

miR-143 is Associated with ER- α 36 Expression Gastric Carcinoma of Xenografted Model

Zou Feng¹, Zhang Shangkun^{1,2}, Li Yan¹, Chen Ying¹ and Liu Lijiang^{1*}

¹Department of Pathology and Pathophysiology, School of Medicine, Jiangnan University, Wuhan, China

²JiangDa Pathology Institution, Wuhan 430056, Hubei Province, China

Abstract

We investigated the expression profile of microRNA (miRNA) in ER- α 36-mediated transplantable tumours. SGC7901 cell lines of higher or lower ER- α 36 expression were constructed with a lentivirus vector. Five million SGC7901 cells were injected into nude mice to form a gastric xenografted carcinoma in the subcutaneous tissue of nude mice. Observation was performed for 30 days. The weight and propagation of the tumours were determined. miRNA expression profiles of the three SGC7901 cell lines were analysed by miRNA microarray. miR-143 expression in transplantable tumours was determined by real-time PCR using TaqMan assay. The expression level of ER- α 36 was proportional to the size and propagation of gastric xenografted carcinomas in nude mice. The expression level of miR-143 significantly decreased in highly expressing ER- α 36 SGC7901 cells and significantly increased in lowly expressing ER- α 36 SGC7901 cells. miR-143 expression results in transplantable tumours amongst nude mice were consistent with the miRNA microarray findings. The expression of miR-143 was suppressed by high ER- α 36 expression. These findings suggest that deregulation of miR-143 plays an important role in the progression of ER- α 36-mediated gastric xenografted carcinoma.

Keywords: Gastric cancer; ER- α 36; miR-143; SGC7901; Gastric xenografted carcinoma

Introduction

The diverse activities of oestrogens and anti-oestrogens mediated by specific receptors designated as oestrogen receptors (ERs) are well documented. Two ERs, ER- α and ER- β , which are ligand-activated transcription factors that stimulate target gene transcription, have been identified. Two forms of ER- α , a full-length form (66 kDa) and a short-length one (46 kDa), have been found on or near the plasma membrane and mediate membrane-initiated oestrogen signalling [1,2]. In 2005, a novel variant of ER- α with a molecular weight of 36 kDa was identified and cloned; this variant was called ER- α 36. ER- α 36 differs from the original 66 kDa ER- α (ER- α 66) in that it lacks two transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding and partial dimerisation and ligand-binding domains. ER- α 36 is predominantly expressed in the plasma membrane of breast cancer cells (ER-positive and -negative), mediates membrane-initiated oestrogen and anti-oestrogen signalling, such as activation of the MAPK/ERK and PI3K/AKT signalling pathways, and stimulates cell proliferation [3-5].

In our previous studies, we examined the expression and localisation of ER- α 36 in established gastric cancer cell lines of SGC7901, BGC823, AGS, and MKN45. Our results show that ER- α 36 is predominantly expressed in the plasma membrane of gastric cancer cell lines. We report the expression of ER- α 36 mRNA in human gastric adenocarcinoma tissues in comparison with non-tumour mucosa. Our data suggest that upregulation of ER- α 36 mRNA is significantly correlated with lymph node metastasis and infiltration of gastric carcinoma. The mechanisms of metastasis and infiltration of gastric carcinoma may be correlated with ER- α 36 protein expression [6-9].

MicroRNAs (miRNAs) are small, endogenously conserved, non-coding RNAs ranging from 20 nt to 22 nt in length. miRNAs can induce mRNA translation inhibition or degradation through regulation of gene expression at the post-transcriptional level by binding to the 3'-UTR of target mRNAs [10,11]. This class of RNAs is believed to regulate about 30% of all human genes. Alterations in the expression of miRNAs are considered a common characteristic of all human

tumours. MicroRNAs have been shown to be involved in a variety of tumour cell biological processes, including cell differentiation, proliferation, apoptosis, metastasis, and invasion [12].

MicroRNAs are frequently located near cancer-associated genomic regions or fragile sites and play a vital role as a novel class of oncogenes or tumour-suppressor genes [13]. Alterations in miRNA expression levels have been demonstrated in several gastrointestinal tumours, and miRNAs have been found to influence the prognostic significance of the tumours. Several miRNAs, such as miR-1, miR-20a, miR-27a, miR-34, and miR-423-5p, can also regulate gene expression in gastric cancer [14,15]. Increasing miRNA expression levels have been associated with gastric cancer.

To the best of our knowledge, the correlation between miRNA expression and ER- α 36 in gastric cancer has yet to be reported. In this study, we detected the miRNA expression of gastric xenografted carcinoma in nude mice and observed the miRNA expression of stable SGC7901 cell lines with lower or higher ER- α 36 expression. The study aimed to investigate the relationship between the expressions of ER- α 36 and miRNA in gastric cancer.

Materials and Methods

Cell culture

The human gastric adenocarcinoma cell line SGC7901 was obtained from the Chinese Academy of Medical Sciences Cell Centre of

***Corresponding author:** Liu Lijiang, Department of Pathology and Pathophysiology, School of Medicine, Jiangnan University, Wuhan, China, E-mail: lulijiang@163.com

Received September 28, 2013; **Accepted** November 11, 2013; **Published** November 18, 2013

Citation: Feng Z, Shangkun Z, Yan L, Ying C, Lijiang L (2013) miR-143 is Associated with ER- α 36 Expression Gastric Carcinoma of Xenografted Model. J Biomim Biomater Tissue Eng 18: 117. doi: [10.4172/1662-100X.1000117](http://dx.doi.org/10.4172/1662-100X.1000117)

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Basic Medicine (Beijing, China). Cell lines were cultured in RPMI 1640 containing 10% FCS at 37°C in a 5% CO₂ atmosphere.

Construction of stable SGC7901 cell lines with lower or higher ER- α 36 expression

ER- α 36-silenced plasmids (PLKO.1-PURO-SP6-ER- α 36-L) and ER- α 36-overexpressing plasmids (PLJM1-ER- α 36-H) were confirmed based on the effective sequences of siRNA targeting of the ER- α 36 gene obtained from Wang Zhaoyi's laboratory. The complementary DNA containing both sense and antisense oligo DNAs of the targeted sequence was designed using the following sequences: ER- α 36F: CCGGTTGATGCCAATAGGTAAGTGAAGTTCGAGTTCAGTACC TATTGGCATCAATTTTGTG; ER- α 36R: AATTCAAAAA TTGATGCCAATAGGTAAGTGAAGTTCGAGTTCAGTACC TATTGG CATCAA. Stable SGC7901 cell lines with lower or higher ER- α 36 expression were constructed by infection of PLKO.1-PURO-SP6-ER- α 36-L and PLJM1-ER- α 36-H after PCR confirmation and sequencing. ER- α 36 low SGC7901 cells stably expressing PLKO.1-PURO-SP6-ER- α 36-L (low ER- α 36 group) and ER- α 36 high SGC7901 cells stably expressing PLJM1-ER- α 36-H (high ER- α 36 group) were established using puromycin for isolation. The ER- α 36 control SGC7901 group was constructed by transferring empty vector (PLKO.1-PURO-scrembled shRNA) at the same time.

Western blot analysis

Cell lysates from SGC7901 cells were prepared in 1 mL lysis buffer (Sigma, USA) on ice in 1.5 mL microtubes. The lysates were solubilised by continuous stirring for 1 h at 4°C and then centrifuged for 10 min at 14000 × g. The supernatants were collected, and protein concentrations were measured using BCA protein assay reagent. Cell lysates were subjected to SDS-PAGE. After blotting, membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary antibodies for 16 h at 4°C. Primary antibodies included those against ER- α 36 (Zhaoyi Wang's laboratory, USA) and actin (Santa Cruz, USA). The membranes were incubated with anti-mouse IgG antibody for 1 h after three washes in Tris-buffered saline containing 0.1% Tween 20. The proteins were detected with an ECL system (KPL Amersham).

Nude mice experiments

Nine male SPF BALB/c-nu mice (20-25 g, 6 weeks old) were supplied by the Experimental Animal Centre of Hubei Province. The experiment was divided into three groups: the control SGC7901 group (ER- α 36 control group, $n=3$), the SGC7901 with high ER- α 36 expression group (high ER- α 36 group, $n=3$), and the SGC7901 with low ER- α 36 expression group (low ER- α 36 group, $n=3$). Five million SGC7901 cells were injected into the nude mice to form a SGC7901 gastric xenografted carcinoma in the subcutaneous tissue, and observation was performed for 30 d. The sizes and weights of the tumours that developed were determined.

HE and immunohistochemical staining of gastric xenografted carcinoma

HE staining was performed according to the conventional method. Immunohistochemical staining was performed on 4 μ m thick tumour sections via a two-step assay. Tissue slides were incubated overnight with the Ki-67 antibody diluted to 1:50 at 4°C in a humidified chamber. Ki-67 staining was scored as described by Couzin: positive were the Ki-67 cell number when counted 100 cell in high power field (×400) [15].

MiRNA microarray of the three SGC7901 cell lines

Total RNA from the three SGC7901 cell lines was isolated using Trizol reagent (Invitrogen, USA). The concentration and quality of RNA were measured by a NanoDrop ND-1000 system at 260 and 280 nm (A260/280). RNA labelling and hybridisation were completed by BGI (Shenzhen, China) according to the manufacturer's instructions. Only those with increases or decreases in expression greater than 2-fold relative to the control were considered significantly changed. Samples were clustered according to their miRNA profile using Cluster 3.0.

TaqMan miRNA real-time RT-PCR quantification of gastric xenografted carcinoma

Real-time RT-PCR analysis of miRNA expression was carried out using TaqMan microRNA assay kits according to the manufacturer's protocol (Applied Biosystems, USA). Briefly, total RNA was extracted using an isolation additive buffer. Reverse transcriptase was synthesised from the total RNA (10 ng) using gene-specific primers. PCR reactions were run on a Step One Plus Real-Time PCR machine (Applied Biosystems) and analysed using Step One Plus System SDS software.

U6 small nuclear RNA (Applied Biosystems) was used as an internal control to normalise RNA input. The Ct value was defined as the fractional cycle number at which fluorescence exceeds the fixed threshold. Fold-changes in tumour expression relative to corresponding normal tissues were calculated using the 2^{- $\Delta\Delta$ Ct} method after normalisation to the endogenous control. All experiments were carried out in triplicate.

Statistical analysis

All values are expressed as mean \pm SEM. Statistical significance was determined using ANOVA followed by t-tests. $P<0.05$ was considered statistically significant.

Results

Establishment of stable SGC7901 cell lines with lower or higher ER- α 36 expression

The ER- α 36 expression of three SGC7901 cell lines was determined by Western blot. This result confirms that construction of stable SGC7901 cell lines with lower and higher ER- α 36 expression was successful (Figure 1).

Formation of distinct gastric xenografted carcinoma in nude mice by injection of the three SGC7901 cell lines

Gastric xenografted carcinoma was observed in all nude mice. After the 30th day, the tumour weight was highest in the high ER- α 36 group, moderate in the control ER- α 36 group, and lowest in the low ER- α 36 group. Significant differences in tumour size were observed amongst groups (Figure 2, $P<0.05$). Nuclear fission and Ki-67 expression showed a similar trend amongst groups (Figure 3, $P<0.05$). ER- α 36 expression was proportional to the size and propagation of gastric xenografted carcinoma.

Differential expression of miRNAs amongst the three SGC7901 cell lines

Several miRNAs were deregulated in ER- α 36-mediated gastric cancer. The expression level of miR-143 significantly decreased in highly expressing ER- α 36 SGC7901 cells and significantly increased in lowly expressing ER- α 36 SGC7901 cells (Figure 4, $P<0.01$).

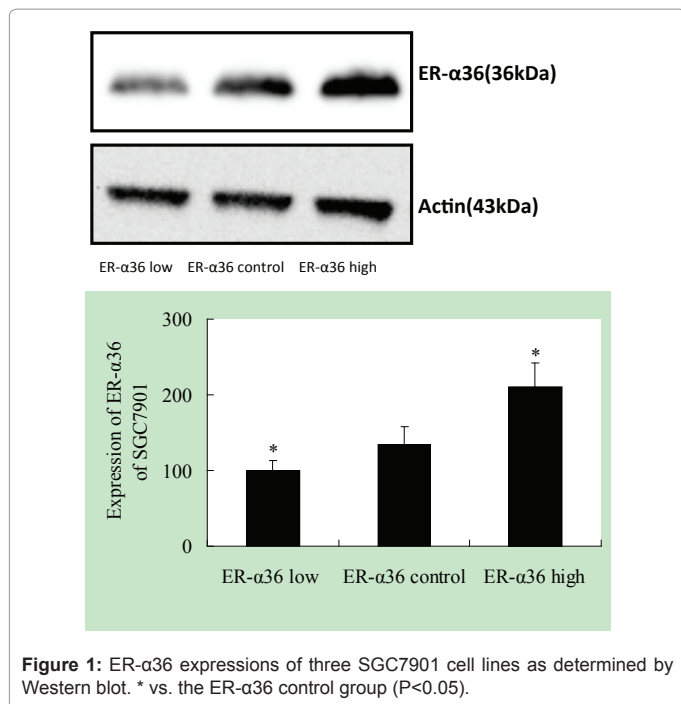


Figure 1: ER- α 36 expressions of three SGC7901 cell lines as determined by Western blot. * vs. the ER- α 36 control group (P<0.05).

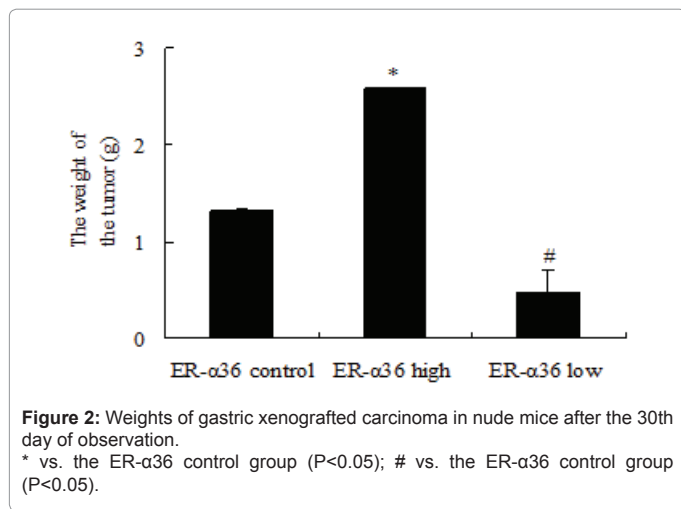


Figure 2: Weights of gastric xenografted carcinoma in nude mice after the 30th day of observation. * vs. the ER- α 36 control group (P<0.05); # vs. the ER- α 36 control group (P<0.05).

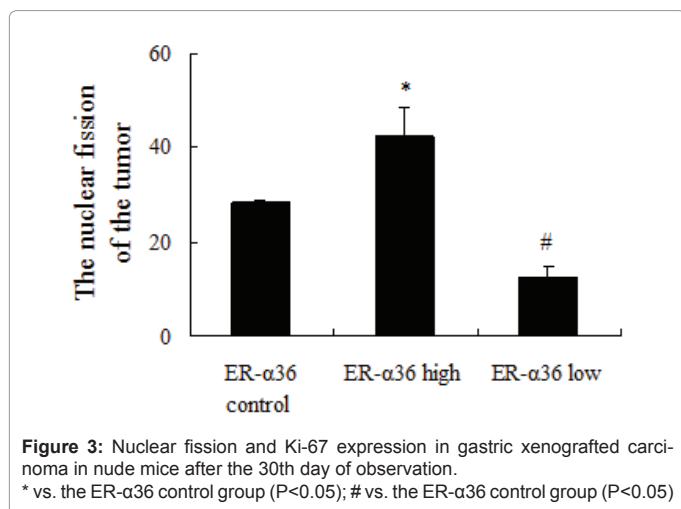


Figure 3: Nuclear fission and Ki-67 expression in gastric xenografted carcinoma in nude mice after the 30th day of observation. * vs. the ER- α 36 control group (P<0.05); # vs. the ER- α 36 control group (P<0.05).

Expression of miR-143 in gastric xenografted carcinoma in nude mice

miR-143 expression levels in transplantable tumours in nude mice were significantly downregulated in the high ER- α 36 group (Figure 5, P<0.05) and upregulated in the low ER- α 36 group (Figure 5, P<0.05) compared with the control ER- α 36 group. These results are consistent with those obtained from our miRNA microarray studies.

Discussion

ER- α 36, a novel variant of ER- α , is identical in most aspects to the ER- α 66 protein encoded by exons 2–6 of the ER- α 66 gene. However, ER- α 36 lacks intrinsic transcriptional activity but can suppress transactivation activities mediated by the AF-1 and AF-2 domains of liganded and unliganded ER- α 66 and ER- α . These properties indicate that ER- α 36 is a potent inhibitor of genomic oestrogen signalling. ER- α 36 was first discovered by Wang in breast cancer and later reported in other cancers. The role of ER- α 36 in gastric cancer remains unclear. In our previous study, we found that gastric cancer cells treated with 17 β -oestradiol respond by the effects of ER- α 36. Tamoxifen could inhibit the growth of BGC823 gastric cancer cells, and ER- α 36 may transmit tamoxifen growth-regulated signals in gastric cancer via a

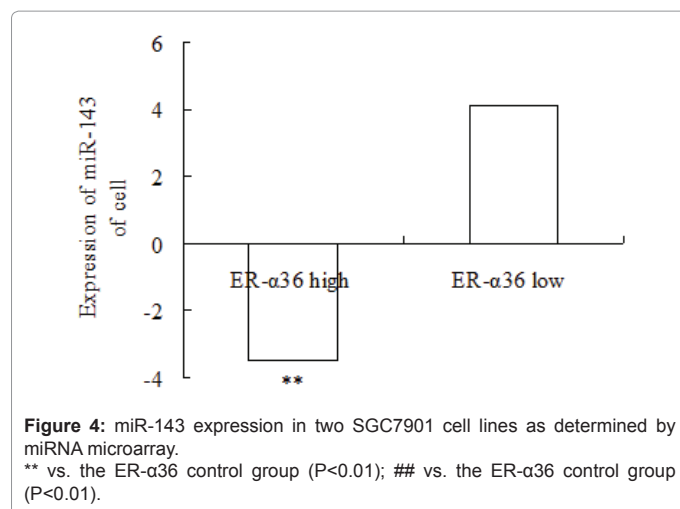


Figure 4: miR-143 expression in two SGC7901 cell lines as determined by miRNA microarray. ** vs. the ER- α 36 control group (P<0.01); # vs. the ER- α 36 control group (P<0.01).

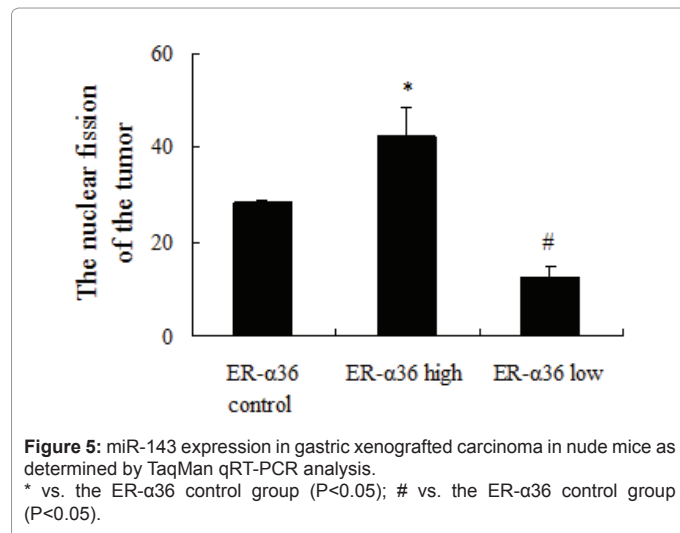


Figure 5: miR-143 expression in gastric xenografted carcinoma in nude mice as determined by TaqMan qRT-PCR analysis. * vs. the ER- α 36 control group (P<0.05); # vs. the ER- α 36 control group (P<0.05).

membrane-based pathway. These results show that ER- α 36 expression is associated with tumorigenesis.

Deregulation of miRNA expression in cancer has been widely reported. However, previous studies focused only on miRNAs expressed in tumour tissues and cells, and the specificity of biomarkers based on a single tumour-specific miRNA was generally poor. Not much is known about the involvement of miR-143 in cancer [16]. The tumour-suppressive effects of miR-143 in squamous cell carcinoma cell lines and in colorectal cancer have been previously reported [17,18]. In this study, our miRNA microarray results indicated that miR-143 is a potentially useful biomarker in gastric cancer, especially in ER- α 36-mediated gastric cancer. In the next phase of our study, we will focus on the regulation of miR-143 target genes in gastric cancer.

In summary, this study determined that miRNA deregulation is associated with ER- α 36-mediated gastric cancer. miR-143 expression is suppressed by high levels of ER- α 36 in gastric cancer cells and gastric xenografted carcinoma in nude mice. Determination of whether or not miR-143 is regulated by oestrogen receptors will be an interesting subject for future research. We believe that miR-143 is involved in oestrogen receptor signal transduction. Our findings provide important insights into the progression of ER- α 36-mediated gastric cancer.

Acknowledgment

We thank Prof. Zhaoyi Wang for providing the ER- α 36 antibody used in this work. This work was supported grants awarded to National Natural Science Foundation of China (Grant No.81272754) and Wuhan Young Chenguang Foundation (Grant No.2013071004010475).

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Citation: Feng Z, Shangkun Z, Yan L, Ying C, Lijiang L (2013) miR-143 is Associated with ER- α 36 Expression Gastric Carcinoma of Xenografted Model. *J Biomim Biomater Tissue Eng* 18: 117. doi: [10.4172/1662-100X.1000117](https://doi.org/10.4172/1662-100X.1000117)

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