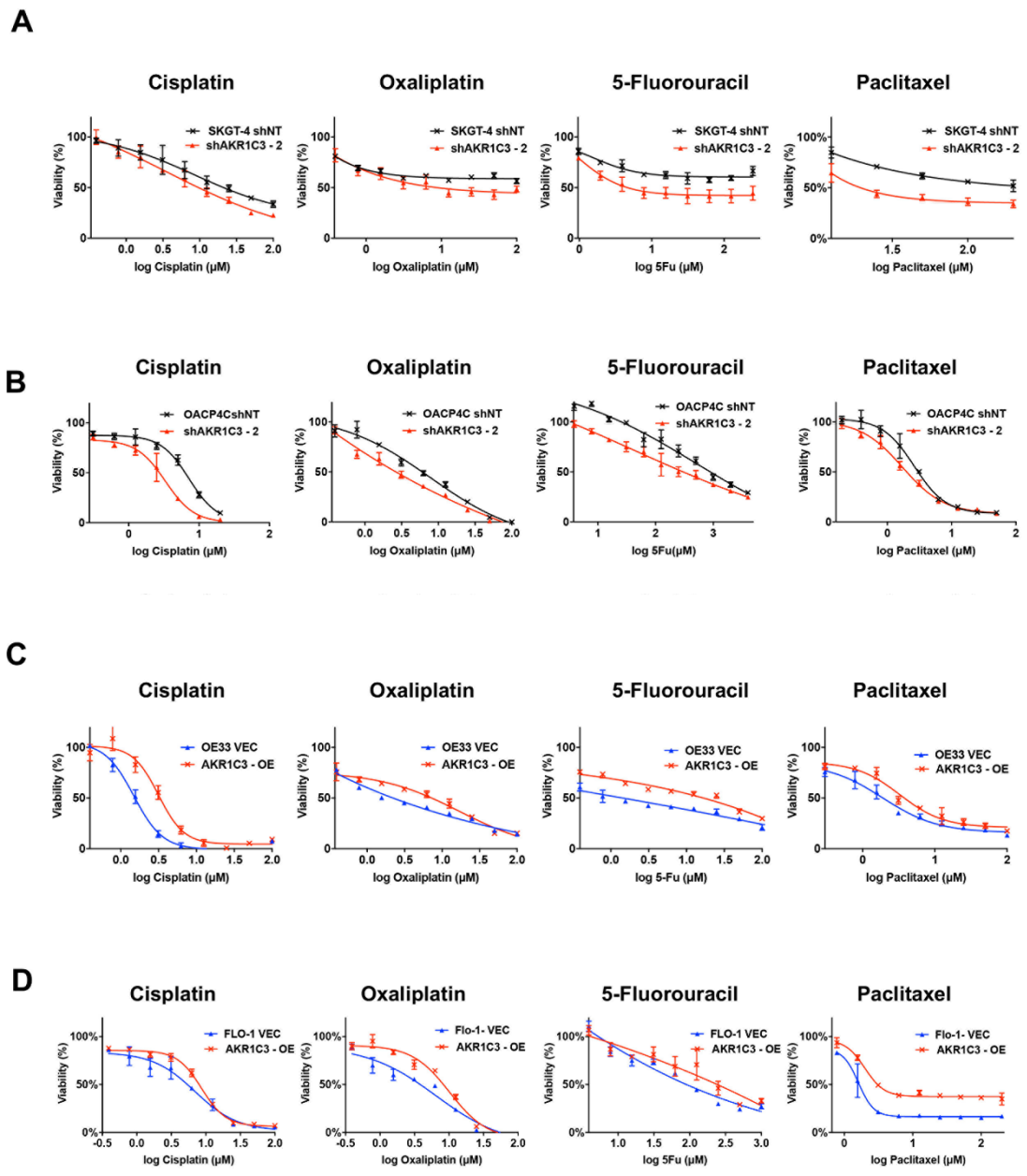


Figure 4.3.1. AKR1C3 protects cells from anti-cancer drugs.

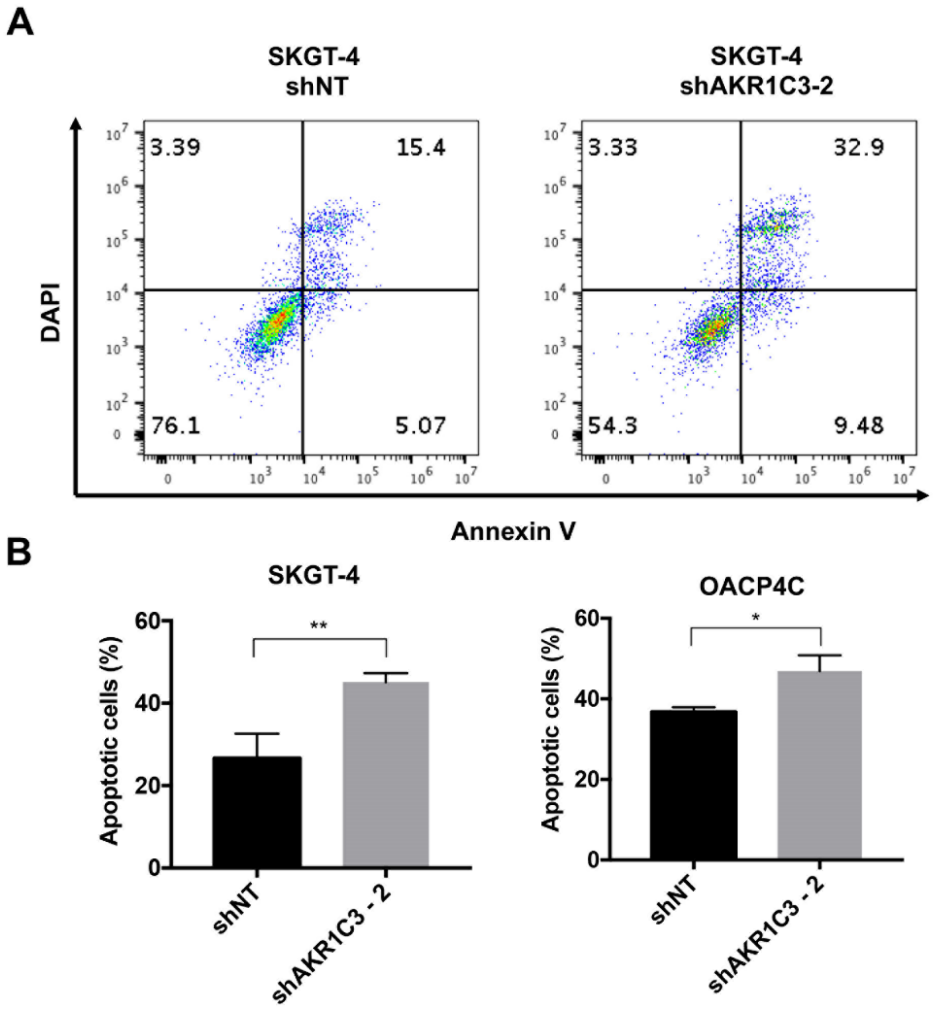
(A and B) Cell cytotoxicity assay was used after CIS, OXA, 5-FU, and PTX treatment in SKGT-4 and OACP4C cells, respectively. (C and D) Cell cytotoxicity assay was used after CIS, OXA, 5-FU, and PTX treatment in OE33 and FLO-1 cells, respectively.

4.3.2. AKR1C3 regulates CIS-induced apoptosis.

We have previously shown that AKR1C3 protects cells from CIS, OXA, 5-FU, and PTX treatment via drug detoxification. In addition, we used another more precise method to verify its effect in tumor cells. The apoptotic cells were determined by Annexin V/DAPI staining via FACS. According to the IC-50 of CIS treatment by MTT, cells with the appropriate concentration of CIS. Results showed that SKGT-4$^{AKR1C3\text{-}KD}$ and

OACP4C$^{AKR1C3\text{-}KD}$ cell lines had more apoptotic cells than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cells upon cisplatin treatment **(Figure. 4.3.2 A and B)**. Rather, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines had less apoptotic cells than OE33 $^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells **(Figure. 4.3.2 C and D)**. Although we only used cisplatin, the results from FACS are consistent with the above results by MTT [101].

Additionally, drug metabolizing enzymes were enriched in AKR1C3^{high} group via GSEA **(Figure. 4.3.2 E)**. Drug metabolizing enzymes take participate in the degradation and metabolism of chemotherapeutic agents. These enzymes contribute to the detoxification of these toxic agents. In conclusion, the elevation of AKR1C3 could protect tumor cells and reduce the damage of anti-cancer drugs to EAC.



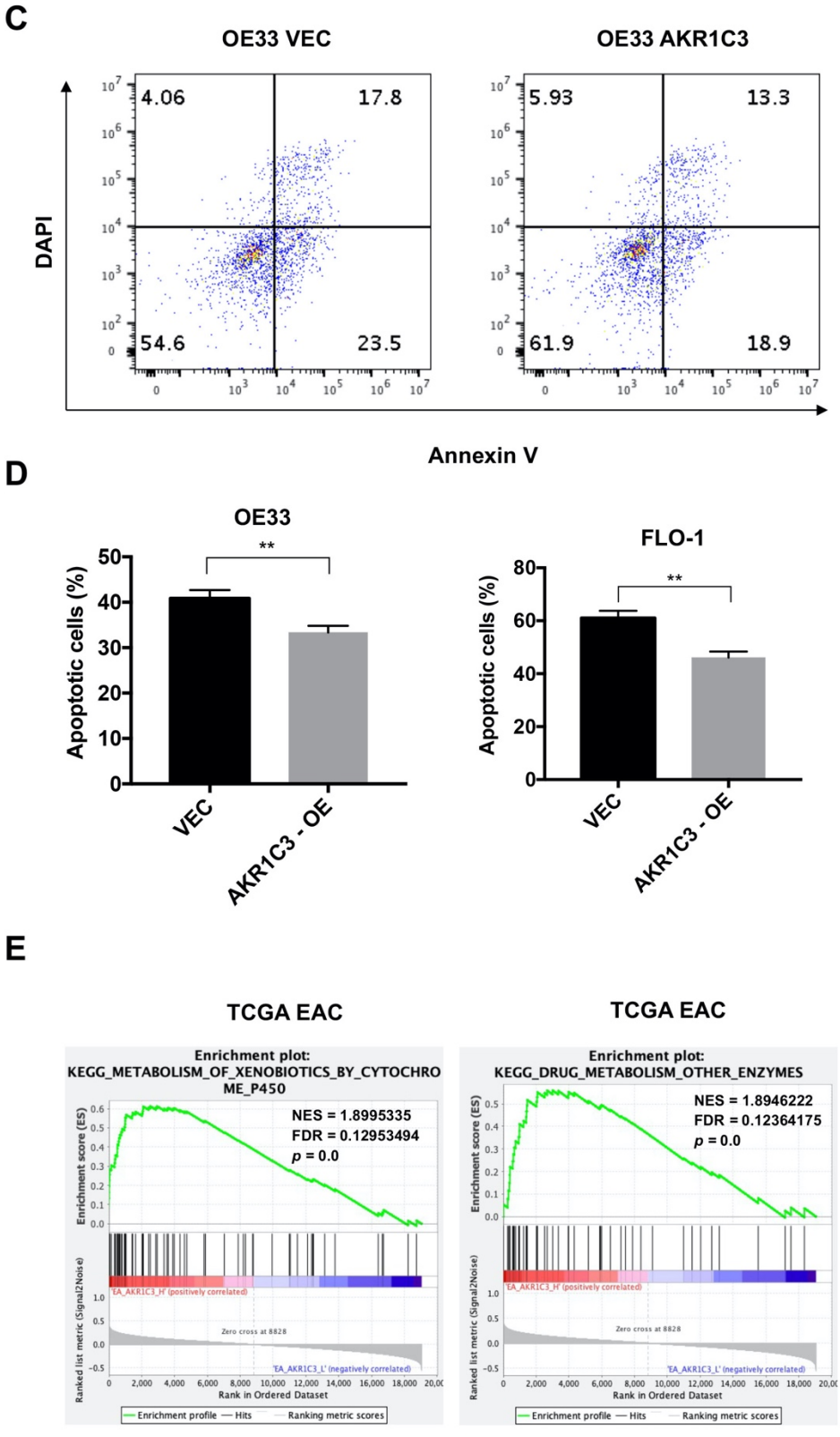


Figure 4.3.2. AKR1C3 mediates CIS-induced apoptosis.

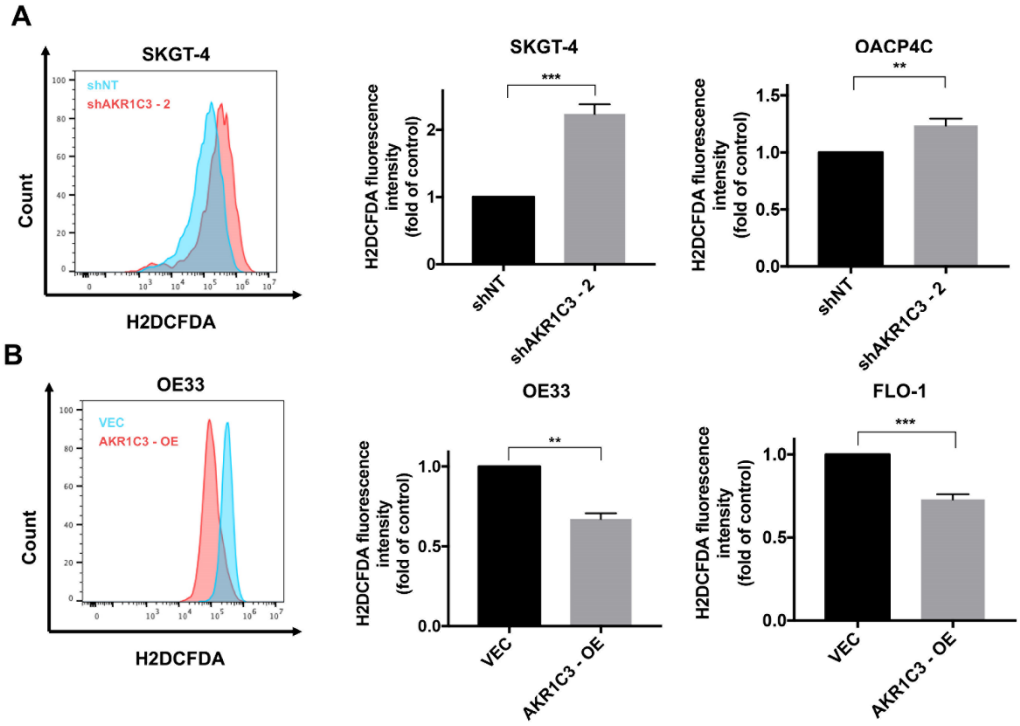
(A and B) Apoptotic cells was checked via FACS. (C and D) Apoptotic cells was checked via FACS. (E) GSEA analysis from TCGA dataset.

4.4.AKR1C3 modulates chemo-resistance through breaking redox-balance

4.4.1. AKR1C3 modulates ROS levels

In general, cytotoxic drugs could increase the ROS level [103]. The current study seeks to elucidate that AKR1C3-mediated chemo-resistance whether rely on ROS. ROS was measured via FACS using H2DCFDA dye. We found that the ROS was higher in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C $^{AKR1C3\text{-}shNT}$ cell lines. Conversely, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines showed decreased ROS than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1 $^{AKR1C3\text{-}VEC}$ cells lines **(Figure 4.4.1 A and B)**.

Hydrogen peroxide (H2O2) was used to perform the rescue experiment for validating the essential role of ROS in AKR1C3-mediated apoptosis. The results showed that the SKGT-4 $^{AKR1C3\text{-}KD}$ and OACP4C $^{AKR1C3\text{-}KD}$ cell lines had more apoptosis than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. upon H2O2 treatment. Conversely, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines had the significant opposite effect **(Figure 4.4.1 C and D)**. The rescue experiment was performed with antioxidant N-acetyl-l-cysteine (NAC) and L-buthionine-S, R-sulfoximine (BSO). The results showed that NAC and BSO reversed AKR1C3's protective effect against CIS **(Figure 4.4.1 E and F)** [101].



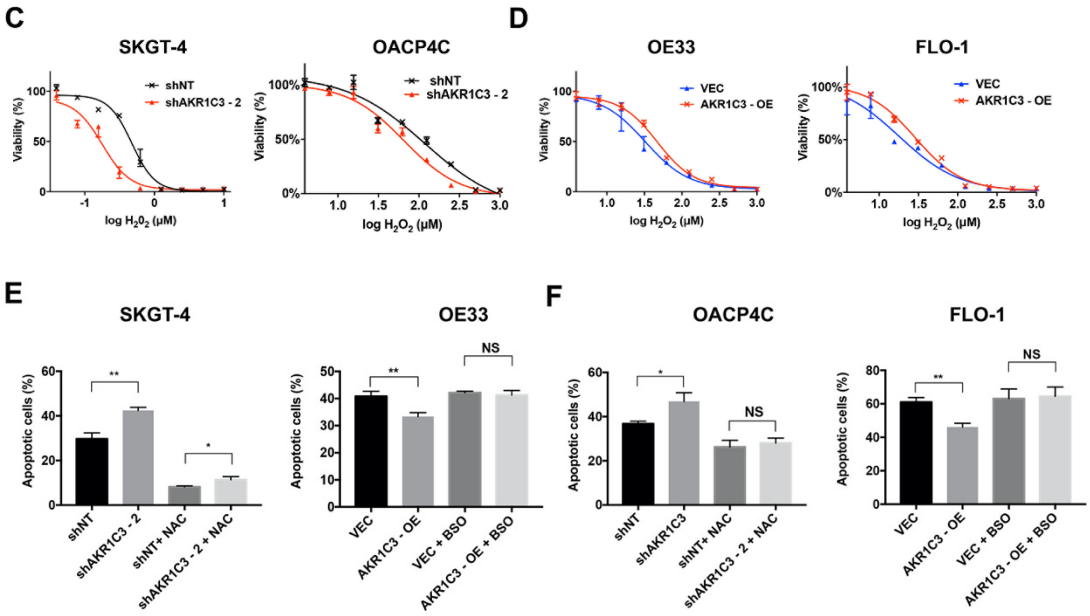


Figure 4.4.1. AKR1C3 protects the EAC cells from chemotherapeutic drugs by regulating ROS levels.

(A and B) ROS was checked via FACS. (C and D) MTT assay was used in SKGT-4, OACP4C, OE33 and FLO-1 cells, respectively. (E and F) Apoptotic cells were checked with CIS and NAC via FACS in SKGT-4 and OACP4C cells; Apoptotic cells were checked with CIS and BSO via FACS in OE33 and FLO-1 cells.

4.4.2. AKR1C3 is regulated by NRF2 in EAC

In the above results, AKR1C3 could not only regulate intracellular ROS levels, but also AKR1C3-mediated apoptosis is ROS-dependent. The transcription factor NRF2 is an important gene regulating the balance of intracellular oxygen stress and has received extensive attention.

Therefore, the relationship between AKR1C3 and NRF2 and ROS is worth exploring. Many articles have reported that NRF2 can bind to AKRs genes' promoter and regulate its transcription, but no one has confirmed its function with AKR1C3 in EAC. So, we validated it by ChIP in SKGT-4 cell line. The results showed that enrichment of NRF2 in the promoter region of AKR1C3 **(Figure 4.4.2 A)**. Both its expression were decreased in SKGT-4$^{NRF2\text{-}KD}$ cells than in the SKGT-4$^{NRF2\text{-}shNT}$ cells **(Figure 4.4.2 B-D)** [101].

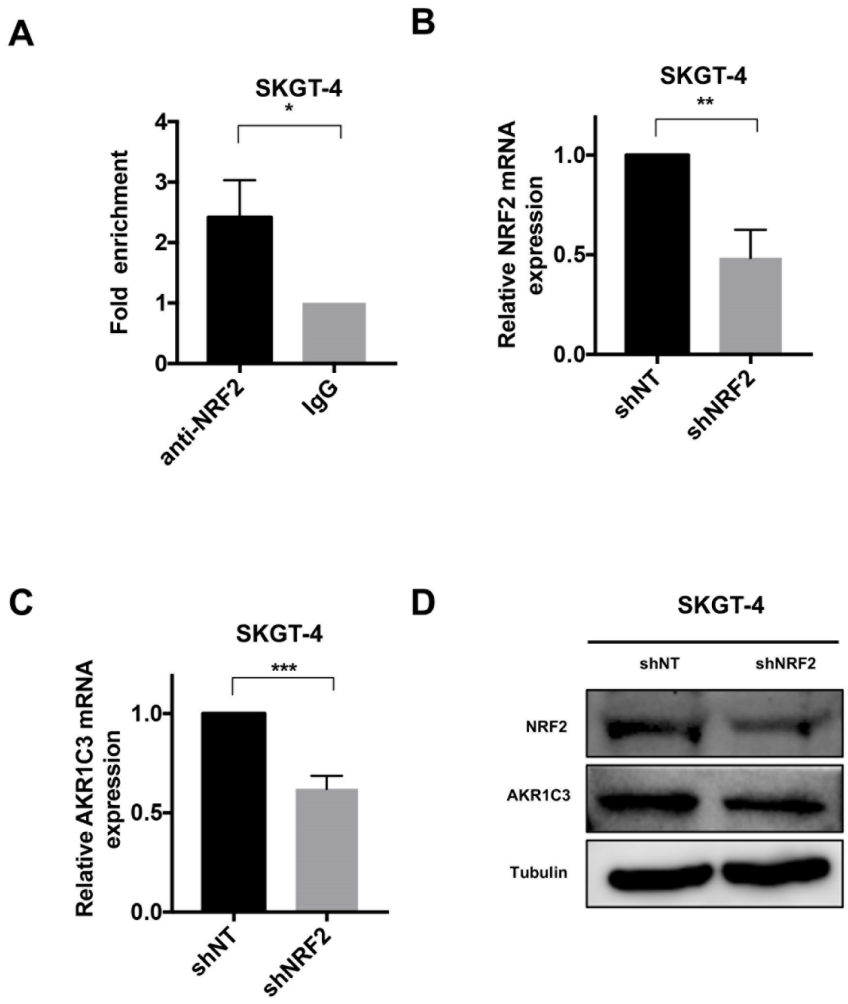


Figure 4.4.2. NRF2 regulates the expression of AKR1C3 in SKGT-4.

(A) The enrichment PCR result from ChIP assay. (B-D) The levels of NRF2 and AKR1C3 were analyzed by PCR and WB in SKGT-4 cells.

4.5.AKT is mediated by AKR1C3 and reduces ROS level.

4.5.1. AKT phosphorylation is regulated by AKR1C3

Our results showed that AKT's phosphorylation was decreased in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. Conversely, it was increased in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells lines **(Figure. 4.5.1 A and B)**.

The above basically proved that this gene regulates AKT. AKT inhibitor VIII was used for further research. The results showed that AKT's phosphorylation decreased in cells treated with AKT inhibitor, but the total AKT protein level did not change. AKT inhibitor

could inhibit the AKT' phosphorylation but had no effect on AKR1C3 expression **(Figure. 4.5.1 A and B)**.

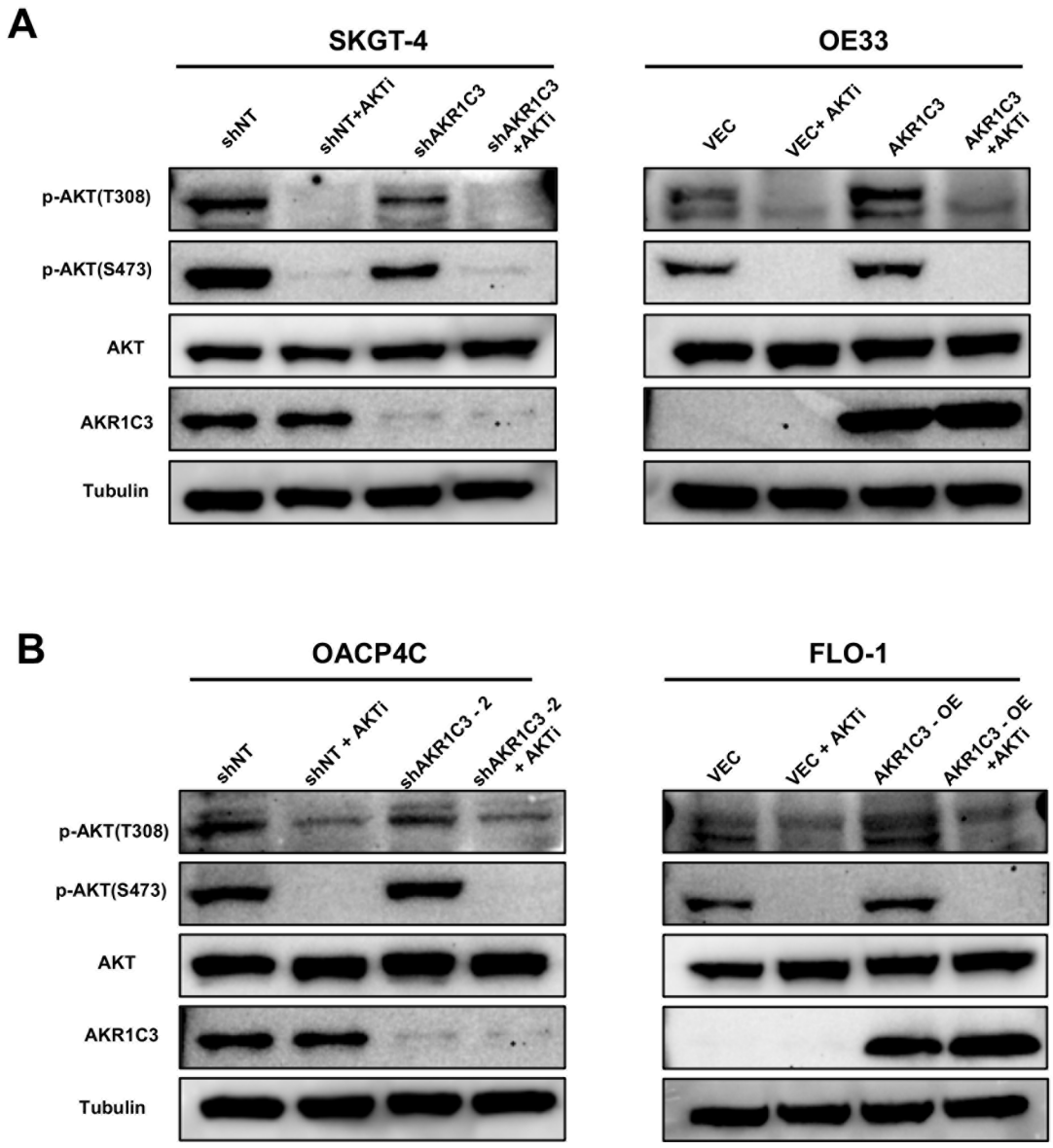


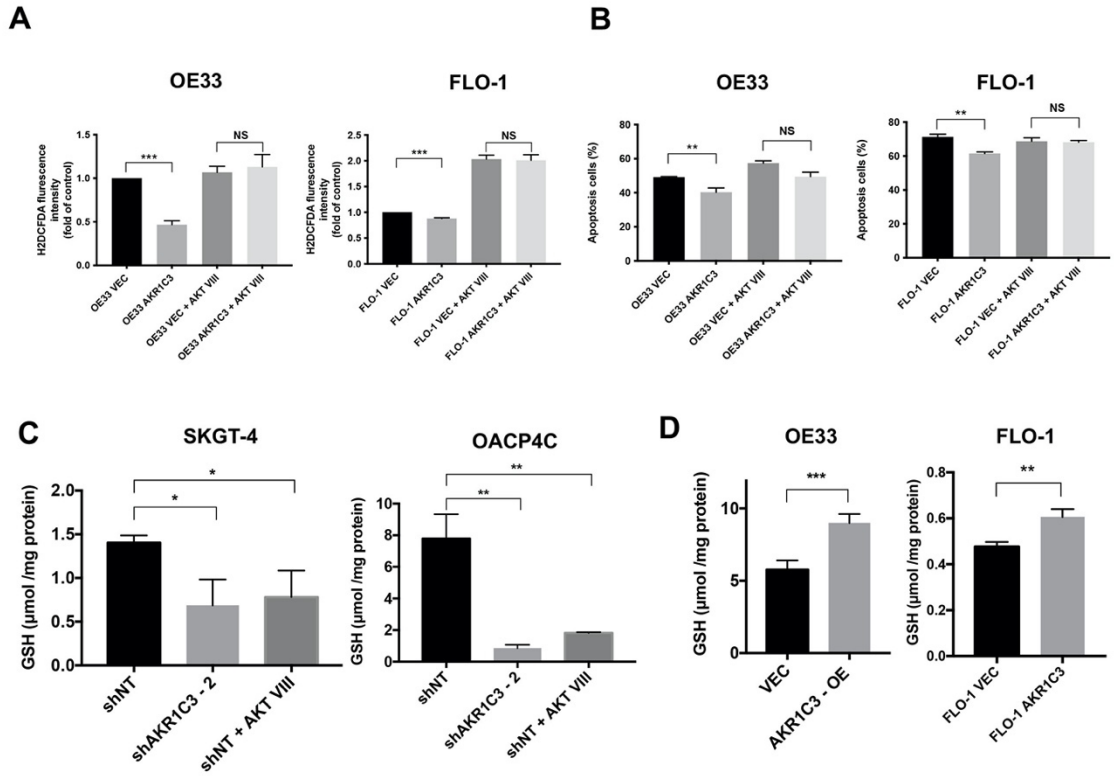
Figure 4.5.1. AKR1C3 mediates the AKT phosphorylation.
(A and B) AKT's phosphorylation was checked by WB and AKT inhibitor had no effect on AKR1C3 expression.

4.5.2. AKT phosphorylation is related to ROS alleviation in EAC

Our previous results indicated that AKR1C3 could regulate AKT expression, so AKT inhibitor was applied. The inhibitor of AKT could not only increase the ROS levels, but also restore the effect of the decrease in ROS levels caused by the upregulation of

AKR1C3 in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines **(Figure. 4.5.2 A)**. At the same time that the inhibitor of AKT could diminish the protective effect of the decrease for apoptotic cells caused by the elevated of AKR1C3 in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines **(Figure. 4.5.2 B)**.

In addition, GSH levels decreased in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. Rather, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines showed increased GSH levels than OE33 $^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells lines **(Figure. 4.5.2 C and D)**. GSH is positively associated with AKR1C3 by GSEA **(Figure. 4.5.2 E)**. Since the relationship between AKR1C3 and AKT has been verified, at the same time, we want to further prove whether there is a connection between GSH and AKT. Then, the inhibitor of AKT was used to clarify its role with GSH. The result showed that GSH levels was decreased and showed that the inhibitor of AKT had the same effect with AKR1C3-knockdown **(Figure. 4.5.2 C)**. Moreover, we observed that GSH accumulation was prevented by adding AKT inhibitor in SKGT-4 and OACP4C cell lines, suggesting that AKT-mediated GSH was involved. These results showed that the AKT's phosphorylation could protect cells from excessive ROS levels and anti-cancer drugs.



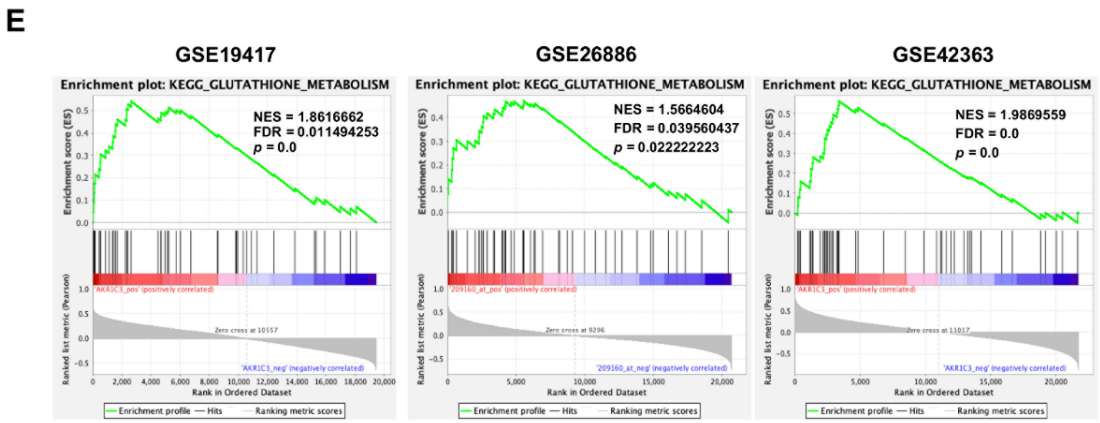


Figure 4.5.2. AKT phosphorylation is responsible for ROS alleviation in EAC.
(A) Cisplatin with inhibitor of AKT was added in OE33 and FLO-1 cells to check ROS via FACS. (B) Inhibitor of AKT was added in OE33 and FLO-1 cells to check apoptotic cells via FACS. (C) GSH levels were decreased in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. GSH was decreased in cells treated with AKT inhibitor. (D) Data indicated that GSH concentration were increased in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines than in OE33$^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cell lines. GSH concentration was normalized to total protein content. (E) GSH enrichment by GESA analysis.

4.5.3. AKR1C3 is associated with lipid metabolism in EAC

We aimed to figure out if AKR1C3 is involved in metabolism of tumor cells. We detected the oxygen consumption rate in OE33$^{AKR1C3\text{-}OE/VEC}$ and SKGT-4$^{AKR1C3\text{-}KD/shNT}$ cells analyzing the mitochondria stress test. We calculated the basal and maximum oxygen consumption level, ATP production, and protein leak in the mitochondria stress test. We found that the oxygen consumption rate is increased in OE33$^{AKR1C3\text{-}OE}$ than in OE33$^{AKR1C3\text{-}VEC}$, while SKGT-4$^{AKR1C3\text{-}sh1}$ and SKGT-4$^{AKR1C3\text{-}sh2}$ showed the opposite results than SKGT-4$^{AKR1C3\text{-}shNT}$ **(Figure. 4.5.3 A)**.

In support of this observation, GSEA analysis also used to show that steroid hormone, reactome β -oxidation of fatty acids, synthesis of bile and bile salts via 7 alpha hydroxycholesterol, retinol metabolism, glycosylphosphatidylinositol gpi anchor biosynthesis, and linoleic acid metabolism were enriched in AKR1C3-high group in EAC data set from public databases **(Figure. 4.5.3 B)**. public databases also indicate that these enriched substances such as fatty acids, steroids are closely related to lipid metabolism, which is also means that AKR1C3 is involved in the metabolism of these substances in EAC cells. There were core enrichment drug metabolizing enzymes with AKR1C3 such

as Thymidine kinase 1 (TK1), N-acetyltransferase 2 (NAT1-2), cytochrome P450 family, and Thiopurine S-methyltransferase (TPMT). So, it can cause cell drug resistance by regulating tumor cell's metabolism.

In conclusion, these results indicated that AKR1C3'expression affects oxidative phosphorylation of EAC cells. At the same time, public databases also indicate that AKR1C3 is involved in the metabolism of many lipids. Whether chemotherapy resistance is caused by regulating lipid metabolism requires further research.

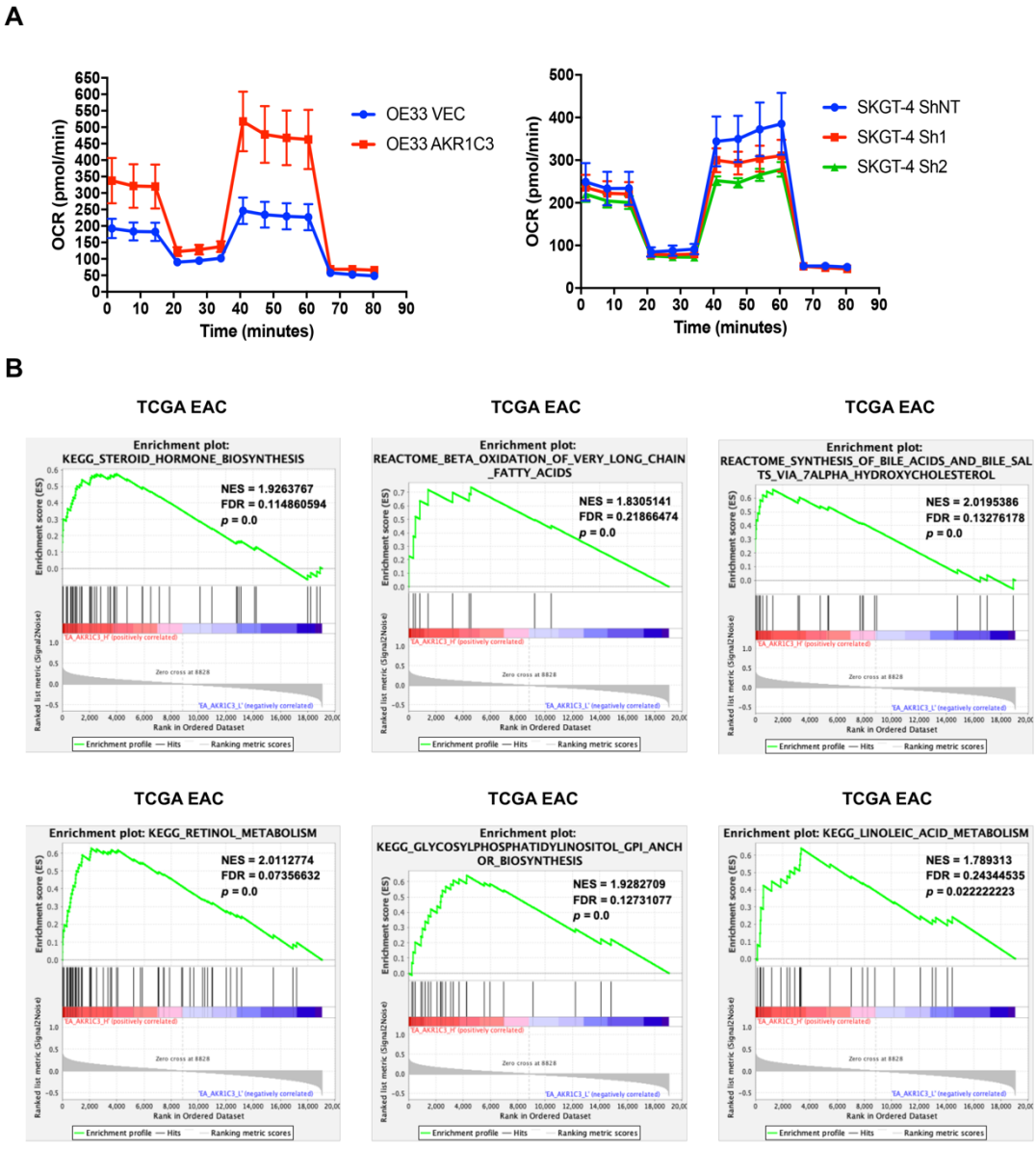


Figure 4.5.3. AKR1C3 is associated with lipid metabolism in EAC.

(A) AKR1C3'expression affects oxidative phosphorylation of EAC cells. (B) GESA analysis showed that substances related to lipid metabolism were enriched in AKR1C3high group in EAC data set.

V. **DISCUSSION**

Advances in combination chemotherapy have improved clinical efficacy and prolonged progression-free and OS of EAC patients, The high resistance rate is still very common, and it also makes the treatment of cancer very complicated [22, 23]. Over recent years, the enzyme AKR1C3 has entered the research field of researchers. This enzyme is closely associated with various diseases. Our work demonstrates that AKR1C3 could lead to chemotherapy resistance in EAC. We report that this enzyme alleviates oxidative stress, protecting EAC cells from chemotherapy-induced cell death via the phosphorylation of AKT. AKR1C3 regulates intracellular GSH levels to clean ROS and inhibit apoptosis via AKT.

The tissue from our cohort showed aberrant expression of AKR1C3 which is inconsistent with the several public datasets. The reason why its expression in our cohort was inconsistent with that in the database may be because of the possibility that the normal esophageal biopsies in our cohort were Barrett's esophagus. It's possible that the 'adjacent normal tissue' may actually be BE, which is the at the same AKR1C3 level with EAC. Indeed, in the study, we still need more samples for discussion. We only used 12 pairs of samples for verification, and obtained different results from the public database, so more sample size needs to be considered for further verification. In addition, this study used public database for survival analysis with 87 EAC patients, and there was no difference in survival between them. Its prediction efficiency is relatively low and is not credible with this sample size. Therefore, clinical data of patients in our research center also need to be collected for further exploration. Whether AKR1C3 has a certain effect on the prognosis of patients, whether the therapeutic effect can be judged according to the change of its expression level during treatment, and whether the its level is related to the clinical characteristics of patients.

The location of the gene expression actually has a certain response to the specific biological function of the gene. So, we used different EAC cell lines such as SKGT-4 and OE33 to localize intracellular protein expression. Firstly, we chose 2 cell lines such as SKGT4 and OE 33 for the immunofluorescence. We found that its was higher in SKGT-4 than in OE33. Then we selected SKGT-4 cell lines with strong fluorescence expression to further examine the location of its expression. We found that anti-AKR1C3

fluorescence was mainly in the cytoplasm. This also indicates that the gene does play a biological role in the cytoplasm. At the same time that we checked its level in six human EAC cell lines such as OE19, OE33, OACP4C, FLO-1, JHesoAD1 and SKGT-4. The result showed 3 cell lines such as OE19, OACP4C and SKGT-4 had high expression, while other 3 cell lines such as OE33, JHesoAD1 and FLO-1 had relatively low expression.

Antiproliferative effects and fewer colonies could be found in the SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C $^{AKR1C3\text{-}shNT}$ cells. Conversely, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cells had the significant opposite effect. These results also suggest that AKR1C3 takes participate in tumor cell development and progression. At the very least, it indicates that the gene can promote the growth and reproduction of the tumor, and it is a pro-oncogene for the tumor cells, which can make the tumor cells renew faster. In addition, the results showed that SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells had reduced wound-healing rate than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cells, whereas OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cells could obviously enhance its rate than OE33 $^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells. The higher migration ability of tumor cells means that the tumor cells have a greater ability to metastasize. Recurrence and metastasis could indicate poor prognosis, so tumor patients are most worried about tumor recurrence and metastasis. A lot of research is going into trying to suppress this as much as possible to improve overall survival.

Four agents such as CIS, OXA, 5-FU, and PTX were applied. The MTT results showed that SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines had more apoptosis than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cells upon chemotherapeutic treatment, whereas OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines had less apoptosis than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells. AKR1C3 could protect the EAC cells from CIS, OXA, 5-FU, and PTX. We also used another more precise method to verify its effect in tumor cells. The apoptotic cells were determined via FACS also showed the same results. Here, AKT's phosphorylation was decreased in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. Conversely, it was increased in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells lines. Previous investigations have indicated that AKT mediates tumor progression and causes cellular resistance [104, 105]. As reported that the activation of

AKT could directly result in resistance to CIS [106-108]. In our present work, we found AKR1C3 protects EAC cells from drugs via AKT.

Furthermore, we have shown that the ROS was higher in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. Conversely, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines showed decreased ROS than OE33 $^{AKR1C3\text{-}VEC}$ and FLO-1 $^{AKR1C3\text{-}VEC}$ cells lines. Excessive ROS is not acceptable in the cell. The stable redox level in the cell is very important, which affects the cell signaling pathways and cellular functions [109]. Therefore, in order to better maintain a stable intracellular redox level, the antioxidant capacity of cells should also be greatly improved for these cells' survival. The various studies confirmed that chemotherapeutic drugs induce ROS [110]. CIS could induce cancer cells death via ROS production [109, 111, 112]. Under the treatment with the anti-cancer drugs, an antioxidant system was evolved [113, 114]. Overproduced ROS induce apoptosis was reported in EAC cells [115]. Meanwhile, the link between AKR1C3 and ROS were reported in ESCC and prostate cancer, however these reports only concentrated on radiotherapy [116, 117]. While the exact role and mechanism of AKR1C3 with ROS was unclear in EAC.

Surprisingly, we observed that overexpressed AKR1C3 could protect cells from apoptosis by neutralizing ROS. This is also easy to understand that AKR1C3 itself has a detoxification effect. When the cells are exposed to chemotherapy drugs, the chemotherapy-induced ototoxicity breaks the redox balance of cells, then excessive harmful substances will accumulate in the cell, and cells with high AKR1C3 expression will survive more easily while cells with low AKR1C3 expression will die more easily.

The NAC and BSO were used for rescue experiment. Furthermore, the GSH results also shown us that AKR1C3 inhibits apoptosis by reducing ROS through the regulation of GSH in EAC. A number of papers have been published investigating the AKT pathway could manipulate GSH [118-120]. Silencing AKR1C3 and AKT inhibitor has the same effect on the GSH levels, which showed a significantly reduce. Meanwhile, the overexpressed AKR1C3 increases GSH levels.

These results indicate that AKR1C3 plays a very important biological role. To sum up, tumor cells exposed to chemotherapy drugs will produce a large number of harmful

substances. In order for cells to adapt to these harmful substances, they have to activate certain defense mechanisms within the cell in response. In EAC, we found that CIS-induced cell death by producing a large amount of ROS, while of course AKR1C3 could play a certain protective role. Cells with higher expression of this gene were more likely to survive. In fact, there are many pathways in the body that protect against oxidative stress, and the pathway involved in this study may be one of them. Although the relevant signaling pathways have been identified and verified, the relationship between the genes can only be described as indirect regulatory relationship, and the specific direct regulatory mechanism has not been tested, which is also a shortcoming of this study.

The tumor microenvironment (TME) is the environment composed of cancer cells, fibroblasts, immunocyte, and stroma etc.. Because of the existence of tumor microenvironment, the interactions between cells are still needs to be taken into consideration. Rewiring of cancer cell metabolism is a characteristic feature of cancer development. Moreover, reprogramming of energy metabolism has been regarded as a very important process for the cancer cells to adapt the environment [121]. The metabolic reprogramming of cancer is closely related to chemotherapy [122].

As an enzyme in the cell, it is greatly influenced by the extracellular environment. When tumor cells face environmental pressure, there will be a lot of responses in the cellular, and these responses will undoubtedly lead to changes in the activity or quantity of the enzyme AKR1C3, thus affecting the biological activities of the whole cell. Drug metabolizing enzymes take participate in the degradation and metabolism of chemotherapeutic agents. These enzymes attached themselves to the detoxification of these toxic agents. There were core enrichment drug metabolizing enzymes with AKR1C3 such as Thymidine kinase 1 (TK1), N-acetyltransferase1-2 (NAT1-2), cytochrome P450 family, and Thiopurine S-methyltransferase (TPMT). So, it can cause cell drug resistance by regulating tumor cell's metabolism.

At this time, we did some experiments to preliminarily check the status of AKR1C3 in cell metabolism. The Mito stress test kit from Agilent Technologies was used. we found that the oxygen consumption rate is increased in $OE33^{AKR1C3\text{-}OE}$ than in $OE33^{AKR1C3\text{-}VEC}$, while SKGT-$4^{AKR1C3\text{-}sh1}$ and SKGT-$4^{AKR1C3\text{-}sh2}$ showed the opposite results than SKGT-$4^{AKR1C3\text{-}shNT}$. There is no doubt that how AKR1C3 regulates tumor metabolism to achieve

therapeutic resistance remains to be further evaluated. Moreover, GSEA analysis also showed that substances related to lipid metabolism were enriched in AKR1C3^{high} group in EAC data set. Indeed, it has been reported that in tumors of the upper gastrointestinal cancers, including esophageal cancer, AKR1C3 regulates intracellular metabolism to resist effective therapy [77, 116]. Of course, whether AKR1C3 participates in tumor cells' lipid metabolism still needs further experimental verification. These are only the enrichment analysis of the data in the network public database. The specific lipid metabolism involved and regulated must be analyzed according to the specific situation.

In fact, the treatment of EAC is not only chemotherapy, but combined radiation therapy is also a commonly used in treatment plan. So, in the later studies, we also included radiotherapy in our study, Whether AKR1C3 can affect the efficacy of radiotherapy in EAC and whether it can affect the efficacy of CROSS trail. Furthermore, the influence of TME on tumor cells is also very important. For example, CAFs have a great influence on tumor, so animal experiments and co-culture experiments are also areas that we need to explore in the future.

Conclusion

This is the first report of AKR1C3 in the treatment of EAC patients. AKR1C3 with its own detoxification function can protect EAC cells from the toxicity of anti-cancer drugs. AKR1C3 can effectively clear the excessive ROS in tumor cells by regulating the AKT/GSH pathway, leading to chemotherapeutic resistance of EAC cells **(Figure 5.1)** Therefore, this study provides rationale for the design of treatment strategies that targeting AKR1C3/AKT/GSH/ROS signaling might potentially prolong survival time.

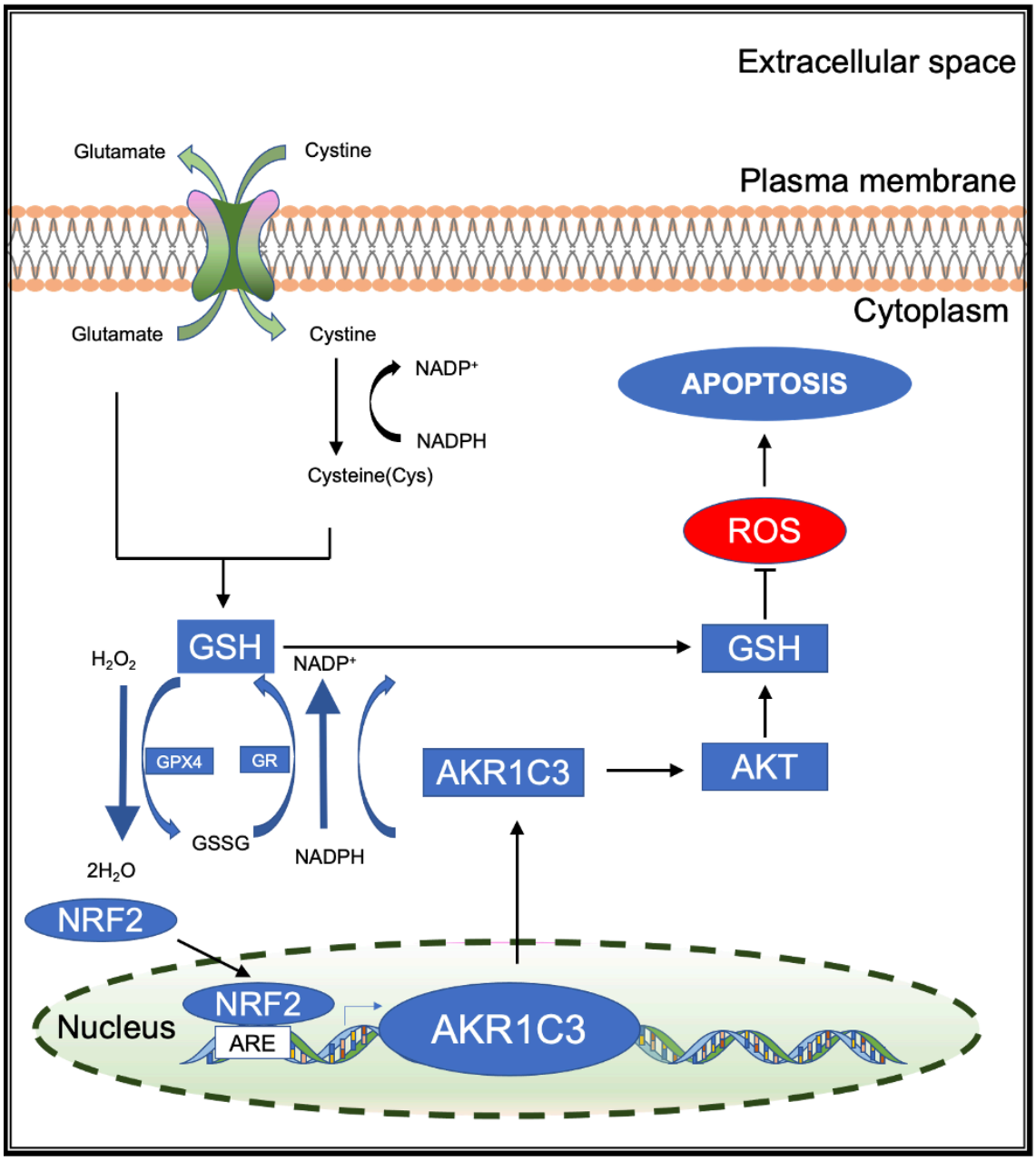


Figure 5.1. AKR1C3's function in EAC cells.

In this study, we validated that AKR1C3 was the downstream of NRF2. AKR1C3 can effectively clear the excessive ROS in tumor cells by regulating the AKT/GSH pathway, leading to chemotherapeutic resistance of EAC cells. Under the pressure of chemotherapeutics, AKR1C3 could protect cancer cells from apoptosis via improving the antioxidant ability of EAC cells. While targeting AKR1C3 could inhibit the AKT signaling and decrease the GSH production, which may further impair the cellular antioxidant ability and cause the elevation of ROS and induce the EAC cell's apoptosis.

VI. **SUMMARY**

EAC incidence has increased sharply in Germany. Due to lack of promising treatments, the overall survival is poor. AKR1C3 has attracted much attention in tumor research. Especially in the treatment of tumors. In many cancers, AKR1C3 seems to show its protective effect on cancer cells against anti-cancer drugs. However, its specific function in the treatment for EAC is unknown.

The tissues from our cohort showed aberrant AKR1C3 level, which is inconsistent with the several public datasets, which shows the tumor tissues has higher AKR1C3 than the normal tissue. Survival analysis of 87 EAC patients in public databases was also not statistically significant. SKGT-4 cell lines with strong fluorescence expression were selected to further examine the location of its expression. We found that anti-AKR1C3 fluorescence was mainly in the cytoplasm. we constructed SKGT-4 (SKGT-4$^{AKR1C3\text{-}KD/NT}$) and OACP4C (OACP4C$^{AKR1C3\text{-}KD/NT}$) cell lines for the loss-function experiment. We constructed OE33 (OE33$^{AKR1C3\text{-}OE/VEC}$) and FLO-1 (FLO-1$^{AKR1C3\text{-}OE/VEC}$) cell lines for the gian-function experiment. Antiproliferative and anti-migration effects and fewer colonies could be found in the SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cells than SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C $^{AKR1C3\text{-}shNT}$ cells. Conversely, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cells had the significant opposite effect.

AKR1C3 protects cells from CIS, OXA, 5-FU, and PTX treatment via drug detoxification. Moreover, ROS was higher in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C $^{AKR1C3\text{-}shNT}$ cell lines. Conversely, OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines showed decreased ROS than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1 $^{AKR1C3\text{-}VEC}$ cells lines. Interesting, AKT's phosphorylation was decreased in SKGT-4$^{AKR1C3\text{-}KD}$ and OACP4C$^{AKR1C3\text{-}KD}$ cell lines than in SKGT-4$^{AKR1C3\text{-}shNT}$ and OACP4C$^{AKR1C3\text{-}shNT}$ cell lines. Conversely, it was increased in OE33$^{AKR1C3\text{-}OE}$ and FLO-1$^{AKR1C3\text{-}OE}$ cell lines than OE33$^{AKR1C3\text{-}VEC}$ and FLO-1$^{AKR1C3\text{-}VEC}$ cells lines. AKT inhibitor was used in rescue experiment to validate that AKR1C3 mediates ROS via AKT. GSH level also checked in EAC cell lines. Silencing AKR1C3 and AKT inhibitor has the same effect on the GSH levels, which showed a significantly reduce.

Here, we presented that AKR1C3 can effectively clear the excessive ROS in tumor cells

by regulating the AKT/GSH pathway, leading to chemotherapeutic resistance in EAC cells.

VII. **ZUSAMMENFASSUNG**

Die Inzidenz von EAC hat in Deutschland stark zugenommen. Da es an vielversprechenden Behandlungsmöglichkeiten mangelt, ist die Gesamtüberlebensrate gering. AKR1C3 hat auf dem Gebiet der Tumorforschung viel Aufmerksamkeit erregt. Dies gilt insbesondere für die Behandlung von Tumoren. Bei vielen Krebsarten scheint AKR1C3 eine schützende Wirkung auf Krebszellen gegenüber Krebsmedikamenten zu haben. Seine spezifische Funktion bei der Behandlung von EAC ist jedoch unbekannt.

Die Gewebe aus unserer Kohorte wiesen einen abweichenden AKR1C3-Gehalt auf, was nicht mit den verschiedenen öffentlichen Datensätzen übereinstimmt, die zeigen, dass das Tumorgewebe einen höheren AKR1C3-Gehalt aufweist als das normale Gewebe. Die Überlebensanalyse von 87 EAC-Patienten in öffentlichen Datenbanken war ebenfalls nicht statistisch signifikant. SKGT-4-Zelllinien mit starkem Fluoreszenzausdruck wurden ausgewählt, um den Ort der Expression weiter zu untersuchen. Wir stellten fest, dass sich die Anti-AKR1C3-Fluoreszenz hauptsächlich im Zytoplasma befand. Wir konstruierten SKGT-4 (SKGT-4$^{AKR1C3\text{-}KD/NT}$) und OACP4C (OACP4C$^{AKR1C3\text{-}KD/NT}$) Zelllinien für das Loss-Function Experiment. Wir konstruierten OE33 (OE33$^{AKR1C3\text{-}OE/VEC}$) und FLO-1 (FLO-1$^{AKR1C3\text{-}OE/VEC}$) Zelllinien für das Gianfunktions-Experiment. In den SKGT-4$^{AKR1C3\text{-}KD}$- und OACP4C$^{AKR1C3\text{-}KD}$-Zellen konnten antiproliferative und migrationshemmende Effekte und weniger Kolonien als in den SKGT-4$^{AKR1C3\text{-}shNT}$- und OACP4C$^{AKR1C3\text{-}shNT}$-Zellen festgestellt werden. Umgekehrt hatten OE33$^{AKR1C3\text{-}OE}$ und FLO-1$^{AKR1C3\text{-}OE}$ Zellen den gegenteiligen Effekt.

AKR1C3 schützt die Zellen vor CIS-, OXA-, 5-FU- und PTX-Behandlung durch Medikamentenentgiftung. Außerdem war die ROS-Konzentration in den Zelllinien SKGT-4$^{AKR1C3\text{-}KD}$ und OACP4C$^{AKR1C3\text{-}KD}$ höher als in den Zelllinien SKGT-4$^{AKR1C3\text{-}shNT}$ und OACP4C$^{AKR1C3\text{-}shNT}$. Umgekehrt zeigten die Zelllinien OE33$^{AKR1C3\text{-}OE}$ und FLO-1$^{AKR1C3\text{-}OE}$ weniger ROS als die Zelllinien OE33$^{AKR1C3\text{-}VEC}$ und FLO-1$^{AKR1C3\text{-}VEC}$. Interessanterweise war die Phosphorylierung von AKT in den Zelllinien SKGT-4$^{AKR1C3\text{-}KD}$ und OACP4C$^{AKR1C3\text{-}KD}$ geringer als in den Zelllinien SKGT-4$^{AKR1C3\text{-}shNT}$ und OACP4C$^{AKR1C3\text{-}shNT}$. Umgekehrt war sie in den Zelllinien OE33$^{AKR1C3\text{-}OE}$ und FLO-1$^{AKR1C3\text{-}OE}$ höher als in den Zelllinien OE33AKR1C3-VEC und FLO-1AKR1C3-VEC. Im Rettungsversuch wurde ein AKT-Inhibitor eingesetzt, um zu bestätigen, dass

AKR1C3 ROS über AKT vermittelt. Auch der GSH-Gehalt wurde in den EAC-Zelllinien überprüft. Das Silencing von AKR1C3 und der AKT-Inhibitor haben die gleiche Wirkung auf den GSH-Gehalt, der sich deutlich verringerte.

Hier präsentierten wir, dass AKR1C3 die übermäßigen ROS in Tumorzellen durch die Regulierung des AKT/GSH-Stoffwechsels wirksam beseitigen kann, was zur Chemotherapieresistenz in EAC-Zellen führt.

VIII. **ABBREVIATION**

1ΔgO2: Singlet oxygen

3α-HSD: 3α-hydroxysteroid dehydrogenase

17β-HSD: 17β-hydroxysteroid dehydrogenase

20α-HSD: 20α-hydroxysteroid dehydrogenase

AKRs: Aldo-keto reductases

AKR1: Human Aldo-keto reductase family 1

AKR1C3: Human Aldo-keto reductase family 1 member C3

AKT: Protein kinase B

AML: Acute myeloid leukemia

ARE: Antioxidant response element

BE: Barrett's esophagus

BSO: L-buthionine-S, R-sulfoximine

CAF: Cancer-associated fibroblasts

ChIP: Chromatin immunoprecipitation

CDDP: Cis-diamminedichloroplatinum (II) (also known as cisplatin)

CRPC: Castration-resistant prostate cancer

CREs: Carbonyl-reducing enzymes

CRT: Chemoradiotherapy

CSCs: Cancer stem cells

CXCR4: C-X-C Motif Chemokine Receptor 4

DAPI: 4 ', 6-diamidino-2-phenylindole, dihydrochloride

DAU: Daunorubicin

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DOX: Doxorubicin

DOX-ol: Doxorubicinol

DTX: Docetaxel

EAC: Esophageal adenocarcinoma

EC: Esophageal cancer

ECF: Epirubicin and Cisplatin plus Fluorouracil

ECX: Epirubicin and Cisplatin plus Capecitabine

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptor

EMT: Epithelial-mesenchymal transition

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

ESCC: Esophageal squamous cancer cells

FBS: Fetal bovine serum

FORMALD: Formaldehyde

5-FU: 5-Fluorouracil

GE: Gastroesophageal

GEO: Gene Expression Omnibus

GERD: Gastroesophageal reflux disease

GSEA: Gene set enrichment analysis

GSH: Glutathione

GSK3: Glycogen Synthase Kinase 3

HCC: Hepatocellular carcinoma

HCL: Hydrochloric acid

HO2▪: Hydroperoxyl

HOCl: Hypochlorous acid

H2O2: Hydrogen Peroxide

HNE: 4-hydroxy-2-nonenal

HSD: Hydroxysteroid dehydrogenase

HRP: Horseradish peroxidase

IGF-R: Insulin growth factor receptor

KRAS gene: Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

PCR: Polymerase chain reaction

PEI: Polyethylenimine, branched

PFA: Paraformaldehyde

PI3K: Phosphatidylinositol 3 kinase

PGD2: Prostaglandins D2

PGF2: Prostaglandin F2

PGF2α: Prostaglandins F2α

PGH2: Prostaglandins H2

PKB: Protein kinase B

PTPs: Protein tyrosine phosphatases

PTX: Paclitaxel

NAC: N-acetyl cysteine

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NADPH: nicotinamide adenine dinucleotide phosphate

NAT1: N-acetyltransferase 1

NAT2: N-acetyltransferase 2

NF-κB: Nuclear factor kappa B

NOXs: NADPH oxidases

NQO-1: NADPH-quinone oxidoreductase-1

NRF2: Nuclear factor erythroid 2-related factor 2

NSCLC: Non-small-cell lung cancer

MTT: 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide

OPSCC: Oropharyngeal squamous cell carcinoma

OS: Overall survival

PBS: Phosphate buffered saline

qRT-PCR: Quantitative Reverse Transcription PCR

RCC: Renal cell carcinoma

RNA: Ribonucleic acid

ROIs: Reactive oxygen intermediates

ROMs: Reactive oxygen metabolites

ROS: Reactive oxygen species

RT: Room temperature

RT-PCR: Reverse transcription polymerase chain reaction

SDS: Sodium dodecyl sulfate

shRNA: short hairpin RNA

SNAIL1: snail family transcriptional repressor 1

SOD2: Superoxide dismutase

TCA: Trichloroacetic acid

TCEP: Tris-(2-Carboxyethyl) phosphine

TCGA: The Cancer Genome Atlas

TK1: Thymidine kinase 1

TME: Tumors microenvironment

TPMT: Thiopurine S-methyltransferase

TSS: transcription start site

WB: Western blot

xCT: Cystine transporter

ZEB-1: Zinc finger E-box-binding homeobox 1

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