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Original article

Expression of Lgr5, a marker of intestinal stem cells, in colorectal cancer and its clinicopathological significance

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ABSTRACT

Cancer stem cells (CSCs) have been the focus of intense investigations in cancer research although the cellular origin of CSCs has not been clearly determined. Lgr5 is a regulated target of Wnt/β-catenin signaling, which was first identified as a marker of intestinal stem cells. However, the expression of Lgr5 in human colorectal cancer (CRC) and its clinical clinicopathological significance in CRC patients as well as its correlation with Wnt/β-catenin pathway are not fully explored. Localization and expression of Lgr5 in CRC tissues was performed by immunohistochemical staining. The correlation between its expression levels and clinicopathological features as well as clinical outcomes of patients was analysed. The quantitative expression levels of Lgr5 in various CRC cell lines were determined using real-time RT-PCR. The relationship between Lgr5 expression and Wnt/β-catenin pathway in CRC was also investigated. Obviously elevated expression of Lgr5 was observed in CRC tissues, compared to the paired nontumor tissues. mRNA expression levels of Lgr5 was positively correlated with the expression of β-catenin in CRC tissues. The expression of Lgr5 was various in different CRC cell lines. Low and high expression levels of Lgr5 were significantly correlated with clinicopathological features such as TNM stage, lymph node metastasis and vascular invasion of CRC patients. More importantly, Lgr5 expression in CRC tissues was also associated with tumor angiogenesis as well as clinical outcomes. Taken together, these results suggest that elevated Lgr5 expression might contribute to the development and progression of CRC, and it could also be used a potential unfavorable prognostic biomarker for CRC. A better understanding of molecule mechanisms and the relevance of potential value for Lgr5 in the progression of CRC will help to identify a novel therapeutic strategy for CRC patients.

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1. Introduction

Colorectal cancer (CRC) is one of the most common malignant cancers in China as well as the western world, which has the third highest incidence and the fourth highest mortality rate [1–3]. Many treatment protocols have been applied to CRC, but they have not resulted in a complete cure, which may be due to CRC stem cells (CSCs) that are resistant to chemotherapy and radiation, and may enable the recurrence of CRC [4]. CSCs comprise small subpopulation of cells displaying stem cell properties, such as the capability of self-renewal, asymmetric cell differentiation, which are thought to play critical roles in the development and maintenance of a malignant neoplasm [5,6]. Thus, a thorough understanding specific biomarker of CSCs is critical for elucidation

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of mechanisms on carcinogenesis and search for curative therapeutic strategies.

It is generally accepted that activation mutations of APC or β-catenin gene in the Wnt pathway plays a pivotal role in the CRC tumorigenesis, which is associated with about 90% of human CRC [7,8]. Meanwhile, recent study also showed the evidence for important roles of Wnt signaling in the stem cells maintenance and development [9]. Leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5), a member of G-protein-coupled receptor (GPCR) family of proteins, is also a regulated target of Wnt signaling, was first identified as a marker of intestinal stem cells [10]. Evidence for the existence of colon CSCs has been the most convincing, with Lgr5+ cells of particular interest in CSCs studies [11,12]. Subsequent studies have demonstrated that Lgr5 protein was overexpressed in several solid malignant tumors and Lgr5 has a close association with initiation and recurrence of different cancer types [13–15]. Moreover, crypt Lgr5+ stem cells in intestine have been observed to be represented as the cells of origin for intestinal cancer [11]. Significantly, research has further demonstrated that Lgr5 plays a role in tumor progression likely due to mutational activation of the Wnt/β-catenin pathway [16].

However, the expression of Lgr5 in human CRC and its clinical clinicopathological significance as well as the correlation between Lgr5 expression and Wnt/β-catenin pathway in CRC are not fully explored. In the current study, first, we examined the expression level of Lgr5 in human CRC tissues and various CRC cell lines. Next, we analyzed the clinical clinicopathological correlation of the Lgr5 expression levels in CRC patients. Finally, the relationship between Lgr5 expression and Wnt/β-catenin pathway in CRC was also investigated.

2. Materials and methods

2.1. Patients and specimens

Tissue samples from 53 CRC patients were collected during surgical resections performed at the Department of General Surgery, the First Affiliated Hospital of Soochow University between 2009 and 2010. The paired non-tumor tissues samples were used as controls. None of the patients had received any preoperative chemotherapy, radiotherapy or immunotherapy. Tumors were staged according to the American Joint Committee on Cancer (AJCC) pathologic tumor-lymph node metastasis (TNM) classification. Fifty patients were followed up for survival. Informed consent was obtained from all study subjects before sample collection and these samples were used according to ethical standards.

2.2. Cell lines and culture

The human CRC cell lines (SW620, Caco-2, SW480, LoVo, and HCT116) were preserved in our institute. Cell lines were seeded in 6-well plate at a density of 1.5×10^5/well and maintained in RPMI1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Hangzhou, China) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Immunohistochemistry (IHC)

Sections were subjected to routine immunohistochemical (IHC) staining as previously described [17]. Samples were fixed in 10% neutral formaldehyde, embedded in paraffin, and sliced. Briefly, the paraffin-embedded tissues were serially cut into 4 μm sections, dewaxed, and rehydrated. Sections were then blocked with peroxide and non-immune animal serum and incubated sequentially with rabbit antibody to human Lgr5 (Abcam, Cambridge, MA, USA) and Mouse anti-human CD34 monoclonal antibody (Beijing Zhongshan Biotechnology Co., Ltd, China), and biotin-labeled goat anti-rabbit IgG. Finally, the sections were stained with DBA, counterstained with hematoxylin, dehydrated, cleared in xylene, and fixed.

2.4. Evaluation of the IHC results

IHC staining was independently examined by two clinical pathologists who were unaware of the patient outcome. Interpretation and evaluation of IHC results was as previously described [18]. Vascular endothelial cells or clusters of brown-stained cells were defined as microvasculature as long as they formed clear boundaries with adjacent capillaries, tumor cells or other connective tissue. The mean value of Lgr5+ cells and the density of CD34 were calculated in the 5 selected fields. The median value of Lgr5+ cells was used to categorize the 53 tumor tissues into Lgr5+ high and low frequency groups.

2.5. Quantitative real-time PCR (qRT-PCR)

The mRNA expression of Lgr5 in CRC cell lines was quantified by qRT-PCR. Total RNA was isolated from cells using Trizol Reagent (Invitrogen) and quantified. cDNA was synthesized from 5 μg of RNA using AMV reverse transcriptase (Fermentas, USA) according to the manufacturer's instructions. Lgr5 was amplified from the cDNA by qRT-PCR. The PCR conditions consisted of 5 min at 95 °C one cycle, 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C, and 7 min at 72 °C 35 cycles. The primer sequences were 5′-CTCCCAGGTCTGGTGTGTTG -3′ (forward) and 5′-GAGGTCTAGG-TAGGAGGTGAAG -3′ (reverse) for Lgr5; 5′-TGCCAAGTGGGTGGTA-TAGAG-3′ (forward) and 5′-TGGGATGGTGGGTGTAAGAG-3′ (reverse) for β-catenin; 5′-TGTGGGCATCAATGGATTTGGV-3′ (forward) and 5′-ACACCATGTATTCCGGGTCAAT -3′ (reverse) for GAPDH.

2.6. Statistical analysis

Statistical analysis was performed with SPSS17.0 software (SPSS Inc, Chicago, USA). Data are expressed as the mean ± standard deviation (SD). The correlation between Lgr5 expression and MVD was determined by Pearson correlation analysis, and the correlation between IHC results and clinical pathological characteristics was determined by Fisher's exact test. Survival was estimated by the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis of prognostic factors was performed using the Cox proportional hazards model. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of Lgr5 was increased in CRC tissues comparing to the paired non-tumor tissues

To elucidate the role of Lgr5 in colorectal tumorigenesis, we firstly investigate the expression of Lgr5 in primary CRC tissues by IHC staining. As shown in Fig. 1, expression of Lgr5 protein was found mostly in the cytoplasm and membrane of cancer cells in CRC tissues. As reports in many studies, Lgr5+ cells moved up to the crypt surface in a patchy distribution pattern and diffused in some cases of CRC tissues. In the 53 cases of CRC patients, 38 cases (78.6%) were positive for Lgr5 expression, among whom 24 (45.3%) were at strong level of Lgr5 expression. On the contrary, Lgr5 was

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