

Association between the ornithine decarboxylase G316A polymorphism and breast cancer survival

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Abstract. Ornithine decarboxylase (ODC) is a significant rate-limiting enzyme in polyamine synthesis, required for normal cell growth, and is highly expressed in various malignancies, including colorectal and breast cancer. In the present study, the associations between the ODC G316A single nucleotide polymorphism (SNP) and breast cancer-specific survival were investigated. In addition, the functional effects of this SNP were examined in the MCF-7 human breast cancer cell line. The present study recruited 300 stage I-III breast cancer cases, which were diagnosed at the Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China) between 2002 and 2003, with follow-up visits conducted until May 2013. ODC G316A was genotyped (ODC GG vs. ODC AG/AA) in the 300 cases and the association of the genotypes with cancer-specific survival was analyzed. In the MCF-7 cell line, the ODC allele-specific binding of E-box transcription factors was determined using western blot and chromatin immunoprecipitation assays. Survival differences were observed between the two genotypes: Compared with the ODC GG genotype, patients with ODC GA/AA exhibited significantly higher survival rates ($P < 0.05$). In cultured cells, the ODC SNP, which is flanked by two E-boxes, appeared to predict ODC promoter activity. Furthermore, the E-box activator c-MYC and repressor MAX interactor 1 were found to preferentially bind to ODC minor A-alleles compared with major G-alleles, in cultured MCF-7 cells. In conclusion, the results of the current

study suggest that the regulation of ODC may affect survival in breast cancer patients and indicate a model in which the ODC SNP may be protective for breast adenoma recurrence and detrimental for survival following a diagnosis of breast cancer.

Introduction

Breast cancer is the most common malignancy in females worldwide and its incidence has increased rapidly over recent decades (1). It is well-established that polyamines are low molecular weight, positively-charged compounds that are highly-expressed in numerous malignancies, including breast cancer (2). Previous studies have indicated that polyamines may affect various processes in transcription, RNA stabilization, ion channel gating and carcinogenesis, while inhibition of polyamine synthesis appears to be important in inhibiting proliferation, decreasing apoptosis and suppressing angiogenesis (3-5). Ornithine decarboxylase (ODC) is a rate-limiting enzyme that contributes to polyamine synthesis (6). Increased polyamine levels and ODC activity have been identified in various types of human cancer, particularly in breast cancer. Therefore, inhibiting ODC activity was able to suppress cancer development in animal models (7-10). ODC expression is regulated by the MYC and MAD transcription factors, which include the MYC-associated factor X (MAX) network transcriptional repressor, MAX interactor 1 (MXI1), MAD1 and MAD4 (11). When MYC forms a heterodimer with MAX, it can bind to DNA at the E-box sequence (CACGTG) and activate ODC gene expression. By contrast, MAD combines with MAX, which can suppress the transcription of the ODC gene (Fig. 1) (12,13).

ODC G316A is a functional single nucleotide polymorphism (SNP) that is located between two promoter region/transcription factor binding sites. Furthermore, the transcription of the ODC gene can be altered by this SNP (14). Previous studies have demonstrated that the ODC G316A polymorphism exhibits prognostic efficacy in colorectal adenoma recurrence and is associated with the survival of patients with colorectal cancer (15,16). However, the effects of this SNP on the clinical outcomes in breast cancer remain unclear. Therefore, the aim of the present population-based study was to

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investigate the association between the ODC G316A SNP and breast cancer-specific mortality by genotyping 300 samples from breast cancer patients that were enrolled at the Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China) between 2002 and 2003. Subsequently, the functional significance of the ODC G316A SNP in the MCF-7 breast cancer cell line was investigated.

Materials and methods

Epidemiological studies

Study population. The present study investigated 300 patients with stage I-III breast cancer that were enrolled at the Affiliated Cancer Hospital of Zhengzhou University between 2002 and 2003, with follow-up visits conducted until May 2013. For all breast cancer patients recruited to the present study, written informed consent was obtained from the patients or the patient's families, allowing the collection of paraffin-embedded tissue samples and the release of the patients' medical data. Furthermore, the present study was approved by the Ethics Committee of the Affiliated Cancer Hospital of Zhengzhou University. Clinical and demographic data, including vital status and follow-up details, were obtained by associating follow-up and regional cancer registry databases. Information regarding lifestyle factors and a detailed family history were collected from all the study participants during face-to-face interviews. Tumor grade was determined according to the 2013 breast cancer guidelines of the National Comprehensive Cancer Network (17). The morphological features (tubule formation, nuclear pleomorphism and mitotic count) of the tumors were assessed and each assigned a score of 1-3. The scores for each category were combined to determine the histological grades of the tumors as follows: Grade 1 (low), score, 3-5; grade 2 (intermediate), score, 6-7; and grade 3 (high), score, 8-9. The 'tumor node metastasis' staging was derived from existing American Joint Committee on Cancer (AJCC) codes, where available, and conversion of the extent of disease codes was performed as previously reported (18,19).

DNA extraction. Subsequently, DNA was extracted from 300 paraffin-embedded breast cancer tissue samples obtained during surgery between May and June 2013, which were previously stored at the Affiliated Cancer Hospital of Zhengzhou University, using a QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) (20), according to the manufacturer's instructions.

Primer design. The ODC sequence was obtained from GenBank (accession no. NC_009142.1) and polymerase chain reaction (PCR) primers were designed using the Premier Primer software (version 5.0; Premier Biosoft, Palo Alto, CA, USA). The forward (F) 1 and reverse (R) 1 primers were used to amplify a 547-bp region, while the F2 and R2 primers were used to amplify a 178-bp region within the first 547-bp amplification. The two PCR primer pairs are indicated in Table I.

Nested PCR-restriction fragment length polymorphism (RFLP). ODC G316A genotypes were generated using nested PCR-RFLP technology. DNA amplification was performed in a 25- μ l reaction volume consisting of 12.5 μ l 2X PCR buffer for KOD FX (Toyobo Co., Ltd., Osaka, Japan),

Table I. Oligonucleotide primers used to amplify the ornithine decarboxylase gene.

Primer	Sequence	Size of DNA product, bp
F1	5'-GGTGCTATAAGTAGGGAGCGGC-3'	547
R1	5'-CGAAGGGTTGGGAAAGAGGC-3'	547
F2	5'-CCTGGGGGCTCTGAGGT-3'	178
R2	5'-AGGAAGCGGCGCCTCAA-3'	178

F, forward; R, reverse.

5 μ l 2 mM deoxynucleotide triphosphate, 2 μ l of each primer, 0.1 μ l KOD, 2.4 μ l water and 1 μ l DNA. The standard conditions for PCR were as follows: 95°C for 2 min; followed by 40 cycles each at 95°C for 30 sec and 62°C for 1 min; and a final extension at 72°C for 5 min. All the reactions were performed in a Perkin Elmer 2400 thermocycler (Perkin Elmer, Foster City, CA, USA). The nested PCR products were analyzed by detecting the polymorphic *Pst*I site using capillary electrophoresis (QIAxcel Advanced System; Qiagen).

Experimental studies

Cell culture. MDA-435 cells and the MCF-7 human breast cancer cell line, which exhibited an ODC AG genotype, were maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) and all media used were supplemented with 10% fetal bovine serum. Furthermore, cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Western blot analysis. Cells were harvested and lysed, and proteins were separated on a 12.5% SDS-PAGE gel (15). Next, the proteins were transferred by electrophoresis onto a Hybond-C membrane (GE Healthcare Life Sciences, Little Chalfont, UK), which was blocked with Blotto A (5% blocking grade dry milk in Tris-buffered saline/Tween-20; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and probed using monoclonal mouse anti-human c-MYC (1:300; cat. no. sc-41; Santa Cruz Biotechnology, Inc.) and monoclonal mouse anti-human anti-MXI1 (1:300; cat. no. sc-130627; Santa Cruz Biotechnology, Inc.) primary antibodies in Blotto A. The primary antibodies were incubated at 4°C overnight, followed by incubation with a monoclonal goat anti-mouse Ig horseradish peroxidase-tagged secondary antibody (1:1,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Chemiluminescent detection was conducted using an electrochemiluminescence western blotting detection reagent (GE Healthcare Life Sciences) and exposing on a Biomax XAR film (Kodak, Rochester, NY, USA).

Chromatin immunoprecipitation (ChIP). ChIP assays were performed using a ChIP Assay Kit, according to the manufacturer's instructions (Cell Signaling Technology, Inc., Danvers, MA, USA). Briefly, the cells were treated with 1% formaldehyde to cross-link the DNA and proteins prior to disruption of the DNA-protein complexes by sonication [25% ultrasound; 14 cycles (4.5 sec each)] to produce fragment

Table II. Descriptive analysis for breast cancer cases overall and based on ODC genotype.

Category	Total breast cancer cases (n=300), n (%)	ODC G316A genotype		P-value
		GG (n=156), n (%)	GA/AA (n=144), n (%)	
Age, years ^a				0.280
≤45	125 (41.67)	68 (43.59)	57 (39.58)	
>45	175 (58.33)	88 (56.41)	87 (60.42)	
Stage at diagnosis				0.482
I	90 (30.00)	48 (30.77)	42 (29.17)	
II	108 (36.00)	56 (35.90)	52 (36.11)	
III	102 (34.00)	52 (33.33)	50 (34.72)	
Family history of cancer				0.948
Yes	106 (35.33)	54 (34.62)	52 (36.11)	
No	194 (64.67)	102 (65.38)	92 (63.89)	
Tumor grade				0.787
1	64 (21.33)	35 (22.44)	29 (20.14)	
2	191 (63.67)	97 (62.18)	94 (65.28)	
3	45 (15.00)	24 (15.38)	21 (14.58)	
ER and PR status				0.848
Positive	187 (62.33)	99 (63.46)	88 (61.11)	
Negative	113 (37.67)	57 (36.54)	56 (38.89)	
Her-2 status				0.675
Positive	128 (42.67)	65 (41.67)	63 (43.75)	
Negative	172 (57.33)	91 (58.33)	81 (56.25)	
Ki-67 status				0.715
Positive	167 (55.67)	85 (54.49)	82 (56.94)	
Negative	133 (44.33)	71 (45.51)	62 (43.05)	
Surgical treatment				0.669
Yes	300 (100.00)	156 (100.00)	144 (100.00)	
No	0 (0.00)	0 (0.00)	0 (0.00)	
Radiation therapy				0.963
Yes	165 (55.00)	86 (55.12)	79 (54.86)	
No	135 (45.00)	70 (44.87)	65 (45.14)	
Chemotherapy				0.981
Yes	246 (82.00)	128 (82.05)	118 (81.94)	
No	54 (18.00)	28 (17.95)	26 (18.06)	

^aMedian age (range) in years of the total cohort, ODC GG and ODC GA/AA patients was 48.6 (30-72), 48.8 (30-72) and 48.4 (30-68), respectively. ODC, ornithine decarboxylase; ER, estrogen receptor; PR, progesterone receptor; Her-2, human epidermal growth factor receptor 2.

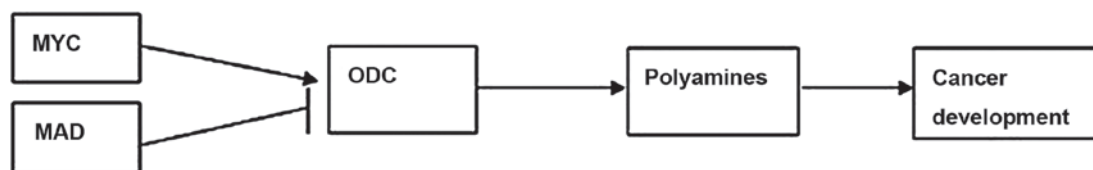


Figure 1. Schematic diagram depicting the differential effects of polyamine regulation by MAD and MYC on ODC. ODC, ornithine decarboxylase.

lengths of 200-1,000 bp. The lysates were then diluted 10 times with immunoprecipitation dilution buffer containing 1 mmol/l phenylmethanesulfonyl fluoride. Antibodies for c-MYC and MXI1 (Santa Cruz Biotechnology, Inc.) were

used to treat the samples, in order to induce chromatin precipitation. However, one sample was left untreated as a minus-antibody control. Immunoprecipitation was performed overnight at 4°C with centrifugation at 234.8 x g for 15 min.

Table III. Breast cancer-specific survival analysis of patients with breast cancer based on the ODC G316A genotype.

ODC G316A genotype	Breast cancer-specific mortality		
	Events, n	At risk, n	HR (95% CI) ^a
GG	32	156	1 (reference)
GA/AA	40	144	1.57 (1.16-3.34)

^aP=0.037. ODC, ornithine decarboxylase; HR, hazard ratio; CI, confidence interval.

Subsequently, immune complexes were obtained by adding 60 μ l salmon sperm DNA/protein A agarose slurry (Merck Millipore, Darmstadt, Germany) and incubating for 1 h at 4°C with rotation, followed by gentle centrifugation (335.4 x g for 1 min). Protein A agarose pellets were washed with low-salt, high-salt, LiCl and Tris-EDTA buffer. The complexes were then eluted twice by adding 250 μ l elution buffer (0.1 mol/l NaHCO₃ and 1% SDS), and DNA-protein cross-links were reversed using 0.2 mol/l NaCl and heating at 65°C for 4 h for all the samples, including the input DNA and minus-antibody DNA controls. Finally, DNA was re-suspended in 30 μ l double-distilled H₂O.

Statistical analysis. All the statistical data were analyzed using SPSS statistical software (version 17.0; SPSS Inc., Chicago, IL, USA). A χ^2 test was used to describe the breast cancer cases overall and in regard to their ODC genotype. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. Furthermore, Cox's proportional hazards model was used to identify prognostic factors for survival. For all data, P<0.05 was considered to indicate a statistically significant difference.

Results

Epidemiological study

Clinicopathological characteristics of breast cancer based on ODC G316A SNP genotypes. In order to enhance sensitivity, nested PCR was used to amplify the products. The first set of primers were designed to amplify a 547-bp region within the ODC gene, while the second primer pair was designed to amplify a 178-bp region within the 547-bp region, and these were used in the nested PCR assay. Capillary electrophoresis technology was subsequently employed to determine the sequence of the PCR products. For the 178-bp region of the ODC gene, *Pst*I enzyme digestion and PCR-RFLP were used to genotype the ODC G316A SNP. The results of capillary electrophoresis were termed C1-C300. For the ODC AG genotype, the 178, 50 and 120-bp regions were obtained. However, only a single region (178-bp) was identified for ODC GG, whereas the 50 and 20-bp regions were detected for ODC AA. A total of 300 stage I-III breast cancer cases admitted at the Affiliated Cancer Hospital of Zhengzhou University were used in the present case-only analysis. Nested PCR-RFLP was used to genotype the patients, and the median follow-up duration was 10 years and 3 months. The cohort included 156 (52%) ODC GG and 144 (48%) ODC GA/AA cases. Clinicopathological data for

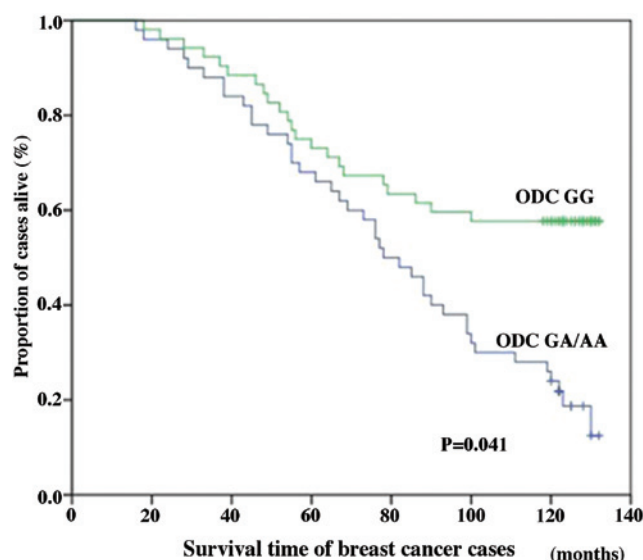


Figure 2. Kaplan-Meier demonstrating the breast cancer-specific survival rate estimates for cases with stage III breast cancer, stratified by ODC G316A genotype. The study included 300 cases of breast cancer, which were enrolled between 2002 and 2003, with follow-up visits conducted until May 2013. The number of breast cancer-specific mortalities in patients carrying the ODC GG and GA/AA phenotypes were 32 and 40, respectively. ODC, ornithine decarboxylase.

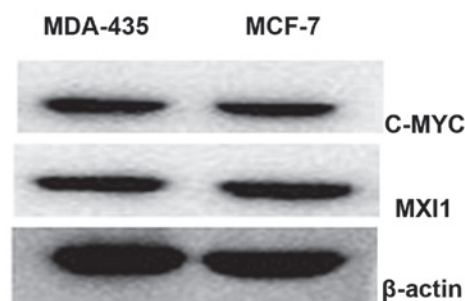


Figure 3. E-box protein expression levels in breast cancer-derived cells. The expression of protein binding at the +316 bp ODC single nucleotide polymorphism was assessed by western blot analysis. Extracts of MDA-435 and MCF-7 cells were evaluated for c-MYC and MXI1 expression, and β -actin was used as the loading control. MXI1, MAX interactor 1.

the breast cancer cases based on ODC genotype are indicated in Table II.

Cancer-specific survival time based on ODC G316A genotypes. Of the 300 stage I-III breast cancer cases, 168 patients (56%) had succumbed to the disease prior to the analysis. In total, 72 mortalities (42.86%) occurred in

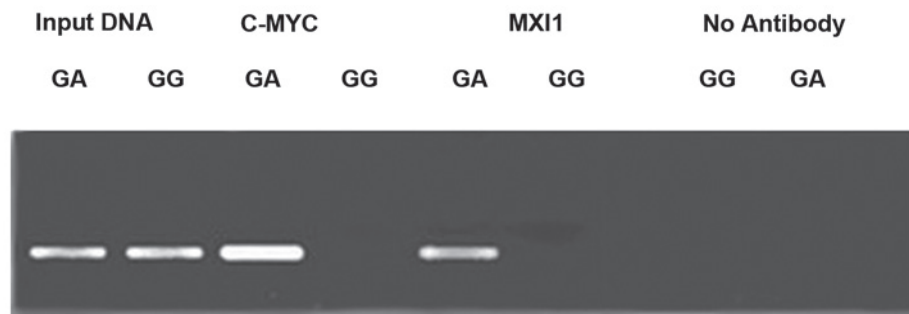


Figure 4. Documentation of allele-specific transcription factor binding by chromatin immunoprecipitation analysis, which was conducted as described in Materials and Methods. MCF-7 cells were a source of ornithine decarboxylase (ODC). A-alleles, as these cells are heterozygous GA at this site. MDA-435 cells were used as a source of ODC G-alleles. MXI1, MAX interactor 1.

patients carrying the ODC GG genotype, compared with 96 mortalities (57.14%) in patients with the GA/AA genotypes. A statistically significant improvement in breast cancer-specific survival was observed among breast cancer cases homozygous for the ODC G-allele (10-year survival, 53.85%) compared with cases exhibiting at least one A-allele (ODC GA/AA; 10-year survival, 33.33%; $P < 0.001$). Furthermore, breast cancer-specific survival analysis by stage revealed no statistically significant differences in the survival of patients with AJCC stages I ($P = 0.537$) or II ($P = 0.482$; data not shown). However, among cases of stage III breast cancer, the ODC GG genotype was significantly associated with improved 10-year breast cancer-specific survival (38.46%) compared with the ODC GA/AA genotype cases (20.00%; $P = 0.041$; Fig. 2). Among all the cases (stages I-III), differences in the genotype-specific breast cancer survival were statistically significant, with the ODC G316A SNP acting as an independent predictor of breast cancer-specific survival. Compared with the ODC GG genotype cases [hazard ratio (HR), 1; 95% confidence interval (CI), 1, reference], the breast cancer-specific risk of mortality was significantly greater for the ODC GA/AA genotype (HR, 1.57; 95% CI, 1.16-4.23; $P = 0.037$; Table III).

Experimental studies

Western blot analysis. The expression of specific E-box binding proteins, including the transcriptional activator c-MYC and the transcriptional repressor MXI1, was established by western blotting of MCF-7 cell proteins (Fig. 3). c-MYC and MXI1 were expressed in the MDA-435 and MCF-7 cells (Fig. 3).

ChIP. To investigate the affinity of the c-MYC and MXI1 proteins for the ODC G316A allele, ChIP analysis of the +316 bp region of the ODC promoter was conducted using antibodies against the aforementioned E-box binding proteins (Fig. 4). The results revealed that protein bands for the c-MYC and MXI1 proteins were present in the MCF-7 cell lines, but not in the MDA-435 cell lines. These results indicate that c-MYC and MXI1 proteins may selectively bind to ODC G316A allele A, rather than the G.

Discussion

Accumulating evidence has indicated that polyamine catabolism is involved in the response to therapeutic agents, apoptosis and the stress response, and is important in the

development of psoriasis, parasitic infection and cancer (21-23). In recent years, a number of anticancer compounds relevant to polyamine biosynthetic single enzyme inhibitors have been developed, including the α -difluoromethylornithine and methylglyoxal bis-guanyldrazone. However, the application of these compounds in a clinical setting is limited due to their poorly-tolerated adverse reactions (24,25). Ornithine decarboxylase (ODC) is the most significant and rate-limiting enzyme in the pathway of polyamine synthesis, which is critical in cell proliferation and highly expressed in a variety of cancer types, including breast cancer (6,26-28). Previous studies have indicated that the overexpression of ODC is common in carcinogenesis and cancer progression (6,26-28). Therefore, certain studies have proposed that it may be an important biological marker in the evaluation of biological behavior and the prognosis of various types of cancer (13,26). Recently, an increasing number of studies have focused on ODC as a potential target for cancer therapy (24,25). In particular, ODC has been proposed to be a promising candidate target for natural products in cancer chemoprevention (29). Thus, future investigation of ODC inhibitors present in nature may facilitate the identification of novel cancer chemopreventive agents. In 2000, Guo *et al* (14) identified a single nucleotide polymorphism (SNP) in the gene regulatory region of ODC, known as ODC G316A. This SNP is located between two significant gene regulatory regions, namely the MYC/MAX/MAD protein binding region, CACGTG E-box, which is known to regulate ODC transcription. However, only a limited number of studies have focused on the association between the ODC G316A genotype and breast cancer survival, as well as the underlying mechanism. In 2003, Martinez *et al* (30) reported that the ODC G316A polymorphism was able to independently reduce the risk of adenoma recurrence by inhibiting synthesis and activating catabolism of the tumor cells. Furthermore, in colorectal cancer, the ODC polymorphism appeared to act as a genetic marker for predicting colon cancer recurrence. Furthermore, the ODC G316A genotype was found to be a prognostic factor in colorectal adenoma recurrence and survival (15,16).

However, thus far, no evidence exists to indicate that this SNP exhibits the same function in breast cancer as in colorectal cancer. In 2009, Brown *et al* (31) reported that the ODC G316A polymorphism (SNP no. rs2302615) may be less important in individuals with an inherited predisposition

for breast cancer than in individuals who develop sporadic breast cancer (32). However, our study involved the ODC G316A polymorphism (SNP no. rs1045900). Therefore, the present study used nested-PCR-RFLP to genotype the ODC G316A SNP in breast cancer. Of the 300 cases of breast cancer investigated, 156 were ODC GG cases and 144 were ODC GA/AA cases. Additional analysis demonstrated that patients with the GG phenotype exhibited significantly higher 10-year survival rates compared with those with the GA/AA phenotype. Furthermore, according to the results of the present study, patients with an A allele (ODC GA/AA patients) exhibited significantly lower 10-year survival rates compared with patients presenting the ODC GG phenotype, indicating that the ODC G316A SNP may be a useful marker for predicting the survival of patients with breast cancer. The results of the present study were in accordance with the report by Hubner *et al* (15); however, the present study was conducted in a Chinese population and used the simpler and more economical technology of nested-PCR-RFLP to genotype breast cancer. Overall, the current results indicated that the ODC G316A genotype is associated with breast cancer survival.

The ODC A allele has been reported to be associated with poor survival in colorectal cancer (16). Martinez *et al* (30) reported that MAD1 (as well as MXI1) selectively suppressed the activity of the ODC promoter containing the A allele, as opposed to the G allele, in the HT29 human colon cancer-derived cell line. Furthermore, Hubner *et al* (15) achieved the same results using ChIP in different human colon cancer-derived cell lines (HCT116 and HT29). However, due to insufficient evidence, it was unclear whether the same outcome would occur in human breast cancer-derived cell lines. A previous study of the current group identified that c-MYC and MXI1 protein expression levels are associated with the prognosis of breast cancer patients (32). Furthermore, the present study employed western blotting and ChIP to examine the association between the two E-box proteins and the ODC SNP. In cultured cells, the E-box activator c-MYC and repressor MXI1 were found to preferentially bind to ODC minor A alleles, rather than major G alleles. Consistent with the results of the present study, Zell *et al* (16) reported that c-MYC, MAD1 and MAD4 preferred binding to ODC minor A-alleles, rather than major G-alleles, *in vitro*.

In conclusion, the present study genotyped ODC G316A in patients with breast cancer and investigated the clinical outcome of the ODC G316A SNP on breast cancer-specific mortality. The current results indicated that MXI1 preferentially binds to the ODC A-allele, thus, contributing to breast cancer progression. Furthermore, the findings of the present study indicated that the G316A SNP may be used to assess the risk of progression in patients with breast cancer.

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