Abstract. Cysteine-rich protein 61 (CCN1/CYR61) is an important marker of proliferation and metastasis in malignant melanoma, making it a potential target for melanoma treatment. In this study, we compared the expression of CYR61 in Chinese patients with malignant melanoma with its expression in patients with other skin tumors or with no skin pathological conditions. We examined the effects of anti-human CYR61 monoclonal antibody on proliferation and evaluated the changes in CYR61 expression and cell proliferation in response to treatment with either epirubicin or interferon (IFN)-α. CYR61 was expressed at lower levels in patients with malignant melanoma than in patients with other skin tumors or with no pathology. Following the treatment of B16 cells with epirubicin and IFN-α, CYR61 levels increased, cell growth was inhibited, and proliferating cell nuclear antigen expression decreased. Thus, CYR61 could become a therapeutic target for malignant melanoma patients with high CYR61 expression.

Introduction

Cysteine-rich protein 61 (CYR61) encodes a protein with 10% cysteine residues and was first described in 1985, as an immediate early gene, which can be induced by serum or platelet-derived growth factor (PDGF) (1). CYR61 is a secreted, extracellular matrix (ECM)-associated signaling molecule that belongs to the CCN-gene family.

The CCN family includes six distinct genes: CYR61/CCN1, connective tissue growth factor (CTGF)/CCN2, nephroblastoma overexpressed gene (NOV)/CCN3, and Wnt-induced secreted proteins WISP-1/CCN4, WISP-2/CCN5, and WISP-3/CCN6 (2). These are matri-cellular regulatory factors involved in internal and external cell signaling. They play a role in angiogenesis, chondrogenesis, and osteogenesis, by stimulating mitosis, adhesion, apoptosis, ECM production, and growth arrest and migration of multiple cell types (3).

Human CYR61 maps to p22.3 on chromosome 1, encodes a 384-residue protein, and yields a 360-residue protein containing 38 conserved cysteine residues after signal peptide cleavage (4-7). To date, a number of studies describe the involvement of CYR61 in many cell functions, such as cell adhesion, migration, proliferation, apoptosis and angiogenesis (8-10).

As integrin receptor, CYR61 is expressed in various tissues and may have different physiological functions (11-14). However, expression profiles of CYR61 in malignant melanoma have been inconsistent. Babic et al and Kunz et al reported CYR61 overexpression in malignant melanoma, possibly related to cell migration (15,16). However, in 2009, Dobroff et al demonstrated that silencing the cAMP-response element-binding protein (CREB) in two human metastatic melanoma cell lines, A375SM and C8161-c9, resulted in suppressed tumor growth and metastasis and increased CYR61 expression (17). They pointed to the possibility of inducing expression of CYR61 as potential therapeutic method for malignant melanoma. To date, the expression profile of CYR61 in Chinese patients with malignant melanoma has not been studied.

Here, we determined the levels of CYR61 in samples obtained from Chinese patients with malignant melanoma or other skin tumors, and compared them with those of patients with no skin pathology. Among these malignant tumors, we analyzed CYR61 expression in samples derived from different clinical stages using anti-CYR61 monoclonal antibody (mAb). Our results showed that CYR61 was expressed at low levels in malignant melanoma, which is consistent with previous studies (17). Furthermore, we examined the role of CYR61 on the growth of B16 cell line. We also explored CYR61 expression in B16 cells, when exposed to either epirubicin or interferon (IFN)-α. The results showed that epirubicin and IFN-α inhibited B16 proliferation and subsequently significantly decreased CYR61 expression. Moreover, the increased apoptosis in...
B16 cells was consistent with the reduced expression of proliferating cell nuclear antigen (PCNA). Taken together, we provide evidence that epirubicin and IFN-α negatively impacted B16 cell proliferation and reduced their expression of CYR61 and PCNA, thus indicating CYR61 as a potential target for malignant melanoma treatment.

Materials and methods

Reagents. Monoclonal anti-CYR61 antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). EliVision™ plus Polyer HRP (mouse/rabbit) immunohistochemistry (IHC) kit was provided by Fuzhou Maxin Biotechnology Co., Ltd. (Fuzhou, China). 3H-TdR was a gift from the Shanghai Institute of Applied Physics, Chinese Academy of Sciences (Shanghai, China). SYBR® Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA) and TRizol was obtained from Invitrogen (Carlsbad, CA, USA). Cyr61 protein was supplied by Peprotech, Inc. (Rocky Hill, NJ, USA).

Tissue microarray (TMA) and IHC assay. The TMA containing malignant melanoma, other skin tumors, and normal skin, was obtained from Xi'an Alena Biotechnology Ltd., Co. (Xi'an, China). IHC studies were performed using the standard EliVision™ method. In brief, TMA sections were deparaffinized, rehydrated, and endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 20 min. For antigen retrieval, TMA slides were microwave-treated in 10 mM citrate buffer (pH 6.0) for 10 min. The slides were incubated with mouse mAb against human CYR61(1:100 dilution), overnight at 4˚C, followed by incubation with an HRP-conjugated goat anti-mouse polyclonal antibody for 60 min, and subsequent reaction with 3,3'-diaminobenzidine (DAB). The nuclei were counterstained with hematoxylin. Negative controls were performed by replacing the primary antibody with mouse IgG. To evaluate the IHC staining of CYR61 in malignant melanoma, other skin tumors, and normal skin, a semi-quantitative scoring criteria for IHC of CYR61 was used, in which both staining intensity and positive areas were recorded as - (negative), + (weak positive), ++ (moderate positive), and +++ (strong positive).

Cell lines and culture conditions. B16, a mouse melanoma cell line derived from spontaneous skin tumors in C57BL/6 mouse, was obtained from Shanghai Institutes for Biological Sciences, CAS, Shanghai, China. B16 melanoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 20% fetal calf serum (FCS), penicillin 100 µg/ml, streptomycin 100 µg/ml (Gibco, North Andover, MA, USA), L-glutamine 2 mM, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) 10 mM, and nonessential amino acids (HyClone). Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂ and were routinely passed every 3-4 days. For passaging cells, parental cells and CYR61-treated cells were released from plastic culture dishes with a trypsin (0.25%)-EDTA (1 mM) solution (Gibco) for 5 min.

B16 cells at a concentration of 1.48x10⁶ were plated and grown in 10-cm Petri dishes with 8 ml of complete culture medium. For the ³H-TdR method, B16 cells were plated at a concentration of 2.0x10⁵ cells/well in complete culture medium, in 96-well flat bottomed culture plates.

Cell proliferation assay. To evaluate the effects of epirubicin (0, 0.075, 0.15, 0.3, 0.6 and 1.2 µg/ml) and IFN-α (0, 10², 10³, and 10⁴ IU/ml) on cell growth, several 96-well plates were plated with 2.0x10⁵ cells/well. After 24 h, cells were incubated with drugs at the above mentioned concentrations, measuring any inhibition in cell growth at 12, 24 and 48 h, by using the MTT test. The methods of Carmichael et al (18) and Alley et al (19) were adapted to our culture conditions. Briefly, the cultures were incubated with 20 µl MTT (5 mg/ml in fresh medium) for 4 h (37°C and 5% CO₂). After a 10 min centrifugation to remove the medium and the non-metabolized MTT, 150 µl of dimethyl sulfoxide (DMSO; Fluka, Milwaukee, WI, USA) were added to each well to solubilize the MITT formazan produced by the cells. After shaking for 10 min at room temperature, the amount of colored formazan metabolite was determined by absorbance at 490 nm.

³H-TdR incorporation in vitro. B16 cells (1x10⁴/ml) were seeded in 96-well plates (200 µl/well) and recombinant human CYR61 protein (Peprotech, Inc.) was added at concentrations of 0, 0.625, 1.25, 2.5, 5.0 and 20 ng/ml for 4 h. Six wells were set for each treatment. Next, the cells were treated with 1 µCi ³H and cultured for another 8 h. They were then washed three times with PBS and digested with 0.125% trypsin (HyClone). A cell suspension was prepared and leached onto a membrane. This was washed with 10% trichloroacetic acid, followed by addition of 0.1 mol/l NaOH. Subsequently, anhydrous ethanol was added for decolorization and dehydration. The membrane was dried at 70°C and placed in scintillation solution for 24 h in the dark. Counts per minute were measured using a Trilux 1450 MicroBeta machine (Perkin-Elmer Wallac Inc., USA).

Cell cycle analysis by FACS. B16 cells were incubated in 50-ml culture flasks in DMEM with or without recombinant human CYR61 protein. After 72 h, cells (1x10⁵/ml) were collected, washed repeatedly with PBS containing 0.1% BSA, centrifuged at 1,000 rpm/min for 10 min, and fixed with Cy5-CD4, Cy5-CD8 antibodies at 4°C for 20 min, respectively. Cells were then washed three times with Annexin V-PI buffer. Five microliters of Annexin V and 5 µl PI (R&D Systems, Minneapolis, MN, USA) were added to the culture for another 15 min. The cell cycle distribution was analyzed using a FACScan flow cytometer (BD FACSCalibur; BD Biosciences, San Jose, CA, USA).

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) analysis. Real-time PCR was performed as previously reported (20). The sequence of primers was as follows: GADPH forward, 5'-GTG AAG GTC GGA GTC AAC G-3' and reverse, 5'-AGC TCA GTA ACG AGA ATG CAG CC-3'; β-actin forward, 5'-TGG CCT TCC AGC AGA TGT-3' and reverse, 5'-AGC TCA GTA ACA GTC CGC CTA G-3'; CYR61 forward, 5'-TCC AGC CCA ACT GTA AAC ATC A-3' and reverse, 5'-GGA CAC AGA GGA ATG CAG CC-3'; PCNA forward, 5'-CCA ATT GTG CCG

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AGA AAA GC-3' and reverse, 5'-GAC AGA GCC AGT ATT GGG AGT TG-3'. The above primers were designed and provided by Takara Biotechnology, Co., Ltd. (Dalian, China). cDNA was amplified with SYBR® Green PCR Master Mix (Takara Biotechnology, Co., Ltd.), by using the 7000 Real-Time PCR system (Applied Biosystems). For target gene quantification, normalization was done based on a Chr.21 assay, C2. Relative copy numbers (RCN) were determined on the basis of comparative \( \Delta \Delta C_q \) (Ct) method, with a normal control DNA as the calibration standard (21). All experiments were repeated three times. A=0.5-fold-change in RCN was considered as benchmark for deletion.

Western blot analysis. CYR61 protein in B16 cells was detected by western blot analysis with specific anti-human CYR61 monoclonal antibodies. Following electrophoresis, proteins were transferred to PVDF membrane at 60 V for 2 h. Membranes were blocked with 5% non-fat milk, washed with PBS, and incubated with mAb at 4°C overnight. Subsequently, they were incubated with HRP-conjugated goat anti-mouse IgG at room temperature for 45 min, followed by washing with PBS. The target protein was visualized by using autoradiography film (Fujifilm LAS-4000).

Statistical analysis. Group measures were shown as mean ± SEM. A Student's t-test was used to analyze the differences between each two groups. A one-way ANOVA was initially performed to assess the overall statistical significance, followed by a two-tailed paired or unpaired Student's t-test. A p<0.05 was considered significant.

Results

Low CYR61 expression in malignant melanoma. Using TMA and IHC, we examined the expression of CYR61 in 78 skin samples from Chinese patients with different clinical diagnoses, stages and pathological types. The expression profile of CYR61 is displayed in Table I. CYR61 levels are low in malignant melanoma (MM) and basal cell carcinoma (BCC) tissues, and high in squamous cell carcinoma (SCC) tissues (p<0.01), when compared with normal skin tissue. This is different from previous studies where CYR61 was found to be overexpressed in malignant melanoma (17). Next, we examined the correlation between CYR61 levels with the clinical stage and pathological diagnosis. There was no direct relationship between CYR61 expression and the pathological diagnosis or TNM stage in either SCC (Fig. 1A and B) or BCC and MM (Fig. 1C and D). The specificity of the anti-CYR61 antibody was concluded from the positive CYR61 expression detected in tissues exposed to antibody as opposed to the negative one in tissues incubated with control IgG (Fig. 1E).

Recombinant human CYR61 inhibits proliferation and promotes apoptosis in B16 cells. Based on the search results from NCBI HomoloGene, human CYR61 has a high degree of homology to the protein in other species (Fig. 2A). We also found 93% sequence similarity between CYR61 in human and mouse, confirming reliability of using human CYR61 in B16 cells in vitro (Fig. 2B). B16 cells appeared to grow well under normal conditions (Fig. 2C). However, \(^{3}\text{H}-\text{TdR}\) incorporation suggested that human CYR61 suppressed B16 proliferation in vitro, an effect enhanced progressively with increasing concentration of the protein added, up to 5 ng/ml (Fig. 2D).

Additional to growth suppression, CYR61 protein also had apoptosis promoting effect, proportional with the concentration of the recombinant protein. At 10 ng/ml, 30% of cells were apoptotic, significantly more than when exposed to low CYR61 concentrations or control (without CYR61) (Fig. 2E).
Anti-tumoral effect of epirubicin and IFNα on B16 cells. Epirubicin and IFN-α were added to B16 cells and the proliferation rate was monitored to determine their effect on cell growth. To evaluate the antitumor effect of different individual concentrations of epirubicin (0, 0.075, 0.15, 0.3, 0.6 and 1.2 µg/ml) and IFN-α (0, 10^2, 10^3 and 10^4 IU/ml), cells (2.0x10^3/well) were seeded into 96-well plates and 24 h later, they were incubated with various doses of drugs. Cell growth was evaluated by the MTT assay after 12, 24 and 48 h of treatment. As shown in Fig. 3, both epirubicin and IFN-α had the ability to inhibit B16 proliferation. After 12 h of treatment with different concentrations of drugs, there was no difference in cell growth, suggesting that cells were probably not in the proliferative stage yet. After 24 h, the 6 different doses of epirubicin did not show a statistically significant cell growth, while 10^4 IU/ml IFN-α clearly inhibited proliferation (p<0.05). Forty-eight hours later, both 0.075-1.2 µg/ml of epirubicin and 10^3-10^4 IU/ml IFN-α strongly inhibited B16 growth, compared with other doses tested. In addition, the number of cells in the 0.3 or 0.6 µg/ml epirubicin treatment groups were decreased (p<0.05 and p<0.01, respectively) (Fig. 3A).

CYR61 and PCNA expression when B16 proliferation is inhibited. We further determined CYR61 and PCNA expression in B16 cells treated with 0.3 µg/ml epirubicin or 10^3 IU/ml IFN-α. To analyze the relationship between cell growth and CYR61 expression, we evaluated CYR61 gene expression at different time-points using quantitative real-time PCR, and CYR61 protein expression using western blot analysis.

In Fig. 4, we show that after 24 h of treatment, neither 0.3 µg/ml epirubicin nor 10^3 IU/ml IFN-α inhibited B16 proliferation or promoted CYR61 expression. However, 48 h later, epirubicin inhibited cell growth and promoted apoptosis, while IFN-α had no effect (Fig. 4B). PCNA is an important indicator for proliferation and differentiation of tumor cells. As shown in Fig. 4C, at the 24 h point, 0.3 µg/ml epirubicin inhibited PCNA expression, although the proliferative status of the cells did not change. After 48 h, both 0.3 µg/ml epirubicin and 10^3 IU/ml IFN-α inhibited the proliferation of B16 cells, a process accompanied by decreased PCNA expression. This suggests that epirubicin had an earlier and sharper effect than IFN-α on PCNA expression. Also, they probably have a different mechanism of action, since solely epirubicin inhibits proliferation and promotes apoptosis by upregulating CYR61.

Discussion

CYR61 has been reported to be overexpressed in different clinical stages of several types of tumors, such as breast cancer (22,23), glioma (24), and pancreatic cancer (25).
However, in other cancers, such as non-small cell lung cancer (NSCLC) (11,26), endometrial cancer, and papillary thyroid carcinoma (27-29), the levels of CYR61 were shown to be reduced. Currently, the expression profile of CYR61 in Chinese patients with malignant melanoma is unclear.

Malignant melanoma has a complex array of pathological features. During early stages, it is often misdiagnosed as hyperpigmentation or nevi, due to their similarities in appearance. The incidence and exact mechanism of malignant melanoma remain unclear, and some of the etiological factors that have been implicated are malignant transformation of nevi, ultraviolet radiation, racial and genetic background, trauma, viral infection, and reduced immunity (30-33). Several studies have reported abnormal gene and protein expression in malignant melanoma, especially of the malignant melanoma growth-related factors (34-37). Tumor suppressor genes may
also play an important role in melanoma progression and aggression.

Here, we analyzed the expression profile of CYR61 in skin samples from 78 Chinese patients with different pathologies, including three common skin tumors (BCC, SCC, and malignant melanoma), inflammatory lesions, and normal skins. Samples were placed on TMA, balanced and normalized by age and gender. The level of CYR61 was examined by IHC and analyzed in correlation with the pathological diagnosis.

CYR61 stained positive in normal skin and skin with chronic inflammation around 80 and 75%, respectively. Compared with levels found in normal skin, CYR61 was reduced in malignant melanoma and BCC tissues, and over-expressed in SCC tissues. Interestingly, we found no direct relationship between the CYR61 expression and the TNM stage. The percentage of CYR61-positive cells was significantly lower in malignant tumors than in normal or chronic inflammation skin. This suggests that CYR61 could become a potential therapeutic target and marker for this cancer. Thus, we used a murine malignant melanoma cell line (B16) to examine the effects of exogenous CYR61 on cell proliferation and apoptosis.

In cancer therapy, proto-oncogenes are targeted to block or reduce cancer cell activities, and tumor suppressor genes are targeted to restore or increase their activity (38-43). CYR61 could be classified as a tumor suppressor gene, since when mutated, the inhibition of tumor growth is lost. While it seems to act similarly to p53 a tumor growth suppressor gene, its role has not been reported yet. This could be a significant finding for the treatment and outcome of malignant melanoma.

Two anti-tumoral drugs, epirubicin and IFN-α, were used to study the B16 cell growth and apoptosis. We first focused on determining the optimal dose and time of action for IFN-α and epirubicin. The results showed that 0.3 µg/ml epirubicin or 10^3 IU/ml IFN-α had negative effects on B16 cells. We also found that no cell inhibition appeared before 24 h of treatment, and that 48 h of treatment was best for suppressing growth. Based on the cell growth curve, at 12 h post-treatment, cells were yet to enter the proliferative phase, and at 24 h, when proliferation reached a peak, the inhibitory effect of IFN-α or epirubicin began to appear. CYR61 expression increased after 48 h of treatment with epirubicin, but not with IFN-α. Western blot analysis confirmed that IFN-α may affect B16 cell growth through a non-CYR61 pathways.

PCNA is an essential protein for eukaryotic DNA synthesis, closely related to several cell cycle regulators (44,45). It expresses abnormally in a variety of malignant tumors. Therefore, PCNA can be an important indicator of cell proliferation and DNA synthesis. Previous studies confirmed that intracellular microinjection of PCNA antisense oligonucleotides or antibodies can inhibit DNA synthesis and cell cycle progression (46-50). Studies on the expression of survivin, an inhibitor of apoptosis, show that it may be associated with
the development of choroidal melanoma. Since PCNA is directly related to survivin protein, the PCNA proliferation index increase parallels the survivin increase in choroidal melanoma. This suggests that PCNA may be an inhibitor of apoptosis during choroidal melanoma development.

Our data indicated that after 24 h of treatment with epirubicin, PCNA expression was inhibited, while B16 growth did not change significantly. When epirubicin and IFN-α inhibited B16 proliferation, CYR61 expression decreased significantly. There was an increase in apoptotic cells, consistent with the low levels of PCNA. At 48 h, the PCNA expression was severely reduced, suggesting that epirubicin suppresses B16 cell growth, by inhibiting either survivin or another apoptosis suppressor gene. The effect of IFN-α on PCNA expression was minimal and discernible only after 48 h. These results showed that both drugs inhibit B16 cell proliferation by decreasing PCNA expression. Epirubicin had an earlier and sharper effect than IFN-α on PCNA expression, probably due to different mechanisms of action. While epirubicin inhibited proliferation and promoted apoptosis by upregulating CYR61, IFN-α is likely to have a different target.

IFN-α, a soluble glycoprotein, is produced by a variety of cells with a variety of anti-viral, anti-tumor and immunomodulatory roles (51). Recent studies have shown that IFN also inhibits tumor angiogenesis (52,54). Currently, IFN type I is widely used in treatment of hematological cancers (55), follicular lymphoma (56), chronic myeloid leukemia (57), multiple myeloma (58) and solid tumors (59), AIDS related Kaposi’s sarcoma (60), renal carcinoma (61), and endocrine pancreatic tumors (62). IFN I inhibits DNA synthesis and slows the mitosis rate in a selective manner, since tumor cells are 500-1,000 times more resilient than normal cells. In our study, IFN-α inhibited cell proliferation, but did not affect the CYR61 expression. CYR61 inhibits B16 cell proliferation and promotes apoptosis, when survivin and PCNA expression is reduced, a mechanism confirmed when using epirubicin. IFN-α did not inhibit cell growth by activating CYR61 and had only a slight inhibiting effect on PCNA expression.

In conclusion, in this study, we found that CYR61 expression was lower in Chinese patients with malignant melanoma compared with that of patients with other skin tumors or normal skin. CYR61 expression was also reduced in proliferative B16 cells. Using epirubicin and IFN-α to inhibit B16 proliferation, we also found increased CYR61 and decreased PCNA expression in arrested B16 cells. In conclusion, our study provides evidence that CYR61 may be a potential therapeutic target for malignant melanoma patients with high CYR61.

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References


