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EXPRESSION OF VACUOLAR H^+-ATPASES AND THE INTRACELLULAR PH VALUES IN THREE ADENOCARCINOMA CELL LINES OF DIGESTIVE SYSTEM

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Abstract – Vacuolar H^+-ATPases function in bone resorption, pH homeostasis and tumor metastasis, play an important role in regulating the extra- and intracellular pH (pHe and pHi) in various eukaryotic cells. Acidity is one of the main features of the tumors. The Vacuolar H^+-ATPases are the primary responsible for the control of tumor microenvironment by proton extrusion to the extracellular medium, which play a crucial role in tumor invasion, metastasis and chemoresistance. Therefore our study aimed to uncover the relationship between Vacuolar H^+-ATPases and the pHi value. Three adenocarcinoma cell lines of digestive system including SGC7901, HT29 and PATU8988 were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics. BCECF-AM pH-sensitive fluorescent probe was used to measure the pHi value of cells. Western blot and immunofluorescent staining were respectively applied to determine the protein expression and intracellular distribution of Vacuolar H^+-ATPases. The pHi value of HT29 was the highest, whereas the pHi value of PATU8988 was the lowest and of SGC7901 was in the midst according to fluorescent intensity of BCECF. Similar results were obtained in the protein expression and the IOD (integral optical density) of Vacuolar H^+-ATPases in them. The pHi value indirectly represented by fluorescent intensity of BCECF was positively correlated with protein expression of Vacuolar H^+-ATPases in an exponential manner.

Key words: Tumor acidification, BCECF, intracellular pH value, Vacuolar H^+-ATPases

INTRODUCTION

The extra- and intra-cellular pH (pHe and pHi) in eukaryotic cell is an exquisitely regulated parameter which is essential to many cellular processes, including cell differentiation, membrane fusion, prohormone processing, transport neurotransmitters, invasion of various viruses to the cells, and tumor metastasis (6,17) . Numerous data indicate that Vacuolar H^+-ATPases play a crucial role in controlling the extra- and intra-cellular pH in eukaryotic cell (20).

Structurally, Vacuolar H^+-ATPases are composed of a peripheral V_1 domain responsible for ATP hydrolysis and integral V_0 domain responsible for proton translocation (19). The V_1 domain is a 570-kDa peripheral complex composed of 8 subunits (subunits A-H) of molecular weight 70-13 kDa which is responsible for ATP hydrolysis. The V_0 domain is a 260-kDa integral complex composed of 5 subunits (subunits a-d) which is responsible for proton translocation (3,5) .

Proton extrusion via proton pumps (7,8) is the major type of pHi regulatory mechanism in tumor cells. Since 1993, it has been known that the Vacuolar H^+-ATPase is an important pHi regulator in tumor cells (1,15). This proton pump is ubiquitously expressed not only in vacuolar membranes (20) but also in the plasma membranes of eukaryotic cells (14).

Vacuolar H^+-ATPases, as a crucial role in homeostatic regulation of pH gradients, have been proposed to be involved in tumor proliferation, tumorigenesis, multidrug resistance (MDR), invasion and metastasis, (29) and they have been found in the plasma membrane of certain tumor cells (14) . The pHi is critical for the cytotoxicity of many anticancer agents, and thus Vacuolar H^+-ATPases have been implicated

in the acquisition of the Multidrug Resistance (MDR) phenotype (16). As reported, Vacuolar H^+-ATPases is increased in chemoresistant cancer cells and can be induced by chemotherapeuticals (18,31). Meanwhile, many tumor cells secrete lysosomal enzymes participating in the degradation of the extracellular matrix, facilitating metastatic invasion. Moreover, a low pH extracellular environment can also enhance the activity of these enzymes. Therefore, Vacuolar H^+-ATPases may represent a potential target for novel anticancer strategy (14).

Currently, little is known on the association between the pHi value and Vacuolar H^+-ATPases. Thus our study aimed to investigate the relationship between them. In addition, several researches revealed over-expression of Vacuolar H^+-ATPases in adenocarcinomas and melanomas (4), and then three adenocarcinoma cell lines of digestive system were involved in our study, which were respectively HT29, SGC7901 and PATU8988.

MATERIALS AND METHODS

Reagents and drugs

Human Caucasian colon adenocarcinoma grade II cell line (HT29) was a gift by Dr. Yan Yu in the Department of Gastroenterology, the affiliated Drum Tower Hospital of Nanjing University, Medical School. Human gastric adenocarcinoma cell line (SGC7901) was kindly donated by Dr. Jing Sun in the department of oncology, the affiliated Drum Tower Hospital of Nanjing University, Medical School. Human pancreatic cancer cell line (PATU 8988) was obtained from the department of Gastroenterology, Chang Hai Hospital. Vacuolar H^+-ATPase $6V_1A$ mouse polyclonal antibody was purchased from Taiwan Abnova Corporation, China. BCECF-AM pH-sensitive fluorescent probe was provided by Beyotime Instititue of Biotechnology, China. Other materials and chemicals were bought from commercial sources.

Cell culture

Three adenocarcinoma cell lines of digestive system including SGC7901, HT29 and PATU8988 were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials, Co. Ltd, China) and antibiotics（100 units/ml penicillin and 100 μg/ml streptomycin）in a humidified 5% CO_2 atmosphere at 37℃ (Thermoforma Direct Heat CO_2, USA).

Intracellular pH measurement

The pH standard buffer solution contained (in mM): 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 10 HEPES, 5 glucose to pH 7.4 at 4 °C. BCECF-AM pH sensitive fluorescent probe（Beyotime Biotec, China）was diluted into 5 mM with DMSO and stored at -20℃ away from the light. 1 μl BCECF-AM solution was added into 1 ml pH standard buffer solution with the final concentration of 5 μM. Intracellular pH was measured in the monolayers using the pH-sensitive fluorescent probe 2',7'-bis-(2-carboxyethyl)-5- carboxyfluorescein (BCECF) as previously described (31) . The fluorescent intensity of BCECF could represent the level of pHi value. Cells were cultured in six-well plates at a density of 1×10^5 cells per well with RPMI-1640 supplemented with 10% fetal bovine serum in humidified 5%CO_2 condition at 37°C. Then after 24-hr incubation, the upper medium was removed and the cells were washed with the pH standard buffer solution twice, five min each time. Then the pH buffer solution containing BCECF-AM (1 μl/1 ml, 5 μM) was added and the cells were incubated for 1 hr. The supernatant was removed and the cells were washed twice by the pH buffer solution. The cells were trypsinized and resuspended with 1 ml solution B. The fluorescence intensity of BCECF was recorded with Flow Cytometry (BD Biosciences, USA). The wavelengths of excitation light and emission light were 490 nm and 530 nm. It is generally accepted that fluorescence intensity of BCECF at 490 nm, which was positively correlated with the pHi value, could represent the pHi level to a certain extent (32).

Western blot analysis of Vacuolar H^+ -ATPases

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis of V-H^+-ATPase 6 V_1A were performed as previously described (13,23). Briefly, cells were lysed in lysate(containing 0.01% PMSF, 150 mmol/L NaCl, 50 mM Tris (pH=8), 0.1%SDS, 0.2% EDTA, 1% Triton X-100, 1% Sodium Deoxycholate) supplemented with protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl fluoride, sodium orthovanadate, Roche) and incubated for 30 min on ice, and centrifuged at 12000 rpm at 4 ℃（Eppendorf centrifuge 5804R, Germany） for 15 min to remove nuclei and cell debris. The protein concentration of the extracts was determined by the BCA Protein Assay Kit (KEYGEN Biotec, Nanjing), following the manufacturer's instructions. Thirty micrograms extracted of each cell extract were electrophoresed on 10% gels and electroblotted to a nitrocellulose membrane using a semidry transfer system (Bio-Rad, America). Nonspecific binding was blocked by incubating the membrane in 1×TBST (Tris Buffered Saline containing 0.05% Tween-20) supplemented with 5% nonfat dry milk for 1 hr. Blots were then incubated with a polyclonal mouse antibody to Vacuolar H^+-ATPase subunit V_1A (1:3000 diluted with TBST) and a monoclonal mouse antibody to β-actin (1:3000 diluted with TBST, Santa Cruz, USA) as a control for protein loading. Antibody binding was detected by incubating the blot with a horseradish peroxidase–conjugated goat anti-mouse antibody for 1 hr (KPL, 1:1000). Antibody staining was visualized by enhanced chemiluminescence (Santa Cruz Biotechnology, USA). The images of Western blot products were collected and analyzed by Quantity One V4.31 (Bio-Rad, USA).

Immunofluorescence staining

Dispersed single cells (2×10^5 cells per well) were grown on 22 × 22×1 mm^3 glass coverslips（pretreated with 0.3% gelatine）in six-well culture plates. After a 36-48 hr culture, cells were fixed in ice-cold acetone for 10 min at 4°C. The cells were blocked with 10% normal goat serum（Boster Biotech , China）for 30 min and probed with V_1A subunit of Vacuolar H^+-ATPase antibodies (1:100 diluted with TBST) at 4℃ overnight. Alexa Fluor Dye Conjugated secondary antibodies (1:100 diluted with TBST, Alexa Fluor 488 goat anti-mouse IgG (H+L) highly cross-

adsorbed, 2 mg/ml, Invitrogen, USA) were used to incubate for 1 hr to visualize under a fluorescent microscope (Imager A_1, Axio, Zeiss). The Image-Pro plus software (6.0 version) was used to quantify the IOD (Integral Optical Density) of Vacuolar H^+-ATPases in the three adenocarcinoma cell lines.

Statistical Analysis

The data were presented as mean ± SD. Statistical analysis was performed with the software package SPSS 13.0. The significant differences among the three groups were evaluated by multiple comparison according to One-way ANOVA, using SNK and LSD methods. Statistical significance was defined as $P < 0.05$ for all tests.

RESULTS

Comparisons of fluorescent intensity of BCECF in the three adenocarcinoma cell lines of digestive system

The fluorescent intensity values of BCECF in SGC7901, PATU8988 and HT 29 were 8916±212, 7323±165 and 9496±265, respectively. The results were summarized in Fig.1. Then the pHi value could be reflected according to fluorescent intensity of BCECF as mentioned above (31). Statistical analysis revealed that fluorescent intensity values of BCECF in HT 29 and SGC 7901 were significantly stronger than in PATU 8988 (P<0.05), whereas there was no obvious difference between HT 29 and SGC 7901(P>0.05). Thus it could be presumably calculated that the pHi value of HT 29 was the highest, whereas the pHi value of PATU8988 was the lowest and the pHi value of SGC7901 was in the midst on the basis of fluorescent intensity of BCECF.

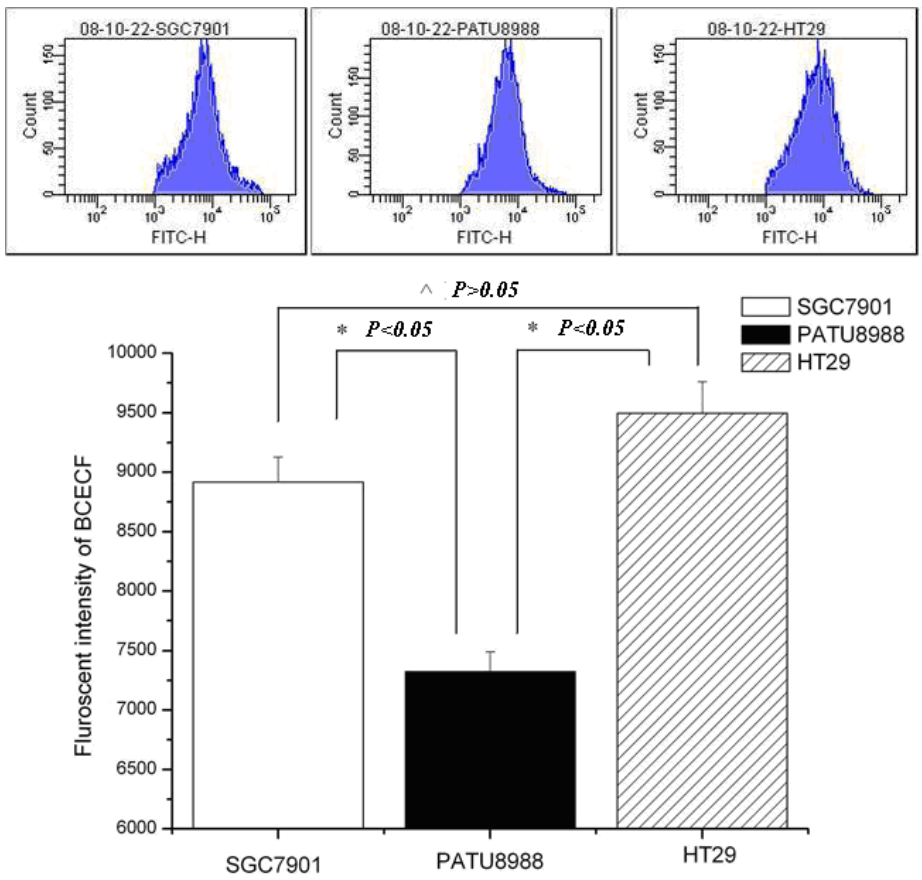

Figure 1. Comparison of fluorescent intensity values of BCECF in SGC7901, PATU 8988 and HT 29
Results were represented as Mean±SD of three independent experiments. One-way ANOVA, including SNK and LSD methods was used for multiple comparisons among the three groups.
* P<0.05, fluorescent intensities of BCECF in SGC 7901 and HT 29 were significantly stronger than in PATU 8988.
^ P>0.05, there was no significant difference in fluorescent intensity of BCECF between SGC 7901 and HT 29.

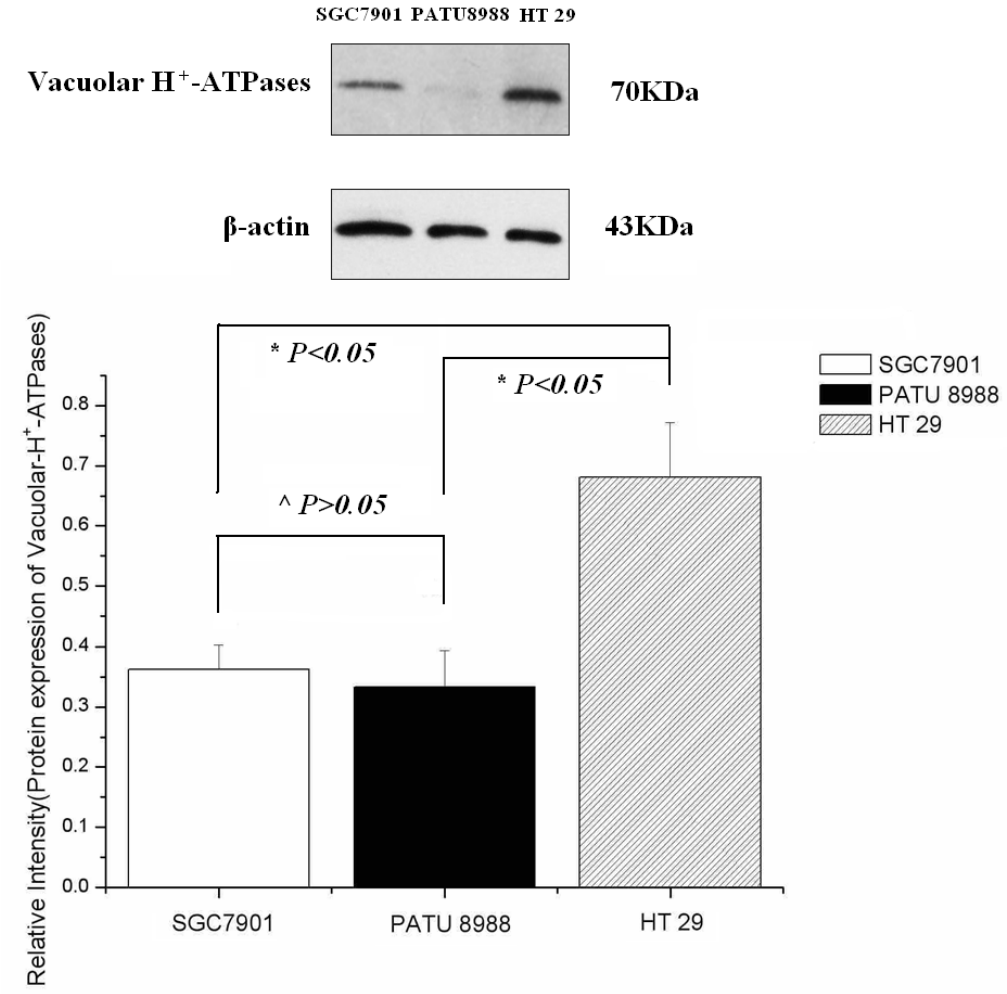

Figure 2. Comparison of protein expression of Vacuolar H^+-ATPases in SGC7901, HT29 and PATU 8988
Results were represented as Mean±SD of three independent experiments. One-way ANOVA, including SNK and LSD methods was used for multiple comparisons among the three groups.
* P<0.05, the protein expression of Vacuolar H^+-ATPases in HT 29 was significantly higher than that in SGC7901 and PATU 8988.
^ P>0.05, no significant difference was found in protein expression of Vacuolar H^+-ATPases between SGC7901 and PATU 8988.

Protein expressions of Vacuolar H^+-ATPases in the three adenocarcinoma cell lines

For immunoblotting analysis, the relative intensities of protein expression of Vacuolar H^+-ATPases in SGC7901, PATU 8988 and HT29 were 0.36±0.04, 0.33±0.06 and 0.68±0.09 respectively (Fig. 2). The protein expression of Vacuolar H^+-ATPases in HT 29 was significantly higher than that in SGC7901 and PATU 8988 (P<0.05), but no significant difference was found in protein expression of Vacuolar H^+-ATPases between SGC7901 and PATU 8988 (P>0.05).

Curve estimation between fluorescent intensity of BCECF-AM versus Relative Intensity of protein expression of Vacuolar H^+-ATPases

It was revealed in Fig. 3 that the more the relative intensity of protein expression of Vacuolar H^+-ATPases was, the higher the fluorescent intensity of BCECF reflecting pHi level would be. Fluorescent intensity of BCECF appeared to be correlated with protein expression of Vacuolar H^+-ATPases. Therefore, curve estimations have shown that there was a kind of exponential relationship between them and the equation was as follows: $Y=-595553.07208\times e^{(-X/0.05621)}+9800.39309$, $R^2=0.85232$.

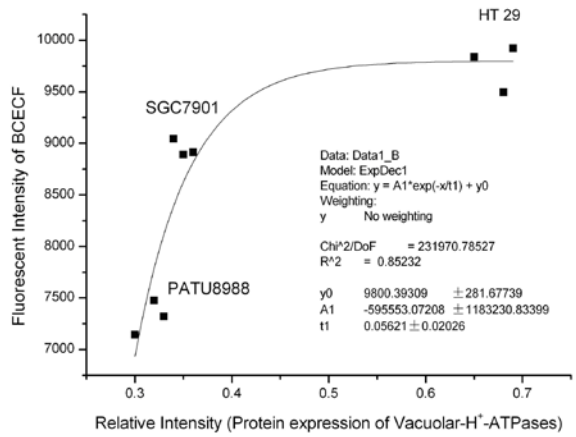

Figure 3. Scatterplot of fluorescent intensity of BCECF versus protein expression of Vacuolar H+-ATPases in SGC7901, PATU 8988 and HT 29

Curve estimation was performed to establish an exponential mathematial model between the fluorescent intensity of BCECF-AM and relative intensity of protein expression of Vacuolar H+-ATPases following the formula: $Y=-595553.07208\times e^{(-X/0.05621))}+9800.39309$, $R^2=0.85232$.

Detailed: Model: Exponential

Equation: y = A1*exp(-x/t1) + y0

Chi^2/DoF= 231970.78527

R^2= 0.85232

y0 9800.39309±281.67739

A1 -595553.07208±1183230.83399

t1 0.05621±0.02026

Intracellular distribution of Vacuolar H+-ATPases via immunofluorescent staining

The fluorescent intensity of Vacuolar H+-ATPases in HT 29 was stronger than those in SGC 7901 and PATU 8988, coincidently consistent with protein expression of Vacuolar H+-ATPases in the three adenocarcinoma cell lines (Fig. 4). Conversely, the fluorescent intensity of Vacuolar H+-ATPases in PATU 8988 was obviously lower than those in other two cancer cell lines. According to the qualified analysis by Image-Pro Plus, the IOD (Mean±SEM) of Vacuolar H+-ATPases in SGC7901, PATU8988 and HT29 were 2209.1382±383.0788, 1970.0476±621.1329 and 12608.288±4815.0245, respectively (Fig.4).

DISCUSSION

In our study we employed BCECF-AM pH-sensitive fluorescent probe to compare the pHi values of three cancer cell lines of digestive system because the fluorescent intensities of BCECF could reflect the intracellular pH level accurately as previously described (32). Then according to Fig. 1, the fluorescent intensities of BCECF in three adenocarcinoma cell lines were clearly displayed.

BCECF is the most widely used fluorescent indicator for intracellular pH (24). Although applications in mammalian cells (11) are predominant, BCECF has also been employed for pH measurements in perfused tissues, intercellular spaces, plant cells, bacteria and yeast (9,28). The absorption maximum of the base form of BCECF is very close to the 488-nm argon-ion laser line, making it ideally suited for the flow cytometry and confocal microscopy applications. Therefore, we chose the flow cytometry to determine the fluorescent intensity of BCECF.

Vacuolar H+-ATPase is an important pHi regulator in tumor cells as mentioned above (14). This proton pump is ubiquitously expressed, not only in vacuolar membranes (20) but also in the plasma membranes (14) of eukaryotic cells. Vacuolar H+-ATPase is a member of a family of ATP-driven proton pumps responsible for the acidification of intracellular compartments such as endosomes, lysosomes, Golgi-derived vesicles and clathrin-coated vesicles (20). Moreover, Vacuolar H+-ATPases, presenting a family of heteromultimeric proteins, translocate protons via eukaryotic plasma and endomembranes. By this process they energize a multitude of secondary transport processes (14,20) .

It is not astonishing that Vacuolar H+-ATPases, which are often overexpressed in the osteoclast or some cancer cell lines, are involved in serious diseases such as osteoporosis or cancer so that the investigation of this enzyme as a potential drug target is one of the most challenging topics in Vacuolar H+-ATPases research (6,17,14,20).

Moreover, in our study the protein expressions of Vacuolar H+-ATPases in the three adenocarcinoma cell lines were also compared by western blot analysis (Fig.2). Interestingly, the results were positively correlated with fluorescent intensities of BCECF in an exponential manner. Meanwhile, immunofluorescent staining analysis revealed that the fluorescent intensities of intracellular Vacuolar H+-ATPases were also consistent with the results of western blotting, providing reliable supports for intracellular distribution of Vacuolar H+-ATPases in the adenocarcinoma cell lines. Moreover, the curve estimation indicated that there was a kind of exponential relationship between fluorescent intensity of BCECF-AM and protein expression of Vacuolar H+-ATPases

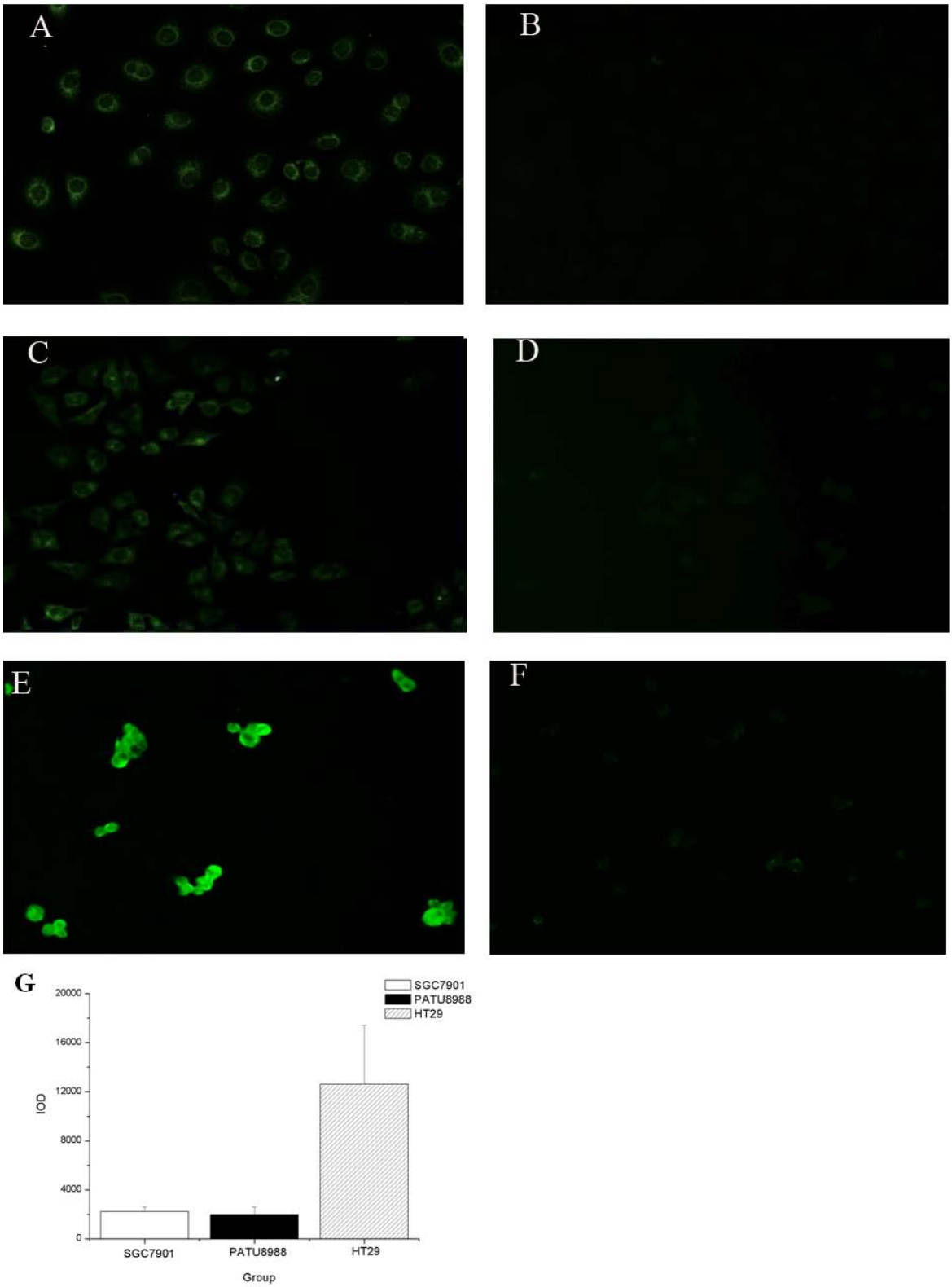

Figure 4. Intracellular distribution of Vacuolar H^+-ATPases in SGC7901, PATU 8988 and HT 29
A. SGC7901 (×200) B. Negative Control (×200) PBS as primary antibody
C. PATU 8988 (×200) D. Negative Control (×200) PBS as primary antibody
E. HT29 (×200) F. Negative Control (×200) PBS as primary antibody
G. The IOD values of Vacuolar H^+-ATPases in SGC7901, PATU 8988 and HT 29

following: $Y=-595553.07208\times e^{(-X/0.05621)}+9800.39309$, $R^2= 0.85232$ (Fig.3). For the limitations of our experiment, we just chose the three adenocarcinoma cell lines of digestive system. Future detailed work would be performed to investigate the relationship between them in more cancer cell lines. Besides, the specific inhibitor of Vacuolar H^+-ATPases and knockdown Vacuolar H^+-ATPase by RNAi also are the feasible ways to testify whether disturbing the Vacuolar H^+-ATPase activity or expression level could result in the pHi value change.

In addition, Vacuolar H^+-ATPases have been proposed to be involved in tumor invasion, metastasis and MDR as mentioned above (14,29) . As Kubota et al. had reported, over-expression of ATP6L (Vacuolar H^+-ATPases 16KDa subunit) in fibroflasts enhanced the invasion activity of the cells (12). Sennoune et al. assessed the effect of bafilomycin A1 inbreast tumor cells and found that cytoplasmic pH recovery was inhibited in response to acid load, in both highly and lowly metastatic cells, although to a greater extent in highly metastatic cells (26). This suggests that V-ATPases in the plasma membrane are involved in the acquisition of a more metastatic phenotype and that the use of V-ATPase inhibitors allows distant metastasis to be minimized.

In tumor cells, Vacuolar H^+-ATPases enhance resistance to antitumor drugs. A series of drug-resistant tumor cells show greater expression of V-ATPase subunits (18,21,30,31) and V-ATPase inhibitors may enhance drug build-up in some tumor cells (27). Murakami et al. found over expression of the ATP6C gene or subunit C in cisplatin-resistant tumors, a fact consistent with the increased number and activity of V-ATPases in cases of chemoresistance and the importance of this subunit in pump regulation (18).

Therefore, it might be a potential target or a prognosis marker in future cancer chemotherapy (2,10,25,26) . Moreover, many specific inhibitors on Vacuolar H^+-ATPases have been utilized to exhibit or improve cytotoxic effects on tumor cell lines (22). To establish the relationship between fluorescent intensity of BCECF and protein expression of Vacuolar H^+-ATPases seemes to contribute to clarify the pHi regulatory mechanism. Besides, it also could provide the concrete indication for utilization of inhibitors of Vacuolar H^+-ATPases on tumor chemotherapy in the future.

In summary, Vacuolar H^+-ATPase would be a potential target in future cancer chemotherapy. Detailed researches on its role in tumor chemotherapy would still be deployed step by step in not far future. Further work would also be done to explore more safe, selective and effective inhibitors on Vacuolar H^+-ATPases in tumor chemotherapy.

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