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Complete List of Authors:	Frycz, Bartosz; University of Medical Sciences, Department of Biochemistry and Molecular Biology Murawa, Dawid; Greater Poland Cancer Centre, ; Regional Specialist Hospital , Borejsza-Wysocki, Maciej; Heliodor Swiecicki Clinical Hospital at the Karol Marcinkowski Medical University, Wichtowski, Mateusz; Greater Poland Cancer Centre Spychała, Arkadiusz; Greater Poland Cancer Centre, Marciniak, Ryszard; Heliodor Swiecicki Clinical Hospital at the Karol Marcinkowski Medical University, Murawa, Paweł; Greater Poland Cancer Centre, Drews, Michał; Heliodor Swiecicki Clinical Hospital at the Karol Marcinkowski Medical University, Jagodziński, Paweł; University of Medical Sciences, Department of Biochemistry and Molecular Biology
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Transcript level of *AKRIC3* is down-regulated in gastric cancer

Bartosz Adam Frycz¹, Dawid Murawa^{2,3}, Maciej Borejsza-Wysocki⁴, Mateusz Wichtowski², Arkadiusz Spychała², Ryszard Marciniak⁴, Paweł Murawa², Michał Drews⁴, Paweł Piotr Jagodziński¹

¹ Department of Biochemistry and Molecular Biology, University of Medical Sciences, Poznań, Poland

² First Department of Surgical Oncology and General Surgery, Greater Poland Cancer Centre, Poznań, Poland

³ Regional Specialist Hospital, Research and Development Centre, Wrocław, Poland

⁴ Department of General, Endocrinological Surgery and Gastroenterological Oncology, University of Medical Sciences, Poznań, Poland

Postal address:

1. University of Medical Sciences, Swiecickiego 6 St., 60-781 Poznan
2. Greater Poland Cancer Centre, Garbary 15 St., 61- 866 Poznan
3. Regional Specialist Hospital in Wrocław, Research and Development Centre, Kamińskiego 73a St., 51-124 Wrocław
4. Heliodor Swiecicki Clinical Hospital at the Karol Marcinkowski Medical University, Przybyszewskiego 49 St., 60-355 Poznan

Corresponding author:

Bartosz Adam Frycz

e-mail: bartekfrycz@gmail.com

tel. +48 (61) 854-65-13

fax: +48 (61) 854-65-10

Abstract

Steroid hormones are shown to play some role in gastric carcinogenesis. Large amounts of them are locally produced in peripheral tissues at both genders. Type 5 of 17 β -hydroxysteroid dehydrogenase, encoded by *AKR1C3* gene, plays a pivotal role in both androgen and estrogen metabolism and its deregulated expression was found in different cancers. In this study we measured AKR1C3 transcript and protein levels in nontumoral and primary tumoral gastric tissues and evaluated their association with some clinicopathological features of gastric cancer (GC). We found decreased AKR1C3 transcript ($p < 0.0001$) and protein ($p = 0.0021$) levels in GC tissues compared to adjacent histopathologically normal appearing mucosa. Lower levels of *AKR1C3* transcript were observed in diffuse and intestinal types of GC whereas AKR1C3 protein was decreased in tumors with multi site localization, in diffuse histological type, T3, T4 and G3 grades. We also determined the effect of histone deacetylases inhibitor, sodium butyrate (NaBu), on AKR1C3 expression in EPG 85-257 and HGC-27 GC cell lines. We found that NaBu is able to elevate both AKR1C3 transcript and protein levels in investigated cell lines. Altogether, our results suggest that decreased expression of AKR1C3 may be involved in development of GC and can be restored by NaBu.

Key words: gastric cancer, steroidogenesis, steroidogenic enzymes, AKR1C3, sodium butyrate

Introduction

Despite a decreasing incidence observed during the last decades, gastric cancer (GC) remains a serious health problem in most populations (Jemal et al 2011). The risk of GC development is mainly associated with *Helicobacter pylori* infections, diet abundant in salty and smoked food, obesity, tobacco smoking and genetic factors (Lee and Derakhshan 2013). Moreover, epidemiological and experimental studies indicate on steroid hormones as a possible factor related to this carcinoma (Camargo et al. 2011). Estrogens seem to have a protective effect in GC etiology, according to the higher incidence of that tumor in males than females (Camargo et al. 2011). Role of androgens in GC etiology is a less elucidated and only a few studies concern that issue (Nakamura et al. 2006; Polimeno et al. 1994). Important findings in the sex steroid biology is the ability of some peripheral tissues to conversion of adrenal derived dehydroepiandrosterone, its sulfate and androstenedione (a-dione) into an active estrogens and androgens (Sasano et al. 1998; Labrie and Labrie 2013). These hormones exert their action on the same cells where its synthesis took place, without a release to the general circulation. The peripheral synthesis rate of steroid hormones is controlled by the local expression of enzymes which synthesize the estrogens and androgens from adrenal derived precursors (Labrie et al. 2000). Aberrant expression of enzymes involved in local steroidogenic pathway may lead to the formation of an abnormal intracellular steroids level and to uncontrolled cells proliferation (Brodie et al. 2009). Some evidence suggest, that changes in the expression of genes encoding steroidogenic enzymes could have a significant meaning in the etiology of hormone dependent tumors, including breast (Sasano et al. 2006), endometrial (Sasano et al. 2000), ovarian (Mungenast and Thalhammerand 2014), prostate (Gianfrilli et al. 2014) as well as in the other tumors not directly related with reproductive features like lung (Drzewiecka et al. 2015), thyroid (Rahbari et al. 2010), colon (Lin and Giovannucci 2010), central nervous system (Park et al. 2010), or gastric (Nakamura et al. 2006; Frycz et al. 2015). One of the most important enzymes family, responsible for the interconversion between the biologically active and inactive forms of estrogens and androgens on the last steps of their biosynthesis, are 17 β -hydroxysteroid dehydrogenases (HSD17Bs). There are 14 type of HSD17Bs reported of which 12 have been found in humans. HSD17Bs isoenzyme differ with the substrate specificity and tissue pattern expression (Moeller and Adamski

2009). Additionally, it was shown that the expression of *AKR1C3* gene encoding type 5 of HSD17B, responsible for the conversion of both, androstenedione to testosterone (T) and estrone (E1) to 17 β -estradiol (E2), is deregulated in multiple human cancers (Dozmorov et al. 2010; Lewis et al. 2004; Zakharov et al. 2010; Martinez et al. 2007). Therefore, according to the emerging role of steroid hormones in GC development, we measured transcript and protein levels of AKR1C3 in nontumoral and in primary tumoral gastric tissues. We also assessed the effect of histone deacetylases inhibitor, sodium butyrate (NaBu), on the AKR1C3 transcript and protein levels in EPG 85-257 and HGC-27 GC cell lines. Additionally, we evaluated the association of AKR1C3 expression with some clinicopathological features of GC.

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Materials and methods

Tissue specimens

Primary tumoral gastric tissues and adjacent histopathologically normal appearing mucosa were collected between December 2012 and March 2015 from fifty five patients who underwent total gastrectomy at First Department of Surgical Oncology and General Surgery, Greater Poland Cancer Centre, Poznań, Poland or Department of General, Endocrinological Surgery and Gastroenterological Oncology, University of Medical Sciences, Poznań, Poland (Tab. 1). The procedures of the study were approved by the Local Ethical Committee of Poznań University of Medical Sciences. Written informed consent was obtained from all participating individuals. Histopathologically unchanged gastric mucosa situated at least 10-20 cm away from the tumoral lesions was obtained from the same patients. A part of the samples was immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA/protein isolation. Histopathological assessment was performed by an experienced pathologist.

Antibodies and Reagents

Goat polyclonal anti-AKR1C3 antibody (Ab) (ab27491) was purchased from Abcam, (Cambridge, UK). Rabbit polyclonal anti-glyceraldehyde-3-phosphate (GAPDH) Ab (SC-25778), goat anti-rabbit horseradish peroxidase (HRP)-conjugated Ab (SC-2004) and rabbit anti-goat HRP-conjugated Ab (SC-2768) were provided by Santa Cruz Biotechnology, (Dallas, USA). NaBu were purchased from Sigma-Aldrich, (St. Louis, USA).

GC cell lines

EPG 85-257 GC cells were kindly provided by Wroclaw Medical University, Poland and HGC-27 GC cells were purchased from CLS Cell Lines Service, (Eppelheim, Germany). EPG 85-257 and HGC-27 GC cells were respectively cultured in DMEM and DMEM/F12 growth media (Sigma-Aldrich, St. Louis, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS),

(Sigma-Aldrich, St. Louis, USA) and 2mM glutamine. MCF7 breast cancer cells were used as a positive control for AKR1C3 transcript and protein identification.

EPG 85-257 and HGC-27 GC cell lines treatment with NaBu

Prior to all experiments, cells were maintained for 24 hours in an appropriate phenol red-free medium, (Sigma-Aldrich, St. Louis, USA) supplemented with 5% charcoal-dextran-stripped FBS, (Sigma-Aldrich, St. Louis, USA). To study the effect of NaBu on the HGC-27 and EPG 85-257 GC cells proliferation and viability as well as on the AKR1C3 protein level, cells were cultured for 24, 48 and 72 hours in appropriate phenol red-free medium, supplemented with 5% charcoal-dextran-stripped FBS, either in the absence or in the presence of NaBu at the concentrations of 2.0, 5.0 and 8.0mM for EPG 85-257 and 0.5, 1.5 and 3.0mM for HGC-27 GC cells. The same conditions were used to evaluate the effect of NaBu on *AKR1C3* transcript level in those cell lines, however incubation time was restricted to 72 hours. Growth media were replaced after every 24 hours of incubation.

Proliferation and viability assaying of the EPG 85-257 and HGC-27 GC cells after NaBu treatment

After respective time of incubation, the cells were harvested and total number of viable and dead cells were counted with trypan blue dye. Total cells number, stained and unstained, was represented as a percent of respective control for proliferation assay. Cells viability was calculated with using following equation: total viable cells (unstained)/total cells (stained and unstained) x 100 and represented as a percent of respective control. Each sample was determined in triplicate and results are presented as the mean \pm SD from three experiments

Reverse transcription and real-time quantitative polymerase chain reaction (RQ-PCR) analysis of the AKR1C3 transcript levels

The total RNA from the primary tissue of patients with GC as well as from EPG 85-257 and HGC-27 GC cells was isolated according to the method of Chomczyński and Sacchi (2006). Purity and integrity of RNA was assessed with using Nano-100 spectrophotometer (Hangzhou Allsheng, China)

and gel electrophoresis respectively. RNA samples were treated with DNase I, quantified, and reverse-transcribed into cDNA. RQ-PCR was carried out in a Light Cycler real-time PCR detection system from Roche Diagnostics GmbH, (Mannheim, Germany) using SYBR® Green I as detection dye. The transcript levels in patient tissues were expressed as multiplicity of these cDNA concentrations in the calibrator. In EPG 85-257 and HGC-27 GC cells, these transcript levels were presented as multiplicity of the respective controls. The calibrator was prepared as cDNA mix from all of the patient samples and successive dilutions were used to create the standard curve as is described in Relative Quantification Manual Roche Diagnostics GmbH, (Mannheim, Germany). For amplification, 1µl of cDNA solution was added to 9µl of Light Cycler®480 SYBR Green I Master mix (Roche, Mannheim, Germany) contained the following primers sequences: *AKR1C3* forward:

5'-GGGATCTCAACGAGACAAAC-3' and reverse: 5'-AAAGGACTGGGTCTCCAAG-3'.

The quantity of *AKR1C3* transcripts in each sample was standardized by the geometric mean of beta-2-microglobulin (*B2M*) and beta-glucuronidase (*GUSB*) cDNA levels and expressed as multiples of these cDNA in the calibrator.

Western blotting analysis

The tissue of patients as well as EPG 85-257 and HGC-27 GC cells were treated with lysis RIPA buffer for proteins isolation. Then, 40µg of protein were resuspended in sample buffer and separated on 12% Tris-glycine gel using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel proteins were transferred to nitrocellulose membrane, which was next blocked with 5% milk in Tris/HCl saline/Tween buffer. Immunodetection of the bands was performed with goat polyclonal anti-AKR1C3 Ab, followed by incubation with rabbit anti-goat HRP-conjugated Ab. To ensure equal protein loading of the lanes, the membrane was re-stripped and incubated with rabbit polyclonal anti-GAPDH Ab and goat anti-rabbit HRP-conjugated Ab. Bands were revealed using ECL kit and Hyperfilm ECL Amersham (Piscataway, NJ). The amount of AKR1C3 protein was calculated as the AKR1C3 to GAPDH band optical density ratio.

Statistical analysis

Normality of the observed patient data distribution was assessed by Shapiro-Wilk test, followed by unpaired, two-tailed t-test or Mann-Whitney test to consider statistically significant differences of compared mean values. Data groups for cell lines were assessed by ANOVA to evaluate if there was significance ($p < 0.05$) between the groups. For the all experimental groups, which fulfilled the initial criterion, individual comparisons were performed by post hoc Tukey test with the assumption of two-tailed distribution and two samples with equal variance at the $p < 0.05$ level. Statistical analysis was performed using STATISTICA 10 software.

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Results

Transcript and protein levels of AKR1C3 in nontumoral and primary tumoral tissues from patients with GC

We found significantly lower levels of *AKR1C3* transcript ($p < 0.0001$) in primary tumoral as compared to adjacent histopathologically normal appearing mucosa of patients with GC (Tab. 2, Fig. 1A), regardless of analysed histopathological features. Moreover, we observed significantly lower levels of *AKR1C3* transcript in diffuse ($p = 0.0003$) and intestinal ($p < 0.0001$), but not in the indetermined histological types of GC. In case of body, fundus, lesser curvature and pylorus GC localization, in T1 grade of tumor depth, M1 grade of distant metastases and G1 histological tumor grade, we not observed significantly lower levels of *AKR1C3* transcript in primary tumoral tissues, probably due to not sufficient cases number. Tissue from the same patients were also used for determination of AKR1C3 protein level (Tab. 3, Fig. 1B). Unfortunately in two cases, amount of the material was not sufficient for western blot analysis. However, we found lower levels of AKR1C3 protein in primary tumoral than in adjacent histopathologically normal appearing mucosa tissues of patients with GC ($p = 0.0021$). The difference in protein level among those types of tissue was small despite the statistical significance. Protein levels of AKR1C3 in primary tumoral tissues were decreased regardless of age and gender, however we observed significantly lower levels of AKR1C3 protein in tumors with multi site localization ($p = 0.0051$), in diffuse histological type ($p = 0.025$), T3 ($p = 0.015$) and T4 ($p = 0.048$) grade of tumor depth as well as in G3 ($p = 0.0013$) histological grading. In case of GC localization, tumor depth and distant metastases we could not observed significantly lower AKR1C3 protein levels probably, due to small number of patients.

Effect of NaBu on the proliferation and viability of EPG 85-257 and HGC-27 GC cells

NaBu significantly inhibited the proliferation (Fig. 2) of both EPG 85-257 (A) and HGC-27 (C) GC cells at the all incubations time and used doses, except, EPG 85-257 cells cultured for 24 hours in presence of 2.0mM ($p = 0.9$) and 5.0mM ($p = 0.55$) of NaBu concentrations. Simultaneously, NaBu

significantly decreased EPG 85-257 cells viability (B) at dose of 8.0mM incubated for 72 hours ($p=0.0072$). In case of HGC-27 cells (D), significant decrease of viability was observed after 48 and 72 hours of incubation with the presence of NaBu at the concentrations of 0.5, 1.5 and 3.0mM.

Effect of NaBu on AKR1C3 transcript and protein levels in EPG 85-257 and HGC-27 GC cells

We were able to detect AKR1C3 protein which was confirmed by positive control- MCF7 protein lysate. However, we also observed an unspecific protein band above AKR1C3 band in some tissues and GC cell lines. This unspecific signal was not analyzed. We observed increase of *AKR1C3* transcript (Fig. 3) in EPG 85-257 (A) and HGC-27 (B) GC cells after 72 hours of incubation with NaBu at the concentrations of 2.0, 5.0, 8.0mM and 0.5, 1.5, 3.0mM respectively. The best effect in the increase of transcript level in EPG 85-257 cells were observed after incubation with 5.0mM and 8.0mM ($p<0.001$) of NaBu. In case of HGC-27 cells all used doses were sufficient for significantly increase of *AKR1C3* transcript level ($p<0.01$). Protein bands of AKR1C3 in EPG 85-257 (Fig. 4A) cells were only observed due to 48 and 72 hours incubation at presence of 8mM NaBu. In HGC-27 (Fig. 4B) cells we observed significantly increased in AKR1C3 protein bands density in all NaBu treated samples according to 24-72 hours of incubation.

Discussion

Local steroidogenesis driven by steroidogenic enzymes is an important source of androgens and estrogens in peripheral tissues and could have significant meaning in cancer development (Brodie et al. 2009). Our work showed reduced transcript and protein levels of AKR1C3 in primary tumoral tissues of fifty five patients with diagnosed GC. Strong positive AKR1C3 immunohistochemical staining was previously demonstrated in nontumoral gastric tissues and positive AKR1C3 staining was shown in 59% of gastric tumoral tissues (Guise et al. 2010). Strong AKR1C3 positive immunoreactivity was also demonstrated in the nontumoral gastric mucosal cells, lining close to the luminal surface but no or weak immunoreactivity was presented in deeply located epithelial cells such as those in the gastric pits (Chang et al. 2013).

Since we used homogenized control mucosa containing both types of these cells, our results could be limited by the method we used. Moreover, in contrast to the previous results, the same study showed strong AKR1C3 positive immunoreactivity in GC tissues. On the other hand, this study was limited by relative small number of examined cases (9 gastric adenocarcinomas). Immunoreactivity of AKR1C3 in tumoral gastric tissues was also demonstrated by Nakamura et al. (2006). However, due to simultaneous presence of this enzyme in nontumoral adjacent mucosa, authors suspected limited AKR1C3 contribution to the development of GC. Up-regulation of AKR1C3 transcript and protein levels were widely shown in breast (Lin et al. 2004; Vihko et al. 2005; Jansson et al. 2006; Suzuki et al. 2007) and prostate (Fung et al. 2006; Stanbrough et al. 2006; Locke et al. 2008; Wako et al. 2008; Pfeiffer et al. 2011; Hamid et al. 2013) tumoral tissues which are known to be steroid sensitive. Thus an over-expression of AKR1C3 could facilitate the growth of those tumors by increasing the intratissue level of active steroids. Additionally, LNCaP prostate cancer cells with AKR1C3 over-expression were shown to synthesize higher amounts of T which results an increased of their proliferation rate (Byrns et al. 2012).

In our study, we showed that decreased *AKR1C3* transcript levels were significantly associated with diffuse and intestinal type of GC. Lowered AKR1C3 protein levels were also significantly correlated with multi site localization, diffuse histological type, T3 and T4 grades of tumor depth and G3,

histological grading. Higher amounts of *AKR1C3* transcript was found in bone metastases in comparison with non-malignant or malignant prostate tissue (Jernberg et al. 2013). An up-regulation of *AKR1C3* transcript and protein levels was also observed in breast cancer and was associated with poor prognosis of patients (Lin 2004; Jansson et al. 2006; Vihko 2006; Suzuki et al. 2007).

AKR1C3 is responsible for both, α -dione conversion to T and E1 to E2 (Penning et al. 2000) thus its down-regulation can result in the reduction of intratissue concentration of biologically potent steroids. Since T and E2 receptors are found in both nontumoral and tumoral gastric tissues (Gan et al. 2012), steroid synthesis mediated by *AKR1C3* could be significant for GC development. Moreover, epidemiological and experimental studies show estrogens, including E2, as protective agents in GC etiology (Freedman et al. 2007; Camargo et al. 2011). Additionally, in our previous study we presented that GC cells are able to convert E1 into E2 by *HSD17B1* action (Frycz et al. 2013). On the other hand, the role of androgens during gastric carcinogenesis still remains unknown. However, there are studies suggesting an oncogenic potential of androgens in GC (Nakamura et al. 2006; Tian et al. 2013). In our recent work, we showed that the amount of *HSD17B2* protein, which acts in opposite to *AKR1C3*, is also reduced in tumoral tissues of patients with GC (Frycz et al. 2015). The decreased expression of *HSD17B2* could result in an elevation of E2 and T in tumoral gastric tissues. Thus, an inhibition of E1 to E2 conversion by down-regulation of *AKR1C3* and an elevation of T level in tumoral tissue maintained by *HSD17B2* down-regulation can enhance gastric carcinogenesis. Although, T synthesis can be also mediated by *AKR1C3* enzyme, *in vitro* studies showed, that MCF7 breast cancer cells with stabled *AKR1C3* expression presented increased steroid conversion leading to pro-estrogenic state and surprisingly, E1 was revealed to be reduced faster than other substrates, including α -dione (Byrns et al. 2009). Moreover, *AKR1C3* possesses reductive activity which partly inactivates 5α -dihydrotestosterone (DHT), the most biologically potent androgen, to the weak androgens 3α -androstane- 17β -diol and 3β -androstane- 17β -diol (Steckelbroeck et al. 2003). Thus, tissues substrate specificity of this enzyme must be clarified for better understanding of its involvement in GC development. Notably, T can be converted into E2 through aromatase (*CYP19*) enzyme which is known to be expressed in nontumoral and tumoral gastric mucosa (Izawa et al. 2008). The aberrant action of steroidogenic enzymes in tumoral tissues, resulting in alterations

of E2/T ratio, could be also caused by the presence of single nucleotide polymorphisms (SNPs) in the sequences of their genes (Rossi et al. 2003). As an example, some SNPs of *CYP19* were significantly associated with increased risk of GC (Cho et al. 2012). So far, we don't know any data showing the association of *AKR1C3* polymorphism with GC risk. However, such correlation was showed in prostate cancer (Schulze et al. 2012).

Except its role in steroid conversion, *AKR1C3* enzyme is able to synthesize prostaglandin F by the conversion of prostaglandin H₂ to prostaglandin F₂ α (PGF₂ α) and prostaglandin D₂ to 9 α , 11 β -prostaglandin F₂ (Matsuura et al. 1998; Suzuki-Yamamoto et al. 1999). Nakamura et al. (1989) observed significantly inhibited the growth of cultured human GC cells KATO III in dose-dependent manner via stimulation of cyclic AMP production. Additionally, the percentage level of PGF₂ α was higher in the metastatic hepatic cells as compared with cells from primary gastric lesions (Nakazawa et al. 1993). Moreover, an elevated PGF₂ α protein level in breast cancer patients was correlated with good prognosis (Vergote et al. 1985). High levels of PGF₂ α were also positively associated with differentiation, positive estrogen and progesterone receptor status, as well as low mitotic index. Thus, disturbances in the level of PGF₂ α , mediated by the altered *AKR1C3* expression could be significant for gastric carcinogenesis, however this must be further clarified.

In our work, we evaluated the influence of HDAC inhibitor - NaBu, on the *AKR1C3* expression level in EPG 85-257 and HGC-27 GC cells. We also investigated its impact on the cell viability and proliferation. Histones acetylation and deacetylation are important processes involved in the regulation of gene expression (Ropero and Esteller 2007). Since altered expression of genes encoding HDAC has been associated with the development of many tumors, including GC (Choi et al. 2001; Song et al. 2005), they become promising targets for cancer therapy (Ropero and Esteller 2007). The potent anti-tumor activity of NaBu was presented in numerous studies, employing various cell lines, what makes that chemical a potentially useful agent in cancer therapy, including GC (Medina et al. 1997; Richon et al. 1998; Shin et al. 2012). It was shown, that NaBu activates the cell cycle inhibitors like p21Waf1/Cip1 and p27Kip1 as well as proapoptotic factors including Bax, Bak and Bik, therefore it induces growth arrest and apoptosis in the human SIIA GC cells (Litvak et al. 2000). Additionally, NaBu treatment of AGS, KATO III, MKN28, MKN45, MKN74, NCI-N87,

SNU1, SNU16 and SNU638 GC cells caused up-regulation of death-associated protein kinase 1/2 but also down-regulation of focal adhesion kinase protein and finally, led to cells apoptosis (Shin et al. 2012). Similarly, in our study NaBu significantly decreased proliferation and viability of both, EPG 85-257 and HGC-27 GC cells in dose and time dependent manner. The decreased proliferation and viability of HGC-27 GC cells, caused by NaBu mediated increase in phosphatase and tensin homolog expression, was previously shown by Pan et al. (2010). We also demonstrated, that NaBu is able to up-regulate transcript and protein of AKR1C3 in EPG 85-257 and HGC-27 GC cells. To the best of our knowledge, there are no studies which have shown the regulation of AKR1C3 by NaBu, however this compound was able to influence the expression of other genes involved in steroidogenesis. The NaBu mediated up-regulation of HSD17B1 mRNA and protein levels was observed in colorectal cancer cell lines - HT29 and SW707 (Rawłuszko et al. 2011). This led to an increased conversion of E1 to E2 in investigated cells. Thus, the intratissue concentration of steroid hormones could be modified by HDAC inhibitors (Martinez-Arguelles and Papadopoulos 2010).

Altogether, we showed reduced expression of AKR1C3 in a group of patients with diagnosed GC as well as in EPG 85-257 and HGC-27 GC cell lines. Along with emerging role of steroid enzymes in GC etiology, an alteration in intratissue synthesis of active steroids mediated by AKR1C3 down-regulation could be significant for the development of this cancer. Lowered transcript and protein levels of AKR1C3 were significantly associated with some clinicopathological features in GC. Moreover, we showed that the expression of AKR1C3 in GC cells could be restored by NaBu activity. Additionally, GC cells treated with NaBu were characterized by reduced proliferation rate and viability. With better understanding the role of AKR1C3 in GC etiology these observations could be helpful for new treatment strategies. Our studies are limited by relatively small patients group, so we are unable to explain what is an exact role of AKR1C3 expression in GC development. We also used homogenized tissues in our studies, therefore we can't exclude the possibility of samples contamination with other cell types like fibroblasts, endothelial cells, smooth muscle or blood cells. Moreover, synthesis of steroid hormones in gastric tissue could be regulated by expression of other genes involved in steroidogenesis process, thus next studies are needed.

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Tab. 1. Clinicopathological classification of GC tissue specimens.

Features	No. of patients
Total no. of patients	55
Gender (male/female)	33/22
Mean (\pm SD) age at radical gastric resection (years)	68.42 \pm 8.93
GC localization	
Multi site	37
Cardia	11
Body	1
Fundus	1
Lesser curvature	3
Pylorus	2
Histological types	
Diffuse	16
Intestinal	23
Indeterminated	12
Tumor depth	
T1	1
T2	9
T3	26
T4	16
Lymph nodes metastases	
N0	15
N1	7
N2	15
N3	15
Distant metastases	
M0	38
M1	4
Histological grading	
G1	1
G2	14
G3	35

Tab. 2. *AKRIC3* transcript levels in nontumoral and primary tumoral tissue samples from patients with GC.

		Nontumoral tissues		Primary tumoral tissues		p*
		Median (range)	Mean±SD	Median (range)	Mean±SD	
All patients	55	3.15 (2.434-3.648)	3.143±0.261	2.601 (0.943-3.64)	2.603±0.519	<0.0001
Age (years)						
≤65	23	3.169 (2.434-3.648)	3.17±0.294	2.783 (1.879-3.401)	2.788±0.445	0.004
>65	32	3.143 (2.623-3.554)	3.123±0.238	2.523 (0.943-3.64)	2.47±0.533	<0.0001
Gender						
Male	33	3.174 (2.535-3.648)	3.142±0.276	2.623 (1.898-3.64)	2.699±0.424	<0.0001
Female	22	3.113 (2.434-3.551)	3.145±0.244	2.554 (0.943-3.294)	2.459±0.618	<0.0001
GC localization						
Multi site	37	3.203 (2.434-3.563)	3.175±0.25	2.584 (0.943-3.64)	2.545±0.546	<0.0001
Cardia	11	3.082 (2.624-3.441)	3.076±0.274	2.623 (1.898-3.401)	2.665±0.457	0.021
Body	1	3.648 (-)	3.648±0	2.985 (-)	2.985±0	-
Fundus	1	2.955 (-)	2.955±0	3.38 (-)	3.38±0	-
Lesser curvature	3	2.983 (2.634-3.02)	2.879±0.213	2.439 (2.022-2.944)	2.468±0.462	-
Pylorus	2	3.162 (3.089-3.235)	3.162±0.1	2.968 (2.822-3.114)	2.968±0.206	-
Histological types						
Diffuse	16	3.141 (2.976-3.648)	3.214±0.217	2.794 (1.879-3.358)	2.679±0.457	0.0003
Intestinal	23	3.15 (2.535-3.555)	3.147±0.263	2.542 (1.253-3.401)	2.497±0.502	<0.0001
Indeterminated	12	3.21 (2.434-3.44)	3.082±0.323	2.697 (0.943-3.64)	2.705±0.693	0.126
Tumor depth						
T1	1	3.022 (-)	3.022±0	2.269 (-)	2.269±0	-
T2	9	3.113 (2.434-3.409)	3.074±0.342	2.275 (0.943-3.401)	2.363±0.716	0.017
T3	26	3.197 (2.624-3.648)	3.181±0.22	2.684 (1.524-3.64)	2.713±0.419	<0.0001
T4	16	3.087 (2.535-3.563)	3.16±0.296	2.604 (1.253-3.358)	2.602±0.56	0.0024
Lymph nodes metastases						
N0	15	3.089 (2.434-3.648)	3.073±0.353	2.439 (0.943-3.114)	2.362±0.621	0.0008
N1	7	3.207 (3.082-3.409)	3.214±0.099	2.783 (2.295-3.401)	2.729±0.395	0.021
N2	15	3.15 (2.816-3.563)	3.205±0.207	2.589 (1.879-3.358)	2.585±0.414	<0.0001
N3	15	3.22 (2.624-3.551)	3.128±0.279	2.715 (1.524-3.64)	2.807±0.515	0.031
Distant metastases						
M0	38	3.205 (2.434-3.648)	3.176±0.276	2.588 (0.943-3.64)	2.582±0.574	<0.0001
M1	4	3.231 (2.751-3.446)	3.165±0.297	2.851 (2.491-2.903)	2.774±0.193	0.19
Histological grading						
G1	1	2.634 (-)	2.634±0	2.439 (-)	2.439±0	-
G2	14	3.191 (2.535-3.555)	3.218±0.267	2.66 (2.263-3.401)	2.712±0.348	0.0014
G3	35	3.187 (2.434-3.648)	3.151±0.257	2.586 (0.943-3.64)	2.561±0.584	<0.0001

The *AKRIC3* transcript levels were standardized by the geometric mean of *B2M* and *GUSB* cDNA and expressed as decimal logarithm of multiples of these cDNA in the calibrator. p<0.05 was considered as statistically significant. *non-parametric, Mann-Whitney test.

Tab. 3. AKR1C3 protein levels in nontumoral and primary tumoral tissue samples from patients with GC.

		Nontumoral tissues		Primary tumoral tissues		p*
		Median (range)	Mean±SD	Median (range)	Mean±SD	
All patients	53	2.931 (2.349-3.343)	2.934±0.15	2.88 (1.942-3.192)	2.827±0.224	0.0021
Age (years)						
≤65	22	2.93 (2.383-3.343)	2.948±0.169	2.9 (1.942-3.089)	2.777±0.27	0.036
>65	31	2.931 (2.349-3.175)	2.925±0.138	2.859 (2.253-3.192)	2.864±0.182	0.026
Gender						
Male	32	2.932 (2.349-3.175)	2.938±0.134	2.906 (2.417-3.192)	2.885±0.153	0.043
Female	21	2.923 (2.383-3.343)	2.929±0.175	2.808 (1.942-3.089)	2.74±0.285	0.017
GC localization						
Multi site	36	2.923 (2.349-3.343)	2.917±0.17	2.83 (1.942-3.175)	2.787±0.252	0.0051
Cardia	10	2.929 (2.802-3.175)	2.947±0.095	2.934 (2.802-3.192)	2.936±0.112	0.91
Body	1	2.997 (-)	2.997±0	2.89 (-)	2.89±0	-
Fundus	1	3.104 (-)	3.104±0	2.953 (-)	2.953±0	-
Lesser curvature	3	3.028 (3.015-3.034)	3.026±0.009	2.969 (2.914-3.009)	2.964±0.047	-
Pylorus	2	2.934 (2.911-2.956)	2.934±0.032	2.706 (2.66-2.752)	2.706±0.065	-
Histological types						
Diffuse	15	2.916 (2.729-3.109)	2.943±0.087	2.89 (2.382-2.969)	2.788±0.191	0.025
Intestinal	22	2.946 (2.349-3.097)	2.929±0.141	2.891 (2.541-3.139)	2.872±0.151	0.098
Indeterminated	12	2.905 (2.383-3.343)	2.908±0.222	2.85 (1.942-3.175)	2.746±0.341	0.24
Tumor depth						
T1	1	2.96 (-)	2.96±0	2.88 (-)	2.88±0	-
T2	8	2.913 (2.383-3.034)	2.871±0.204	2.923 (1.942-3.009)	2.698±0.397	0.87
T3	25	2.936 (2.349-3.109)	2.93±0.144	2.859 (2.417-3.175)	2.862±0.163	0.015
T4	16	2.924 (2.729-3.343)	2.957±0.134	2.81 (2.382-3.089)	2.807±0.194	0.048
Lymph nodes metastases						
N0	14	2.972 (2.383-3.1)	2.917±0.177	2.841 (1.942-3.086)	2.734±0.319	0.051
N1	7	2.936 (2.863-3.017)	2.942±0.054	2.817 (2.752-3.175)	2.89±0.146	0.2
N2	14	2.923 (2.349-3.012)	2.888±0.163	2.901 (2.382-3.139)	2.855±0.176	0.32
N3	15	2.931 (2.802-3.343)	2.991±0.147	2.859 (2.417-3.192)	2.846±0.205	0.074
Distant metastases						
M0	36	2.931 (2.349-3.343)	2.922±0.168	2.853 (1.942-3.175)	2.799±0.249	0.0061
M1	4	2.88 (2.802-3.009)	2.893±0.095	2.796 (2.583-2.989)	2.791±0.203	-
Histological grading						
G1	1	3.034 (-)	3.034±0	3.009 (-)	3.009±0	-
G2	14	2.93 (2.349-3.097)	2.912±0.172	2.952 (2.581-3.139)	2.912±0.24	0.76
G3	33	2.916 (2.383-3.343)	2.928±0.144	2.808 (1.942-3.175)	2.767±0.247	0.0013

The amount of AKR1C3 proteins was presented as the decimal logarithm of AKR1C3 to GAPDH band optical density ratio.

P<0.05 was considered as statistically significant. * non-parametric, Mann-Whitney test

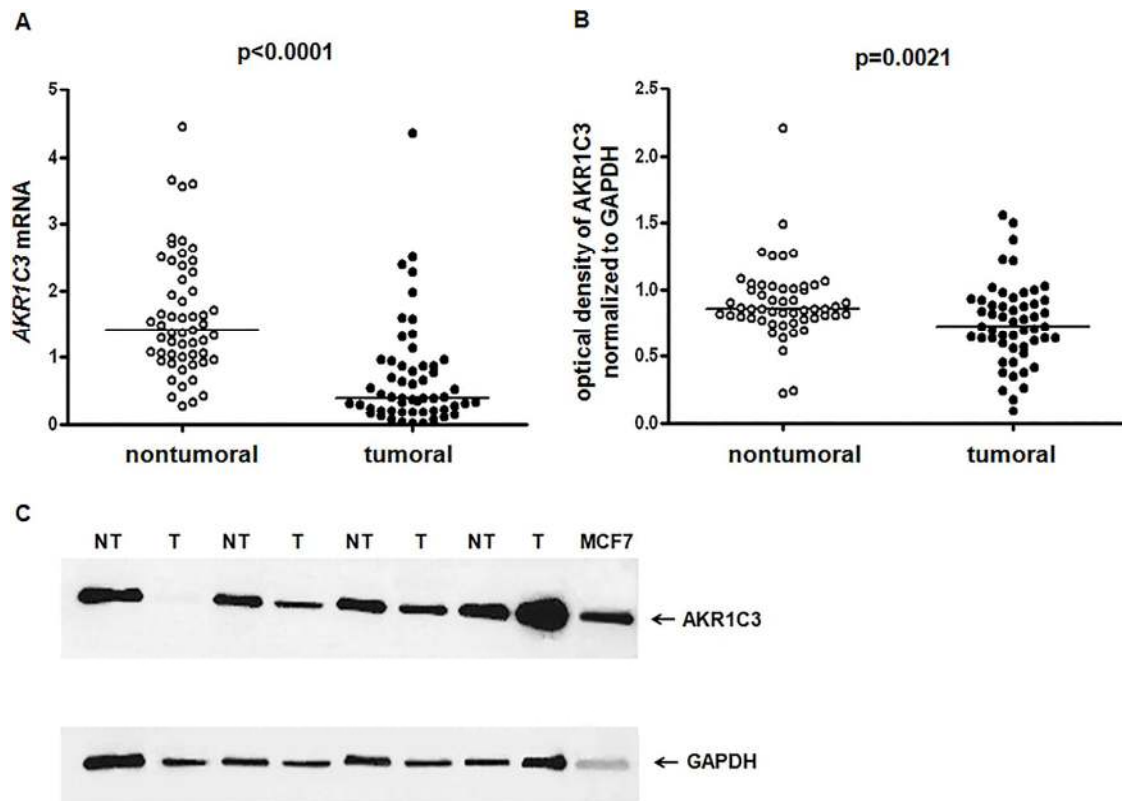


Fig. 1. AKR1C3 transcript (A) and protein (B) levels and a representative image of western blot analysis (C) of AKR1C3 presence in nontumoral (NT) and tumoral (T) tissues of patients with GC. The quantity of *AKR1C3* transcripts in each sample was standardized by the geometric mean of *B2M* and *GUSB* cDNA levels and expressed as multiples of these cDNA in the calibrator. The amount of AKR1C3 protein was calculated as the AKR1C3 to GAPDH band optical density ratio. The "p" value was evaluated by Mann-Whitney Test.

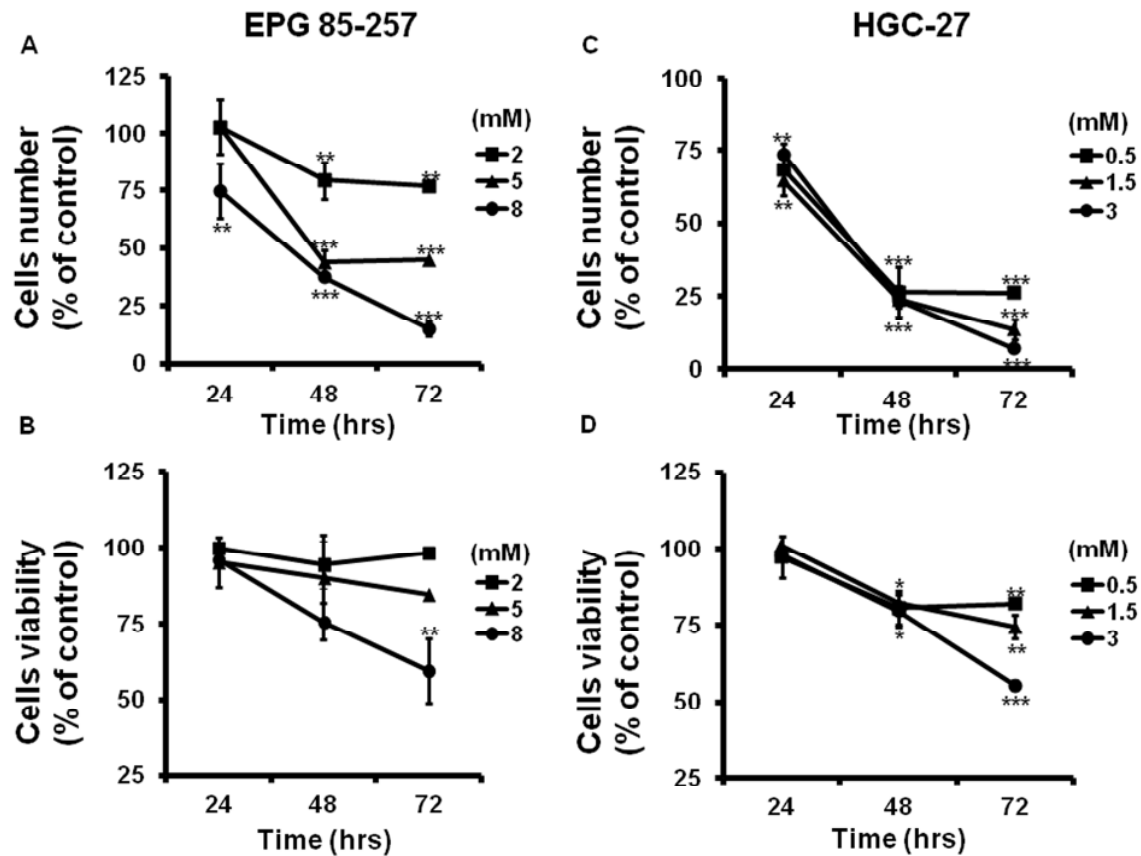


Fig. 2. NaBu inhibits proliferation (A,C) and viability (B,D) in EPG 85-257 and HGC-27 GC cells respectively. The cells were incubated for 24-78 hours and treated with the different concentrations of NaBu. After incubation, the cells were harvested and the total number of both, viable (unstained) and unviable (stained) cells were calculated with using trypan blue dye. Proliferation rate of EPG 85-257 (A) and HGC-27 (C) cells was represented as percent of total (stained and unstained) cell numbers of respective control. EPG 85-257 (B) and HGC-27 (D) GC cells viability was calculated with using following equation: total viable cells (unstained)/total cells (stained and unstained) x 100 and represented as a percent of respective control. Each sample was determined in triplicate and results are presented as the mean \pm SD from three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

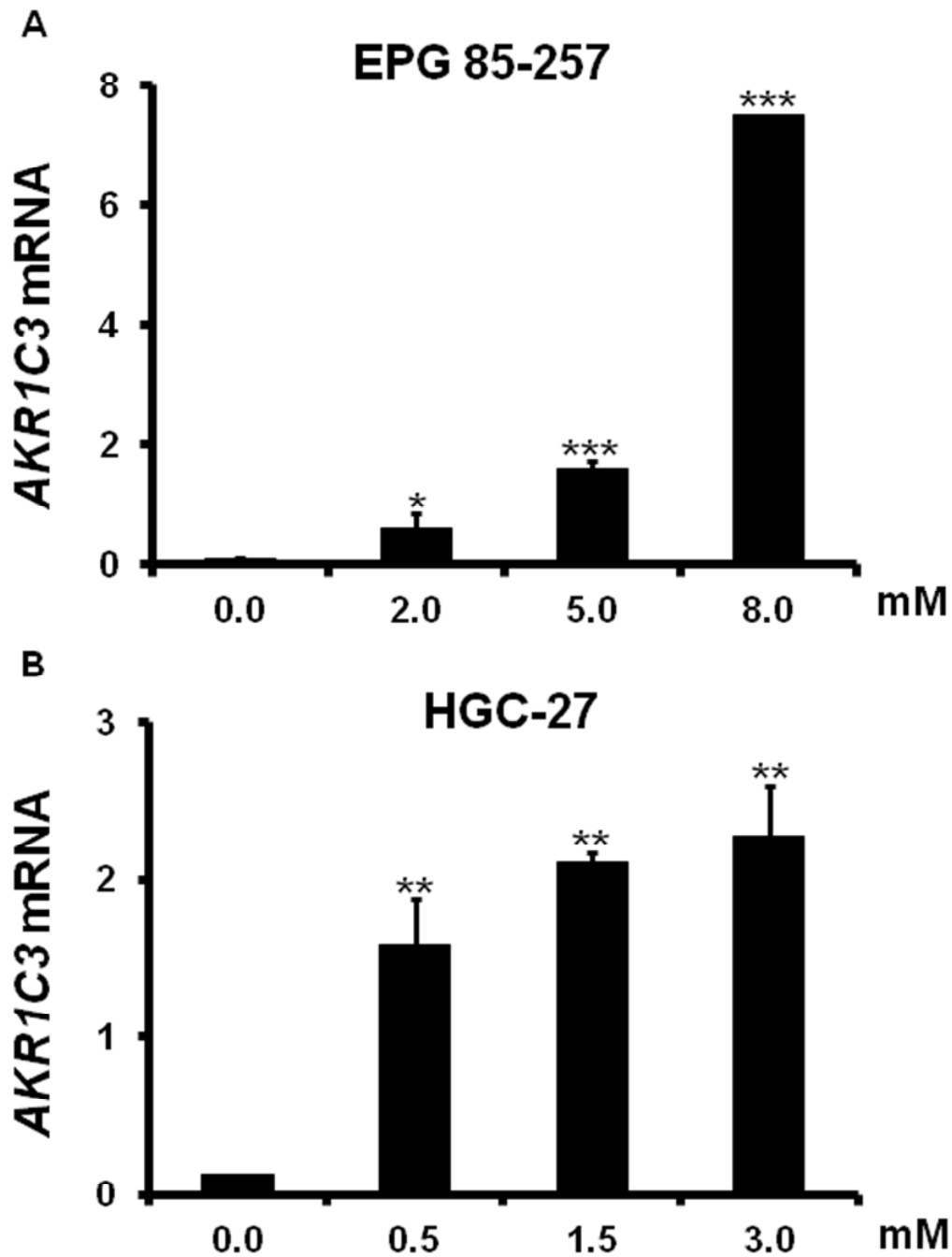


Fig. 3. NaBu increases *AKR1C3* transcript levels in EPG 85-257 (A) and HGC-27 (B) GC cells. The cells were cultured for 72 hours in an appropriate phenol red-free medium either in the absence (0.0) or in the presence of NaBu at the concentrations of 2.0, 5.0 and 8.0mM for EPG 85-257 and 0.5, 1.5 and 3.0mM for HGC-27 GC cells. After incubation, the cells were used for total RNA isolation and reverse transcription. The *AKR1C3* cDNA levels were determined by RQ-PCR relative quantification

analysis. RQ-PCR results were standardized by the geometric mean of *B2M* and *GUSB* cDNA levels. *AKR1C3* cDNA levels were expressed as a multiplicity of the respective controls. Each sample was determined in triplicate and results are presented as the mean \pm SD from three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

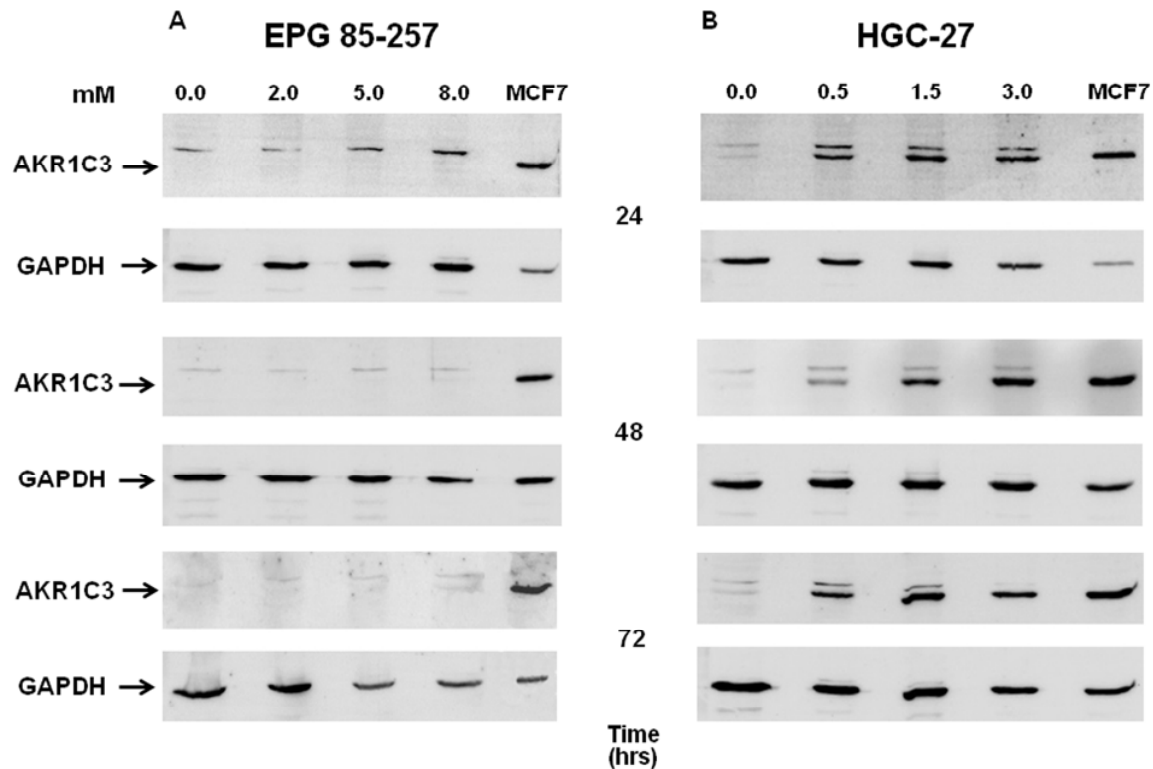


Fig. 4. NaBu increased AKR1C3 proteins level in EPG 85-257 (A) and HGC-27 (B) GC cells. The cells were cultured for 24, 48 and 72 hours in an appropriate phenol red-free medium either in the absence or in the presence of NaBu at the concentrations of 2.0, 5.0 and 8.0mM for EPG 85-257 and 0.5, 1.5 and 3.0mM for HGC-27 GC cells. The cells protein were separated by 12% SDS-PAGE, and transferred to a membrane that was then immunoblotted with rabbit polyclonal anti-AKR1C3 Ab and incubated with goat anti-rabbit HRP-conjugated Ab. After stripping, the membrane was reblotted with rabbit polyclonal anti-GAPDH Ab and goat anti-rabbit HRP-conjugated Ab. The band densitometry readings were normalized to GAPDH loading control.

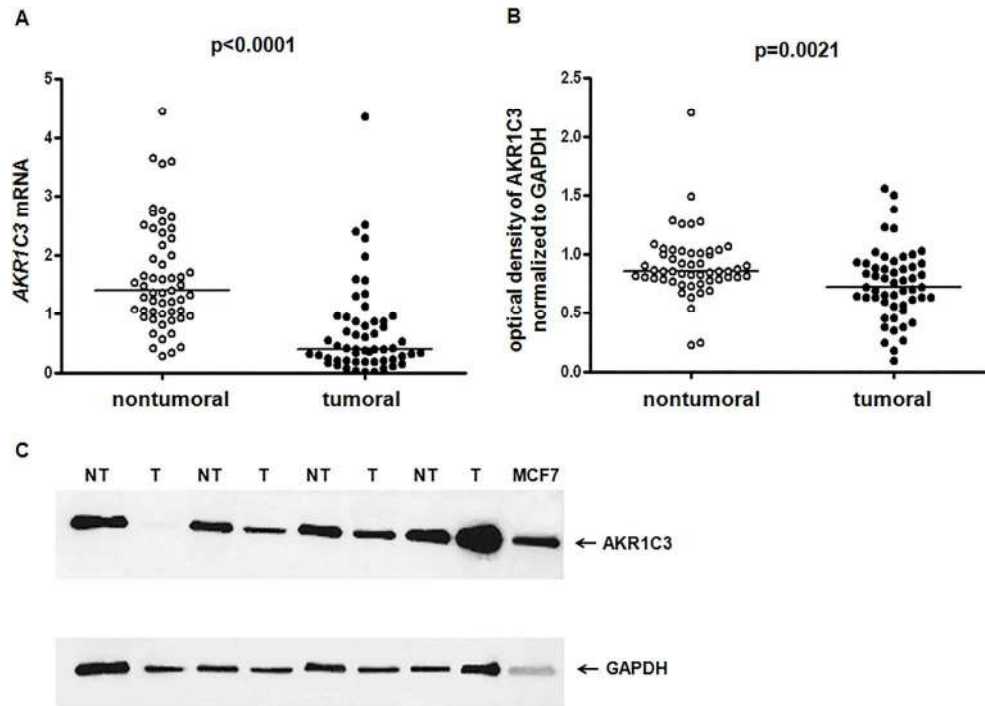


Fig. 1. AKR1C3 transcript (A) and protein (B) levels and a representative image of western blot analysis (C) of AKR1C3 presence in nontumoral (NT) and tumoral (T) tissues of patients with GC. The quantity of *AKR1C3* transcripts in each sample was standardized by the geometric mean of *B2M* and *GUSB* cDNA levels and expressed as multiples of these cDNA in the calibrator. The amount of AKR1C3 protein was calculated as the AKR1C3 to GAPDH band optical density ratio. The "p" value was evaluated by Mann-Whitney Test. 263x186mm (300 x 300 DPI)

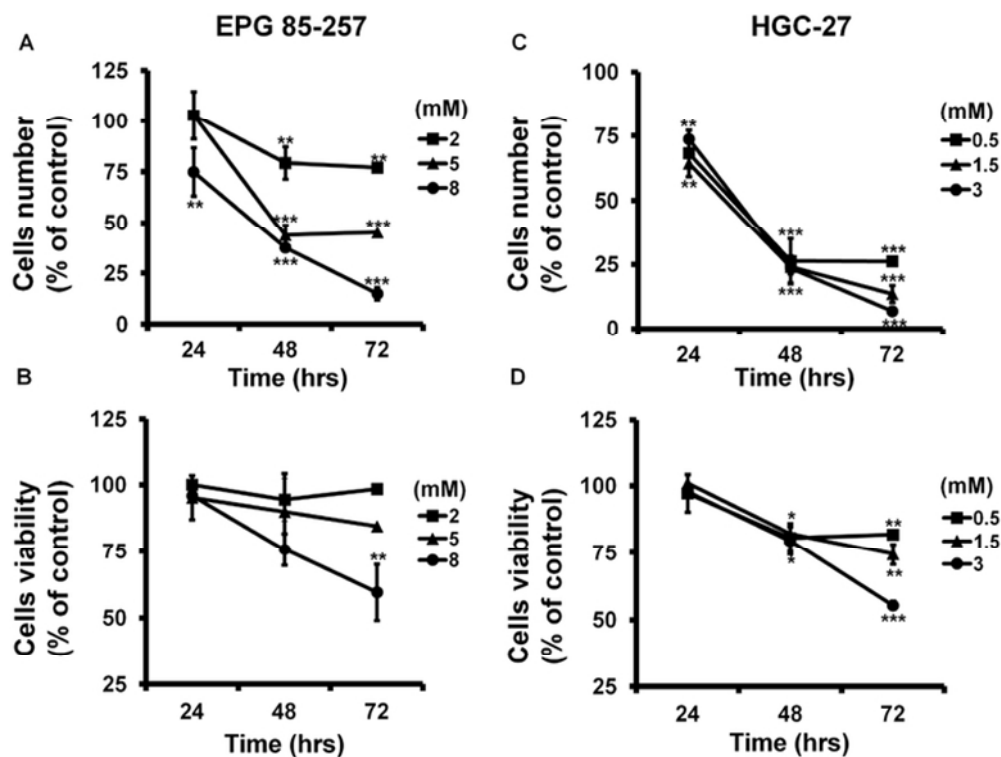


Fig. 2. NaBu inhibits proliferation (A,C) and viability (B,D) in EPG 85-257 and HGC-27 GC cells respectively. The cells were incubated for 24-78 hours and treated with the different concentrations of NaBu. After incubation, the cells were harvested and the total number of both, viable (unstained) and unviable (stained) cells were calculated with using trypan blue dye. Proliferation rate of EPG 85-257 (A) and HGC-27 (C) cells was represented as percent of total (stained and unstained) cell numbers of respective control. EPG 85-257 (B) and HGC-27 (D) GC cells viability was calculated with using following equation: total viable cells (unstained)/total cells (stained and unstained) x 100 and represented as a percent of respective control. Each sample was determined in triplicate and results are presented as the mean \pm SD from three experiments.

*p<0.05; **p<0.01; ***p<0.001.

64x48mm (300 x 300 DPI)

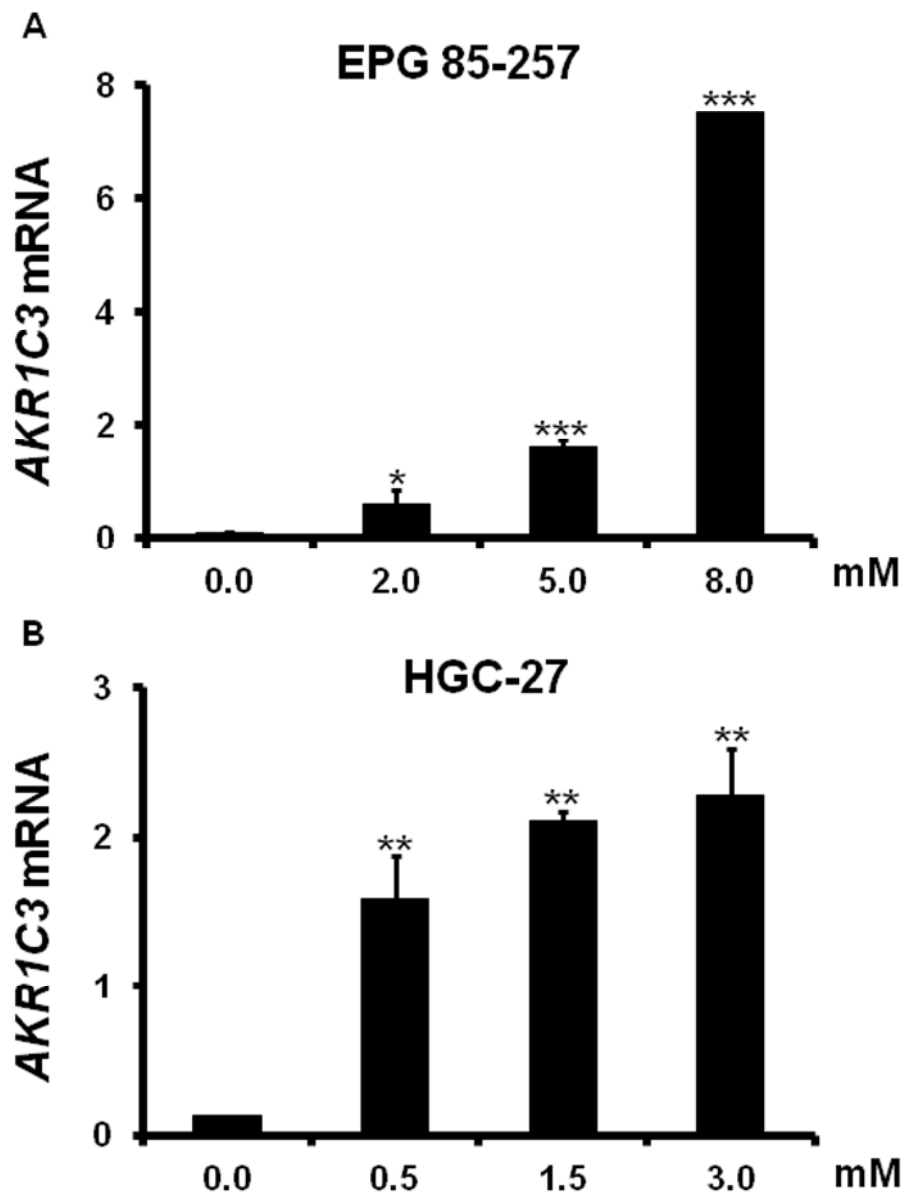


Fig. 3. NaBu increases *AKR1C3* transcript levels in EPG 85-257 (A) and HGC-27 (B) GC cells. The cells were cultured for 72 hours in an appropriate phenol red-free medium either in the absence (0.0) or in the presence of NaBu at the concentrations of 2.0, 5.0 and 8.0mM for EPG 85-257 and 0.5, 1.5 and 3.0mM for HGC-27 GC cells. After incubation, the cells were used for total RNA isolation and reverse transcription. The *AKR1C3* cDNA levels were determined by RQ-PCR relative quantification analysis. RQ-PCR results were standardized by the geometric mean of *B2M* and *GUSB* cDNA levels. *AKR1C3* cDNA levels were expressed as a multiplicity of the respective controls. Each sample was determined in triplicate and results are presented as the mean \pm SD from three experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

190x247mm (300 x 300 DPI)

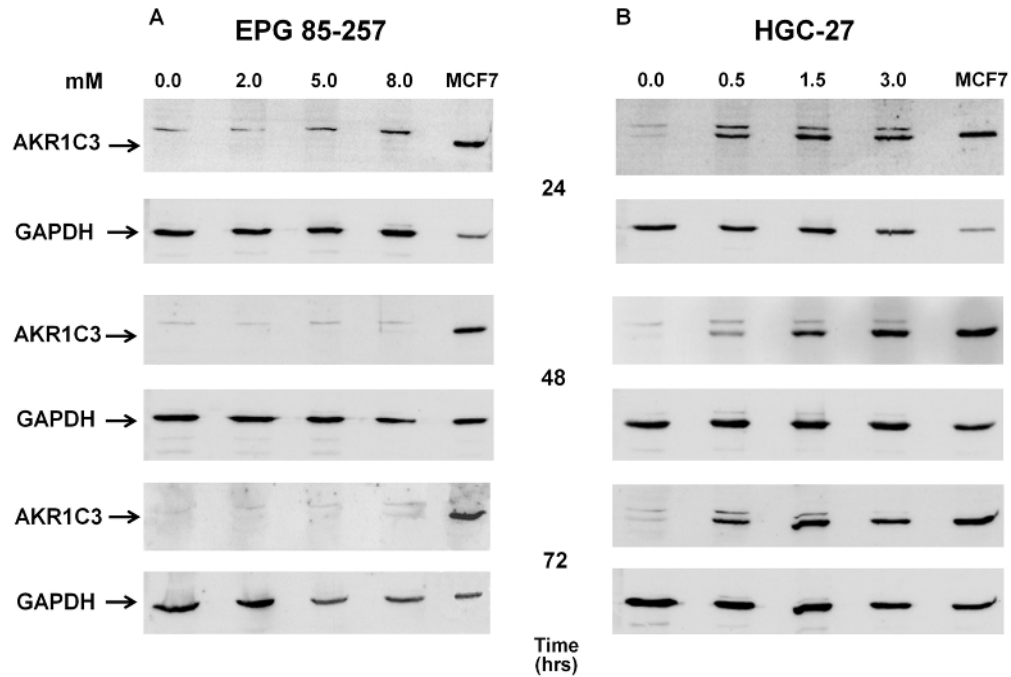


Fig. 4. NaBu increased AKR1C3 proteins level in EPG 85-257 (A) and HGC-27 (B) GC cells. The cells were cultured for 24, 48 and 72 hours in an appropriate phenol red-free medium either in the absence or in the presence of NaBu at the concentrations of 2.0, 5.0 and 8.0mM for EPG 85-257 and 0.5, 1.5 and 3.0mM for HGC-27 GC cells. The cells protein were separated by 12% SDS-PAGE, and transferred to a membrane that was then immunoblotted with rabbit polyclonal anti-AKR1C3 Ab and incubated with goat anti-rabbit HRP-conjugated Ab. After stripping, the membrane was reblotted with rabbit polyclonal anti-GAPDH Ab and goat anti-rabbit HRP-conjugated Ab. The band densitometry readings were normalized to GAPDH loading control. 122x81mm (300 x 300 DPI)