Original Article
Androgen receptor expands the population of cancer stem cells in upper urinary tract urothelial cell carcinoma cells

Chi-Cheng Chen1,2, Teng-Fu Hsieh1,2, Chi-Ping Huang1, Al-Lin Yu1, Wen-Lin Chang1, Chih-Rong Shyr1

1Sex Hormone Research Center, Departments of Urology/Surgery and Medical Laboratory Science and Biotechnology, Graduate Institute of Clinical Medical Science, China Medical University/Hospital, Taichung 404, Taiwan; 2Department of Urology, Taichung Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taichung 404, Taiwan

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Abstract: Androgen receptor (AR) affects the development and progression of upper urinary tract urothelial cell carcinoma (UUTUC). However, the regulatory mechanism exerted by AR to affect UUTUC cells remains unclear. Here we investigated whether AR promotes UUTUC development and progression, possibly by expanding the population of cancer stem cells (CSCs), which are a particular population of cells within cancer cells responsible for tumor initiation, drug resistance and metastasis. We compared UUTUC cells with or without the addition of AR on their CSC population with flow cytometry, colony formation and sphere formation assay to determine the effect of AR on CSC activity, and real-time PCR was used to detect the expression stemness genes and miRNAs. In vivo tumor formation was evaluated with the implantation of cancer cells in nude mice. We found that the addition of AR in UUTUC cells, significantly increased the population of CSC, clonogenicity, sphere formation and the expression of stemness genes (Oct4, Bmi1 and Nanog), altered CSC-related miRNA profile, as well as promoted epithelial mesenchymal transition (EMT). And AR inhibitor, enzalutamide was shown to suppress AR’s effect on tumorsphere formation. Furthermore, in an immune-deficient mouse model, the addition of AR in UUTUC cells also increased the tumor formation capacity. This study will help us better understand the extent to which AR contributes to UUTUC progression by expanding their CSC population and capacity. Our findings could explain high incidence of UUTUC observed in males. And targeting AR may lead to novel therapeutic approaches for genetically diversified urothelial carcinomas in precision medicine era.

Keywords: Androgen receptor, upper urinary tract urothelial cell carcinoma, cancer stem cells, tumorsphere

Introduction

UUTUC originating in the urothelium, is an aggressive and lethal urologic malignancy since patients with primary stage T4 tumors have 5-year disease-specific survival rate less than 10% and a median survival of 6 months only [1-3]. UUTUC patients initially respond to surgery and chemotherapy, but the long-term prognosis is generally unfavorable with recurrence and metastasis [4, 5]. Therefore, it is critical to identify new therapeutic targets that prevent recurrence and prolong survival for UUTUC patients.

CSCs are a small population of cells in heterogeneous tumor tissues, which could self-renew and differentiate [6]. CSCs could initiate tumors and are more resistant to chemotherapy and radiotherapy and linked to metastasis and poor prognosis [7]. CSCs have been reported to be present in multiple types of solid tumor and cultured cancer cell lines, including brain, breast, colon, prostate and urothelial tumors [6, 8-11]. The existence of CSCs in urothelial tumors is further substantiated by the presence of urothelial stem cells in the basal cell layers of urothelium, which are responsible for regenerating the damaged urothelium caused by physical, chemical or biological stresses [12]. Urothelial CSCs are similar to normal urothelial stem cells with the ability to self-renew and generate all of the heterogeneous cells that comprise a tumor [11]. The expression of CSC makers have been linked to the recurrence, invasive progression and worse prognosis of urothelial carcinomas [13-17], suggesting CSCs are involved in the progression and recurrence of UUTUC. The
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understanding of how CSCs arise is critical to develop a novel therapeutic approach to block their presence and provide long-term disease-free survival to afflicted patients.

UUTUC afflicts more men than woman with a male-to-female ratio of 2~3 to 1 [1, 18, 19], and females were associated with a better survival [4], indicating that male hormones, androgens may play an important role in the progression of UUTUC. Our previous studies have demonstrated the androgen receptor (AR), which mediates androgen actions, affects the UUTUC cell migration and invasion as well as the response to anti-cancer drugs [20, 21]. Other study also indicates that the actions of AR could be linked to the regulation of CSCs since AR was shown to occupy the CD44 loci [22], which is a urothelial CSC maker [11]. Therefore, the objectives of our study were to examine whether CSC population and capacities in UUTUC cells could be altered by AR.

In this study, we firstly aimed to determine whether AR could affect the CSC population and functions by evaluating CD44 population, sphere and colony formation as well as stemness gene expression under the influence of AR. We also examined the expression of miRNAs and EMT makers affected by AR. In this study we identify that AR is an important factor to affect the development and progression of UUTUC.

Materials and methods

Cell culture

The human UUTUC cell line, BFTC 909, (from a UUTUC of renal pelvis patient) was a generous gift from Dr. Tzeng CC [23] and 7630 cells (also established from a UUTUC of renal pelvis patient) [24] were cultured in Dulbecco’s modified Eagle’s medium, containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. The generation of BFTC pWPI and BFTC hAR cells from parental BFTC 909 cells or the 7630 pWPI and 7630 hAR cells from parental 7630 cells was described previously [20, 21].

Flow cytometry of CD44+ cells

Cell suspensions obtained from BFTC pWPI and BFTC hAR cells were tested for fluorescent labeled monoclonal antibodies for CD44 antigens and respective IgG isotype controls. The antibodies were incubated for 30 min at 4°C. After washing, the labeled cells were analyzed by flow cytometry using a BD LSR II flow cytometer (Becton & Dickinson, Mountain View, CA, USA).

Quantitative reverse transcriptase-polymerase chain reaction (Q-RT-PCR)

Total RNA was extracted from cells using TRizol (Invitrogen, Carlsbad, CA, USA) and used for first-strand cDNA synthesis. The mRNA levels were measured using CFX96™ real-time system (Bio-Rad Laboratories) using KAPA SYBR® fast qPCR kits (Kapa Biosystems, Inc., Woburn, MA, USA). The mRNA and miRNA expression level were determined using the 2^(-ΔΔCt) method.

Colony formation assay

The cells were plated at a density of 1 × 10³ cells on 6 cm plate. After a 10-day incubation at 37°C in a humidified atmosphere containing 5% CO₂, cell monolayers was washed with saline, fixed with 100% methanol, and stained with 0.5% crystal violet solution. Colonies, defined as being more than 50 cells, were counted with the use of a light microscope.

Sphere formation assay

The cells were plated as single cells in ultra-low attachment six-well plates at a density of 5,000 viable cells/well. Cells were grown in a serum-free medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA), 20 ng/ml human recombinant epidermal growth factor (hrEGF), 10 ng/ml human recombinant basic fibroblast growth factor (hrbFGF), 5 μg/mL insulin and 0.4% BSA (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator (5% CO₂) at 37°C. For secondary tumorsphere culture, the primary tumorspheres were dissociated into single cells filtered through a 40 μm cell strainer to ensure the single cells suspension. 2,500 cells were seeded into each well of six-well plate. Tumorsphere was observed under microscope 10-15 days later.

The subcutaneous xenografts in nude mice

BALB/c nude mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl) purchased National Laboratory Animal Center (Taipei, Taiwan) were injected with 1 ×
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10⁶ and 5 × 10⁶ BFTC pWPI or BFTC hAR cells on both the right and left flanks were injected with BFTC pWPI or BFTC hAR cells respectively, for a total of 12 injection sites per condition. Mice were monitored for tumor formation up to 3 months post-injection, and each injection site was scored as either positive (palpable tumor) or negative (no palpable tumor). All animal procedures followed the Guide for the Care and Use of Laboratory Animal of the Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, U.S.A and were approved by the animal care and use committee of the China Medical University, Taiwan.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on paraffin embedded sections (5 μm thick). Paraffin sections were dewaxed and rehydrated through a graded series of ethanol washes, and maintained in phosphate buffered saline (pH 7.4; PBS). Sections were treated with primary anti OCT4 or anti-MMP9 antibodies, followed by biotin conjugated secondary antibody. Vectorstain ABC kit from Vector Laboratories (Burlingame, CA, USA) was used for immunohistochemistry, followed by DAB to visualize immunocomplexes. For quantification of immunostaining, the number of positively stained cells was counted in 4 random fields at a magnification of 40 ×.

Statistical analysis

For in vitro cell based experiments, data are expressed as mean ± SD from at least three independent experiments. Results were analyzed using two-tailed paired t-tests. For animal studies, tumor-free survival in BFTC pWPI and BFTC hAR cells implanted mice was determined via Kaplan-Meier analysis with comparison of curves using the survival log-rank test. A P-value < 0.05 was considered to be statistically significant.

Results

AR enriches the CD44 population and increases stemness gene expression of UUTUC cells

Since AR has been demonstrated to affect the migration, invasion and chemoresistance of UUTUC cells [20, 21, 25], we suspected that these actions could be linked to CSCs because CSCs are involved in cancer progression and chemoresistance. First we examined the proportion of CD44 population in UUTUC cells, BFTC 909 cells with or without the addition of AR. CD44+ cells are found in the basal layer of normal urothelium as well as urothelial carcinoma and it has been used as a CSC marker [8, 11]. Flow cytometry was performed to determine the CD44+ population of BFTC pWPI and BFTC hAR cells. The BFTC hAR cells contained higher cell fraction of the CD44+ cells than BFTC pWPI cells (Figure 1A), suggesting that AR in BFTC 909 cells is able to expand CSC population. To further confirm that AR is able to increase the CSC population in UUTUC cells, we examined the expression of several CSC stemness maker genes (Oct4, Bmi1 and Nanog), which were shown to be expressed in urothelial carcinomas [11]. The quantitative RT-PCR (Q-RT-PCR) analysis showed that the BFTC hAR cells expressed higher mRNA levels of Oct4, Bmi1 and Nanog (Figure 1B). This result showed that the stemness genes in UUTUC cells could also be enhanced by the addition of AR, which is consistent with the flow cytometry data, indicating that higher expression of CSC makers in BFTC hAR cells.
Figure 2. The clonogenicity and tumorsphere formation were increased in BFTC hAR cells. A. The colony formation of BFTC pWPI cells or BFTC hAR cells was photographed after being cultured for 10-14 days. BFTC pWPI cells or BFTC
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hAR cells were seeded on 6 cm tissue culture dishes and incubated for 10-14 days. At the end of incubation period, numbers of colonies were counted. The relative colony number was calculated by setting the control (BFTC pWPI cells) as one-fold. B. The tumor sphere formation analysis of BFTC pWPI or BFTC hAR cells. The BFTC pWPI or BFTC hAR cells were grown in six-well ultralow attachment plates at a density of 5,000 cells/well in defined medium at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ for 10-15 days. At the end of incubation period, spheres were counted. The relative sphere number of primary spheres and secondary spheres was calculated by setting the control (BFTC pWPI cells) as one-fold. C. The size of spheres was also measured. The relative sphere size was calculated by setting the control (BFTC pWPI cells) as one-fold. Scale bar, 20 μm. D. The tumor sphere formation analysis of 7630 pWPI or 7630 hAR cells. E. Effect of enzalutamide (MDV3100) on tumorsphere formation of BFTC hAR and 7630 hAR cells. BFTC hAR or 7630 hAR cells were treated with 10 μM enzalutamide in media and assayed for tumorsphere formation and the relative sphere number was calculated after 10-15 day incubation. F. The relative size of tumorspheres from BFTC hAR or 7630 hAR cells with or without enzalutamide treatment were compared. Data are expressed as mean ± SD from three independent experiments (n = 3) (**P<0.01 vs control).

Figure 3. The CSC-related miRNA expression profile and EMT marker expression in BFTC pWPI and BFTC hAR. A. CSC-related miRNAs in BFTC pWPI and BFTC hAR were determined using Q-RT-PCR. Data are expressed as mean ± SD from three independent experiments (n = 3) (*P<0.05, **P<0.01. vs control). B. The expression of E-cadherin and Vimentin in BFTC pWPI and BFTC 909 hAR cells as well as 7630 pWPI or 7630 hAR cells. The cells were lysed. Equal amounts of protein were analyzed by SDS-PAGE and immunoblotted with anti-E-cadherin, anti-Vimentin, and anti-β-actin antibodies. The representative blot from three independent experiments is shown.

AR affects the colony and tumorsphere formation of UUTUC cells

To determine whether the clonogenicity of UUTUC cells are affected by AR, colony formation assay was performed to examine cell ability to repopulate themselves. The result showed that by the BFTC hAR cells increased their capacity to form colony with higher number of colonies when compared to BFTC pWPI cells (Figure 2A). Another characteristic of stem cells is the ability to form spheres. We therefore, further examined the function of AR in promoting the capacity of CSCs to survive and proliferate in anchorage-independent conditions and ability to form tumorspheres. Our results demonstrated that BFTC hAR cells had higher tumorsphere forming capacity both for primary and secondary tumor spheres (Figure 2B) and produced larger size of tumorsphere (Figure 2C) than BFTC pWPI cells. Similarly, when we used another UUTUC cells generated from a UUTUC of pelvis patient, 7630 cells, the addition of AR also increased the ability of 7630 cells to form primary and secondary tumorspheres (Figure 2D). Furthermore, since the presence of AR affects the colony and tumorsphere formation in UUTUC cells, the inhibition of AR could suppress the colony and tumorsphere formation. To test this notion, we treated BFTC hAR and 7630 hAR cells with a potent AR inhibitor, enzalutamide (MDV3100) which binds to the androgen receptor with greater relative affinity than the clinically used antiandrogen bicalutamide and reduce the efficiency of its nuclear translocation as well as impair both DNA binding to
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androgen response elements and recruitment of coactivators [26]. And we found that both the number and size of tumorspheres were decreased by enzalutamide treatment (Figure 2E, 2F), indicating that enzalutamide antagonized the effects of AR in increasing tumorsphere formation of UUTUC cells. Therefore, we demonstrated that AR in UUTUC cells could increase cellular capacity of CSC in UUTUCs.

Differential miRNA expression and EMT transition in BFTC hAR and BFTC pWPI cells

To examine the potential mechanism by which AR expand CSC population and promote their capacities of clonogenicity and tumorsphere formation, we looked at whether AR could affect the expression of micro RNAs (miRNA), which are 21-25 nucleotides long, non-coding RNAs that post-transcriptionally regulate gene expression [27]. The functions of miRNAs are not only involved in the initiation and progression of cancer, but also essential in regulating self-renewal and differentiation of CSCs [28, 29]. Therefore, we used Q-RT-PCR to compare the expression several CSC-related miRNAs in BFTC pWPI and BFTC hAR cells. As the result shown, the BFTC hAR cells had higher expression of miR-27a and miR-125b, but lower expression of miR-145, miR-200b and miR200c (Figure 3A). There was no significant difference on miR-21 expression between BFTC pWPI and BFTC hAR cells. Up-regulation of microRNA-125b has been also shown to expand the population of CSCs and increases chemoresistance [30]. miR-27a function as an antiapoptotic and proliferation-promoting factor in liver cancer cells [31]. miR-145 was shown to suppress tumor sphere formation and expression of CSC surface markers and stemness genes in prostate cancer cells [32]. In the other hand, miR-200 family were significantly downregulated in both breast CSCs and normal mammary stem and/or progenitor and furthermore, the overexpression of miR-200c reduced the clonogenic and tumor-initiation activities breast CSCs [28]. Therefore, from the miRNA expression patterns between BFTC pWPI and BFTC hAR cells, the result demonstrated that AR upregulates miRNAs that promote CSC population and downregulates miRNAs that suppress CSC population in UUTUC cells. Besides investigating the expression of miRNA, we also examined whether the addition of AR in BFTC cells would promote epithelial-mesenchymal transition (EMT), another characteristic of cancer stem cells [33]. We determined the expression of the epithelial cell marker, E-cadherin and mesenchymal cell marker, vimentin [34] in BFTC pWPI and BFTC hAR cells as well as in 7630 pWPI and 7630 hAR cells. And the result showed that lower E-cadherin, but higher vimentin expression were observed in BFTC hAR cells comparing to those expressions in BFTC pWPI cells and this finding was also demonstrated in 7630 cells when 7630 hAR cells had higher vimentin expression, but lower E-cadherin expression that 7630 pWPI cells (Figure 3B), further supporting the role of AR in promoting the expansion of CSC population in UUTUC cells.
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To examine the effect of AR on CSC population in vivo, we compared the tumorigenic potential of BFTC pWPI and BFTC hAR cells since inside cancer cells, there are a heterogeneous collection of cell types including differentiated cancer cells and CSCs that are tumorigenic, resulting in higher tumor take rates [9]. Nude mice were injected subcutaneously with either 1×10^6 or 5×10^6 of BFTC pWPI and BFTC hAR cells in left and right flanks, respectively and tumor formation were observed. Tumors were detected when both 1×10^6 and 5×10^6 cells had been injected. Both number of cells injected, BFTC hAR cells were significantly more tumorigenic than BFTC pWPI cells at 5×10^6 injected cells (Figure 4B). However, at 1×10^6 injected cells, the significant difference was not reached between the two groups, but BFTC hAR cells still showed higher tumorigenic potential (Figure 4A). The result demonstrated that BFTC hAR cells were more tumorigenic in nude mice than BFTC pWPI cells, indicating that there are more tumorigenic CSCs in BFTC hAR cells. This finding that the addition of AR in BFTC 909 cells, compared to vector controls, resulted in higher tumor incidence in low cell number or high cell number implantation, correlates well with in vitro results. To further confirm the above in vitro cell line data in the in vivo mouse model, we examined the expression of OCT4 and MMP9 in BFTC pWPI and BFTC hAR cell xenograft tumors in nude mice. IHC staining of OCT4 expressing (brown-staining) cells in BFTC pWPI or BFTC hAR cell xenograft tumor in nude mice with the activity to proteolize the extracellular matrix components for cell mobilization is a key molecular for cancer stem cells in the process of cancer cell metastasis key molecules involved in the process of cancer cell metastasis [35, 36]. The IHC staining data indicated that BFTC hAR tumors had more OCT4 (Figure 5A, 5B) and MMP9 (Figure 5C, 5D) expressing cells than BFTC pWPI tumors did. These in vivo results further imply that AR may play an important role in expanding the CSC population.

Discussion

The current CSC model states that CSCs in cancer cells are responsible for tumor initiation, recurrence, metastasis and chemoresistance [6]. However, the regulation on how CSCs arise remains unclear. In this study, we found that the CD44+ population, stemness gene expression, and the capacity to repopulate of BFTC hAR cells are all higher than to BFTC pWPI cells, indicating that the CSC properties in UUTUC cells are enhanced by AR. These findings demonstrate the role of AR in UUTUC cells as a key regulator to increase CSC population, suggesting a potential therapeutic target to contain the population of CSCs by targeting AR actions. In addition to our finding that AR regulates CSC
population in UUTUC cell, the study on genome-wide distribution of the AR binding sites using chromatin immunoprecipitation (ChIP) studies coupled with genomic microarray analysis (ChIP-chip) identify that AR occupancy of the CD44, CD49f/integrin-α6, CD133 and PTEN loci, which have been shown to be involved in regulating the rise of CSC population [22].

Furthermore, we also identified several stemness genes (Oct4, Bim 1 and Nanog) were expressed higher in BFTC hAR cells (Figure 1B). The transcription factors Oct4 and Nanog form a core regulatory network that coordinate the capacity to self-renew and the multipotential differentiation of embryonic stem cells and cancer cells [37]. Bmi1, a polycomb group repressor, plays a key regulatory role in the self-renewal of maintenance of both normal and cancer stem cells [38]. We have also demonstrated that the addition of AR increase the self-renewal capacity of UUTUC cells as measured by colony and sphere formation (Figure 2) as well as tumor formation in nude mice (Figure 4). These data showed that AR in UUTUC cells can enhance CSC characteristics in gene expression and function, suggesting AR could be a regulator on the expansion of CSC-like cells through a set of transcription factors involved in maintaining the pluripotency of stem cells.

Beside changes in the expression of the stemness genes by AR, we also found that the profile of microRNAs was also altered by AR (Figure 3A). The ability of miRNAs to regulate expression of hundreds of target mRNAs gives miRNAs the control over a variety of cell functions including cell proliferation, stem cell maintenance, and differentiation in normal tissues and cancers [39]. In this study, we have found that there are two cluster of miRNA affected by AR in our miRNA analysis; one (miR-145, miR-200b and miR-200c) was downregulated, but the other (miR-27a and miR-125b) is upregulated by AR. In the downregulated group, miR-145 has been demonstrated to suppress tumor sphere formation and expression of CSC markers and ‘stemness’ factors including CD133, CD44, Oct4, c-Myc and Klf4 in PC-3 cells [32]. miR-200b expression in breast cancer cells was shown to block the formation and maintenance of mammospheres [40]. miR-200c decreased the expression of BMI1, and inhibited the clonal expansion of breast cancer cells [28]. In the upregulated group, miR-125b was shown to be required for Snail-induced breast cancer stem cell enrichment [30]. miR-27a was reported to promote cell proliferation and suppress apoptosis in liver cancer cells [31]. Since the population of CSCs could be regulated by miRNA networks, and AR was found in this study to control the profile of miRNA, how AR modulates CSCs through the regulation of miRNA networks warrants further investigation.

The acquisition of EMT characteristics in BFTC hAR and 7630 hAR cells (Figure 3B) and increased expression of OCT4 and MMP-9 (Figure 5) in BFTC hAR cell xenograft tumors imply that the roles of AR in UUTUC cells grant the cells with higher ability to migrate and invade since EMT is a key step for cancer cell migration, invasion, and metastasis [33] and MMP-9 also play an important role in facilitating cancer cell migration and invasion [41]. Indeed, our previous findings have demonstrated that AR promotes the migration and invasion of UUTUC cell through the up-regulation of MMP9 [20], which could be explained that the addition of AR in UUTUC cells could promote EMT in UUTUC, which is also a characteristic of cancer stem cells [33, 42]. Furthermore, CSCs in bladder cancer have been linked to chemoresistance due to the CSC repopulation [43]. In our previous study, we demonstrated that the addition of AR in UUTUC cells increased chemoresistance of UUTUC cells to anti-cancer drugs [21]. The similar finding was reported that in AR expressing bladder cancer cells, androgen/AR signaling promoted cell growth and resistance to doxorubicin [44]. These results corroborate the findings in this study to claim the role of AR in modulating CSC population in UUTUCs.

Current anticancer therapies, such as chemotherapy, radiotherapy, or target therapy, fail to suppress CSCs and develop resistant clones [45]. The therapy to prevent the expansion of the CSC population could improve the outcome of cancer patients. Our findings that AR in UUTUC cells promotes the expansion of CSC population and increases clonogenicity and sphere formation through modulating the stemness genes and miRNAs provide a novel and potential approach to treat urothelial carcinomas through the blockade of AR signaling with antiandrogens such as enzalutamide used in our study. This could be a breakthrough in cancer treatment by adding the hormonal therapy in current treatment regime to provide a syner-
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gistic combination therapy for genetically diversified urothelial carcinomas [46] in precision medicine era.

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Disclosure of conflict of interest

None.

Address correspondence to: Chih-Rong Shyr, Sex Hormone Research Center, China Medical University Hospital, Cancer Center Building, 6 Hsiuh-Shih Rd, Taichung, 40454, Taiwan. Tel: 886-4-22052121 Ext. 7514; Fax: 886-4-22057414; E-mail: t20608@mail.cmuh.org.tw

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