RESEARCH COMMUNICATION

Expression of Proteasome Activator REGγ in Human Laryngeal Carcinoma and Associations with Tumor Suppressor Proteins

Li-Ping Li[1], Wei-Bo Cheng[2], Hong Li[3], Wen Li[1], Hui Yang[1], Ding-Hou Wen[1], Yue-Di Tang[1]*

Abstract

The functional significance of the proteasome activator REGγ in the regulation of cell proliferation and apoptosis has been recognized. However, pathological contributions to tumor development remain to be elucidated. Both oncogenic proteins and tumor suppressors are targeted by REGγ for proteasomal degradation. It has been proposed that the role of the REGγ in the pathogenesis of cancer is cell- and context-specific. In this study, we aimed to explore the potential involvement of REGγ in laryngeal carcinomas, comparing protein expression in tumor and adjacent tissues by immunohistochemical staining and Western blot analysis. We also characterized the correlation between the expression of REGγ and the previously identified substrates p53 and p21. We showed that REGγ was abnormally highly expressed in cancer tissues. Statistical analysis revealed that there was a positive relationship between the level of REGγ and the expression of p53 and p21. Our study suggests that REGγ overexpression can facilitate the growth of laryngeal cancer cells.

Keywords: REGγ - laryngeal carcinoma - p53 - p21

Asian Pacific J Cancer Prev, **13**, 2699-2703

Introduction

The 20S proteasomes are large complexes that play crucial roles in protein quality control and in regulating many cellular functions in eukaryotic cells such as the cell cycle, transcription, cell signaling, cell death (Ciechanover et al., 2000). At least two families of proteasome activator complexes have been found, the 19S proteasome (also known as PA700) and the 11S proteasome (also known as REG or PA28) (Mao et al., 2008; Luo et al., 2010). The 19S activator binds to 20S proteasome to form the 26S proteasome in an ATP-dependent manner, which mainly performs degradation of proteins in a ubiquitin-dependent manner. In contrast, the REG activates the 20S proteasome in an ATP-independent manner and is primarily responsible for ubiquitin-independent protein degradation (Realini et al., 1997). There are three REG homologs, called α, β and γ. The α and β subunits form a heteroheptamer and is mainly cytoplasmic, whereas γ has a nuclear-restricted expression pattern and can be found independently or associated with 20S proteasomes as a homoheptameric lid (Ahn et al., 1996; Soza et al., 1997; Wojcik et al., 1998). REGα/β is induced by interferon IFN-γ and infection and its main function has been implicated in MHC class I antigen presentation. REGγ is unaffected by IFN-γ and can be markedly reduced during infection (Mao et al., 2008; Luo et al., 2010).

Although the functional significance of REGγ has not been fully defined, previous studies suggest a role for REGγ in the regulation of cell cycle progression and apoptosis. REGγ-deficient mice showed reduced body size and defects in cell-specific mitosis (Murata et al., 1999; Barton et al., 2004). Moreover, the expression of REGγ has been found abnormally high in human breast cancer cells and the metastatic lymph nodes (Wang et al., 2009). Recently, there has study showed that REGγ was a strong candidate for the regulation of cell cycle, proliferation and the invasion in poorly differentiated thyroid carcinoma cells (Zhang et al., 2012). Despite these interesting observations, the pathological contributions of the REGγ to the development of cancer remain unclear. Several cell cycle/apoptosis regulating proteins, including cyclin-dependent kinase inhibitors p21, p16, and p19 (Chen et al., 2007; Li et al., 2007), and tumor suppressor p53 (Zhang et al., 2008), have been identified to be the substrates of the REGγ. In addition, early studies demonstrated that REGγ also targets several oncogenic proteins, including steroid receptor coactivator 3 (SRC-3) (Li et al., 2006) and pituitary tumor-transforming 1 (PTTG1) (Ying et al., 2006), for proteasomal degradation. Thus, the overall pathological outcome of REGγ-proteasomal activity is complicated by the fact that it may play either tumor-promoting or tumor-suppressive roles. The distinct function of REGγ in cancer development is

[1]*Department of Otorhinolaryngology and Translational Neuroscience Center, West China Hospital of Sichuan University,* [2]*Department of Pathology, West China School of Public Health, Sichuan University,* [3]*The Joint Research Center, West China Second University Hospital and University of Hong Kong, Chengdu, China *For correspondence: tangyd@hotmail.com*

likely determined by multiple factors, including cell-type or context-specific.

Laryngeal cancer constitutes up to 2% of all cancers, and 90-95% are squamous cell carcinomas (Powell and Robin, 1983). The etiology of laryngeal cancer has yet to be clarified, although it seems to be closely related to alcohol and tobacco abuse. Only now are the molecular changes associated with laryngeal cancer being elucidated. Several cell cycle/apoptosis regulating proteins have been identified overexpression in larynx cancers (Jin et al., 1998), including cyclin-dependent kinase inhibitors p21, p16, and tumor suppressor p53, and the oncogene bcl-2. The process of tumorigenesis involves multiple molecular events involving both activation of proto-oncogene products that stimulate growth and inactivation of tumor suppressor genes, the products of which normally inhibit cell proliferation. Tumor suppressor p53 is involved in controlling cell growth and differentiation and in the maintenance of genomic integrity, with high levels of p53 activity inducing apoptosis, cell cycle arrest, or senescence (Lane, 1992). Decreased levels of p53 allow cells to pass through theG1/S checkpoint during cell cycle progression. The cyclin-dependent kinase inhibitor, p21 is induced in various biological situations including cell cycle arrest, differentiation, and apoptosis. The effects of targeted deletion of p21 in mice and its expression patterns in some human cancers are consistent with a role for p21 as both a tumor suppressor and an oncogene (Roninson, 2002). Increasing evidence has suggested that REGγ plays either tumor-promoting or tumor-suppressive roles in the development of human cancers, however, the role and expression pattern of REGγ in human laryngeal cancer is not clear. In addition, REGγ has been reported recently to be involved in the degradation of p53 and p21 proteins (Li et al., 2007; Zhang and Zhang, 2008). To better understand the function of REGγ in human laryngeal carcinoma, we examined the expression levels in patient samples and analyzed the relationship with p53 and p21.

Materials and Methods

Human subjects

All human tissues were obtained from the Department of Otolaryngology Head and Neck Surgery at the West China Hospital. Laryngeal carcinoma tissues and the tissues adjacent to the laryngeal tumor (the dysplastic epithelium surrounding the cancer) were from 36 male patients respectively with different stages of cancer (T1 (12 cases), T2 (12), T3 (6), and T4 (6)) and ages ranging from 45-73 years. Control laryngeal tissues were obtained from 12 male patients with benign lesions.

Antibodies

The monoclonal rabbit anti-p53 (7F5) and the anti-p21 WAF1/CIP1 (12D1) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal anti-REGγ antibody was purchased from Zymed Laboratories (San Francisco, CA). The mouse monoclonal anti-β-actin antibody was from Proteintech Group (Chicago, IL).

Immunohistochemical staining

Formalin-fixed, paraffin-embedded serial tissue sections (3 μm thick) were deparaffinized and immunostained following a standard protocol. Briefly, tissue sections were dewaxed and dehydrated. Endogenous peroxidase activity was then blocked with 0.3% hydrogen peroxide in absolute methanol. The sections were then incubated with either REGγ antibody (1:100) or p53 (1:50) or p21 (1:100) for overnight at 4° C. After incubation with biotinylated rabbit anti-IgG (1:3000 from ZSGB-BIO, China) for 15 min at 37 °C, the sections were immersed in a solution with the avidin-biotin complex (ZSGB-BIO, China) for 15 min at 37 °C, developed with diaminobenzidine and counterstained with hematoxylin. The images were scanned at a magnification of 200 using light microscopy. The expressions of REGγ, p53 and p21 in the tissues were semi-quantified in regard to the proportion of positive areas (0-100%) and intensity (five grades: none (0), slight (1), moderate (2), strong (3), extremely strong (4)). The positive areas were scored by integrated optical density (IOD = area×density). Three samples in each condition were analyzed and averaged for comparison.

Western blot analysis

Human laryngeal carcinoma, the adjacent tissues, and control laryngeal tissues were homogenized in 300 μl RIPA buffer [50 mM Tris(PH7.4),150 Mm Nacl, 1% Triton-X-100, 1% sodium deoxycholate, 0.1% SDS and sodium orthovanadate, sodium fluoride, EDTA (from Keygen Biology, China) and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and protein concentration was measured using a Bradford assay kit (Bio-Rad, Hercules, CA). A total of 100 μg of each sample was separated on 10% SDS–PAGE and transferred to PVDF membranes (Millipore, UK), followed by blocking with Tris-buffered saline and 0.05% Tween-20 containing 5% non-fat dried milk for 2 h at room temperature, and incubation with the appropriate primary antibody (REGγ, p53, p21, or β-actin) for overnight at 4 °C. These membranes were then exposed to the secondary anti-rabbit antibody (1:1000) or anti-mouse (1:2000) antibody for 1.5 h at room temperature. The detection was performed using enhanced chemiluminescence (ECL; Beyotime - BIO, China). The density of each protein band was analyzed by Volume Contour measurement (Molecular Image Gel Doc XR System170-8170; Bio-Rad).

Statistical analysis

The results shown are the means ± standard deviations (SD), and statistical analysis was performed using the analysis of variance (SPSS 16.0 statistical software). The difference is considered statistically significant when the p value is less than 0.05.

Results

The expression of REGγ in laryngeal carcinoma

Immunohistochemical staining revealed that the

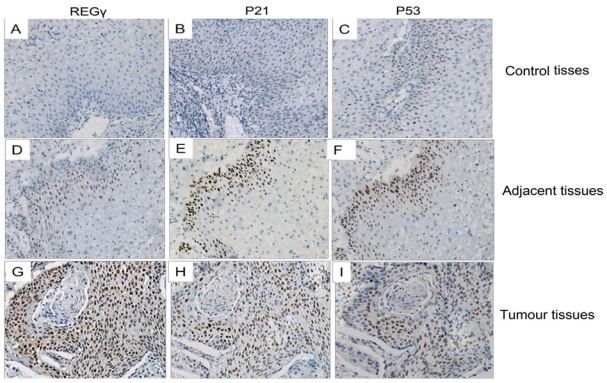

Figure 1. Representative Immunohistochemical Staining of REGγ (A, D, G), p21 (B, E, H), and p53 (C, F, I) in Serial Sections of Laryngeal Carcinoma Tissues (A, B, C), Adjacent Tissues (D, E, F), and Control Laryngeal Tissues (G, H, I) ×200

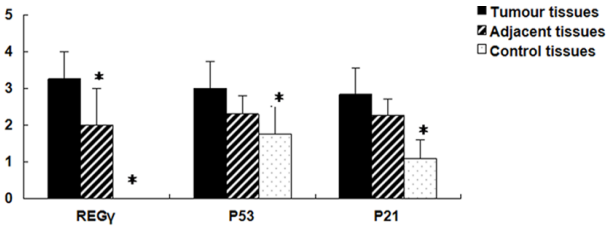

Figure 2. Statistical Results of the Immunohistochemical Staining Data. Expression of REGγ, p53, and p21 proteins in laryngeal carcinoma, the tissues adjacent to the laryngeal cancer and control tissues was detected by IHC analysis and the results were semiquantified according to description in Method and Materails. The results are presented as means ± SD (n=36). *, P < 0.01 compared with tumor tissues

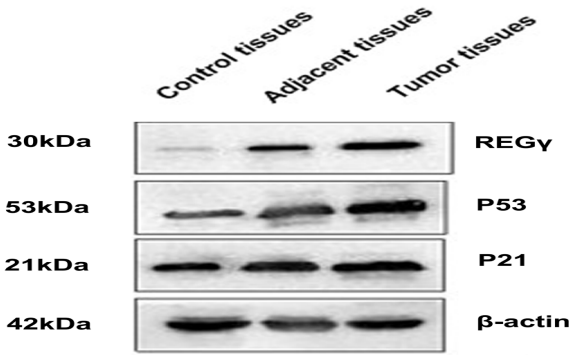

Figure 3. Western Blot Analysis of Protein Expression of REGγ, p21, and p53 in Laryngeal Carcinoma, Adjacent Tissues and Control Tissues. β-actin was used as loading control

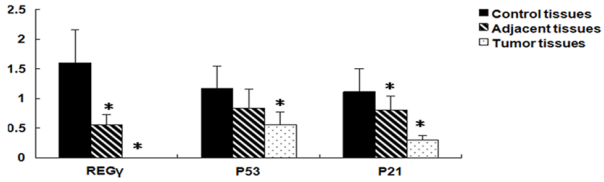

Figure 4. Quantification Results of the Western Blot Data. The results are presented as means ± SD (n=36). It displays the normalized results against β-actin. *P < 0.01 compared with tumor tissues. The expression of REGγ in adjacent tissues was significantly higher compared with control tissues (P < 0.01) and significantly lower compared with laryngeal carcinoma tissues (P <0.01); The expression of p53 and p21 was higher in laryngeal carcinoma tissues than in the adjacent tissues (P < 0.05) and control tissues (P < 0.01)

level of REGγ was higher in the tumor tissues than in adjacent tissues and the control tissues (Figure 1A, 1D, 1G). In control (Figure 1A) and adjacent tissues (Fig. 1D), the expression of REGγ was almost undetectable or at a low level. However, it was observed that REGγ was abnormally overexpressed in laryngeal carcinoma tissues (Figure 1G).

To compare the expression of REGγ, we classified into five categories (0, negative; 1, weak; 2, moderate; 3, strong; and 4, extremely strong) according to the intensity and extent of the staining. We revealed that the intensity of REGγ expression of cancer mass (classes 1–4) is higher than that of the adjacent laryngeal cancer tissues (class 0-2; Figurre 2; p < 0.01) and control laryngeal tissues (class 0; Figure 2; p < 0.01). Quantitative analysis of Western blot (Figure 4) results also demonstrated that the differences in REGγ protein expression between control laryngeal tissues and the adjacent laryngeal cancer tissues or between the adjacent laryngeal cancer tissues and the laryngeal cancer tissues are statistically (p < 0.01). Immunohistochemical staining revealed that REGγ was predominantly expressed in the nucleus (Figure 1A, 1D, 1G). Such a peculiar property of REGγ distribution in laryngeal cancer leads us to the idea that REGγ expression may reflect the growth property of cancer cells.

Relationship between the expression of REGγ, p53, and p21 in laryngeal carcinoma

To further establish a mechanistic link between REGγ and laryngeal carcinoma, we examined the levels of p21 and p53, two identified REGγ substrates critical for the regulation of cell cycle progression and apoptosis (Chen et al., 2007; Li et al., 2007; Zhang and Zhang, 2008).

We found that three different tissues all contained detectable amounts of p21 (Figure 1B, 1E, 1H; Figure 3) and p53 (Figures 1C, 1F, 1I; Figure 3). Quantitative results of immunohistochemical staining (Figure 2) and Western blot analysis (Figure 4) showed that the levels of p53 and p21 in tumor tissues were significantly higher than those in the adjacent tissues (p < 0.05) and control tissues (p < 0.01), parallel to the expression levels of REGγ. Therefore, there was a positive relationship between the level of REGγ and the expression of p53 and p21.

Discussion

How the protein is recognized and selective degraded in the cellular life process is very important segment. The proteasome system is one of the most important protein degradation systems in eukaryotes and has the ability to regulate cellular processes such as the cell cycle, transcription, cell signaling, cell death, and immune responses (Ciechanover, 1994; Hochstrasser, 1995; Ciechanover, 1998). The 19S proteasomal activator (or PA700) is a well-studied proteasomal activator that binds to the 20S, forming the 26S proteasome, an ATP- and ubiquitin-dependent protease complex. Most cellular proteins are degraded through the 26S proteasome after ubiquitination, a way for 26S to recognize proteins

that need to be degraded. The alternative proteasomal activator, REG (also known as 11S proteasome) activates the 20S proteasome in an ATP-independent manner and is primarily responsible for ubiquitin-independent protein degradation (Realini et al., 1997). REGα/β can be induced by interferon(IFN)-γ and play an important role in MHC class I antigen presentation. REGγ, however, is not responsive to IFNγ and does not appear to be heavily involved in the immune system (Mao et al., 2008; Luo et al., 2010). REGγ null mice have phenotypes that include decreased cell proliferation and increased apoptosis, implying that the REGγ complex may regulate the stability of specific proteins that act in these pathways. Increasing evidence has also suggested that overexpressed REGγ could positively contribute to cell cycle progression in the development of various human cancers. For instance, REGγ has been reported to be highly expressed in thyroid cancer (Okamura et al., 2003) and in colorectal cancer serum (Roessler et al., 2006). Recently, the overexpressed REGγ has been also found in human breast cancer cells and the metastatic lymph nodes (Wang et al., 2009). On the basis of these observations, we presumed that REGγ may be highly expressed laryngeal cancer and have a role in the development of laryngeal cancer.

First, we detected laryngeal cancer tissue and its adjacent tissues, control laryngeal tissues. We found surprisedly that REGγ was overexpressed in laryngeal cancer. There was a lesser amount expression of REGγ in the adjacent tissues. Relatively, in control laryngeal tissues, there was no apparent expression of REGγ was detected. In addition, the immunocytochemical examination revealed that REGγ was predominantly distributed in the nucleus of cancer cells. Tomohisa et al. (Tomohisa et al., 2003) observed significant correlation between PCNA and REGγ expression. the key transition factors for cell cycle progression, PCNA is overexpressed in laryngeal cancer tissues (Sarafoleanu et al., 2009). Bianchi et al. (1993) found more or less PCNA positive cells could reflect tumor cells growth rate. Tumor is known to be a cellular cyclic disease (Shunqian and Qimin, 2002), and regulatory disbalance of cell cycle is a primary reason for malignant tumors proliferation. Whether cell cycle can initiate and result in proliferation relies on whether it can pass G1/S check point or not. Once pass the check point, cell cycle can be accomplished under the driver of cyclins and CDK even though the stimulation from growth factor is absent. It was showed that REGγ could promote HBL-100 cell to go from G1 into S phase (Wang et al., 2009). Therefore, these findings suggest that REGγ overexpression can promote laryngeal cancer cells proliferation and accelerate laryngeal cancer cells growth.

REGγ has been shown to be involved in the degradation of p53 and p21 proteins (Li et al., 2007; Zhang and Zhang, 2008). The tumor suppressor protein p53 is well-documented to have the ability to stimulate apoptosis and cell cycle arrest in the event of DNA damage and strongly suppress oncogenesis (Sharpless and DePinho, 2002). p21, a broad-acting cyclin-dependent kinase inhibitor, occupies a central position in the regulation of the cell cycle progression in many tissues (Weinberg and Denning, 2002). Defects or downregulation of p21 have been linked to the development of various cancers and contribution to tumour progression (Roninson, 2002). To our knowledge, there is no report on the relationship and interaction between REGγ and p53 and p21 in human laryngeal cancer tissues. Our study showed a positive correlation between REGγ and the expression of p53 and p21 in all three laryngeal tissues on the first time. The levels of p53 and p21 in tumor tissues were significantly higher than in the adjacent tissues ($P < 0.05$) and control tissues ($P<0.01$), which are parallel to the expression of REGγ. We explain the phenomenon as follows. The wild-type p53 protein is kept at a low concentration by rapid degradation in normal cells. Therefore, the wild-type p53 protein is usually undetected or detected at a very low level. However, mutations in the p53 gene, which accounts for more than half of tumor development, result in a dysfunctional protein product with a prolonged half-life that enables them to accumulate in the cell and mutation of p53 was dominantly expressed in tumors (Reich et al., 1983; Bosari et al., 1995; Finlay et al., 1988). Our result also suggests that the behavior of p53 protein in laryngeal carcinoma may differ from that in other cancers. But it is needed to further confirm. p21, another tumor suppressor gene involved in the regulation of the cell cycle G1-S transition, is a downstream p53 effector gene and seems also adapted to this explanation (Bianchi et al., 1993; Harper et al., 1993; El-Deiry et al., 1994). What's more, The biological function of p21 is complex, as p21 appears to have both tumor suppressor and oncogenic roles, depending upon the cellular context. Because of this, REGγ may also play either tumor-promoting or tumor-suppressive roles in different cellular context. It has been also proved that degradation of p53 protein are both in ubiquitin-independent and other manners. Therefore, we speculate that REGγ fails to efficiently degrade mutant p53 and its downstream gene p21. What's more, p53- and p21-unrelated mechanisms may be involved in the function of REGγ in tumor pathogenesis or REGγ-proteasome system regulated the degradation of p53 and p21 protein with other protein degradation systems.

In summary, our results demonstrate that REGγ overexpression can facilitate the growth of laryngeal cancer cells. In addition, we know that a mechanism independent of p53 and p21 may be associated with the role of REGγ in cancer development. Indentification of the mechanism of REGγ overexpression in laryngeal cancer and examination of the proteolytic and pro-survival activity of the nuclear proteasome assembled with REGγ warrant further investigation.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (30872844) and the Project of Scientific and Technical Supporting Programs of Sichuan Province (2010SZ0172) to Dr. Yuedi Tang. We would like to thank Dr. Qingjie Xia at the West China School of Public Health, Sichuan University, China for his advice and Miss. Juan Huang and Miss. Ting Feng at the Joint Research Center, West China Second University Hospital and University of Hong Kong for their assistance.

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