Original Article
Expression level and clinical significance of low-density lipoprotein receptor-related protein 1B gene in cervical squamous cell carcinoma

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Abstract: Objective: To quantitatively measure the expression of low-density lipoprotein receptor-related protein 1B gene (LRP1B) in normal and cervical squamous cell carcinoma tissues and explore the correlation between LRP1B gene and age, degree of tumor differentiation, clinical staging, degree of infiltration, lymph node metastasis and the infection of human papilloma virus (HPV) 16/18. Methods: Forty patients diagnosed with cervical squamous cell carcinoma and 20 healthy subjects were recruited. The expression of LRP1B mRNA was quantitatively measured by *in situ* hybridization using LRP1B oligonucleotide probe. The expression of HPV16/18 in the cervical squamous cell carcinoma specimen was investigated. Results: Among 40 cervical carcinoma samples, the expression level of LRP1B mRNA was down-regulated in 22. Among 20 healthy controls, low expression of LRP1B mRNA was observed in 3. The negative rate of LRP1B mRNA expression in the cervical carcinoma group was significantly lower compared with that in the normal tissues ($P<0.05$). The positive rate of HPV16/18 in patients with negative LRP1B was significantly higher than that in their counterparts positive for LRP1B ($P<0.05$). In the cervical carcinoma group, the expression of LRP1B mRNA was not correlated with age, degree of tumor differentiation or clinical staging (all $P>0.05$). The expression level of LRP1B mRNA in patients with serous membrane infiltration and lymph node metastasis was significantly down-regulated (both $P<0.05$). Conclusion: LRP1B is lowly expressed in cervical squamous cell carcinoma tissues, suggests LRP1B gene probably acts as a new tumor suppressor gene. LRP1B gene alteration and HPV16/18 infection play a coordinating role in the incidence of cervical cancer. LRP1B expression is intimately correlated with the incidence and metastasis of cervical squamous cell carcinoma.

Keywords: Cervical squamous cell cancer, LRP1B, *In situ* hybridization, HPV16/18

Introduction

Cervical cancer is one of the most prevalent malignant tumors in the female with the 2nd highest incidence rate and mortality rate secondary to breast cancer. Globally, it has been estimated that approximately 500,000 cases are newly diagnosed with cervical cancer with an increasing incidence at a younger age. The incidence of cervical cancer accounts for approximately 5% of all categories of carcinomas, and 80% of cases are reported in the developing countries [1]. Therefore, understanding of the mechanism underlying the incidence, metastasis and recurrence of cervical cancer is of clinical significance. LRP1B is a novel candidate tumor suppressor gene inacti-

vated by genetic and transcript alterations in almost 50% of non-small-cell lung cancer cell lines. The gene-encoded protein is highly homologous to the gigantic (LRP1) that belongs to the family of low-density lipoprotein receptors [2]. Previous investigations have demonstrated that LRP1B is extensively expressed in both normal and tumor cells [3], and it is responsible for regulating the adherence, proliferation, differentiation and angiogenesis of cancer cells. Abnormal LRP1B gene transcription and loss of expression have been reported in multiple types of malignant tumors, such as esophageal squamous cell carcinoma, oral squamous cell carcinoma, glioma and acute leukemia. The exact mechanism underlying these events is associated with loss of mRNA

homozygote and enhanced CpG island methylation. There are 2 types of cells on the surface of the cervix, squamous and columnar cells. Most cervical cancers are derived from squamous cells. In addition, abnormal expression of LRP1B has been reported in esophageal squamous cell carcinoma and oral squamous cell carcinoma [4-6].

Consequently, patients diagnosed with cervical squamous cell carcinoma were selected in this study. *In situ* hybridization technique was performed to statistically compare the expression levels of LRP1B in cervical squamous cell carcinoma and normal tissues, aiming to investigate the potential correlation and clinical significance between LRP1B expression, age, tumor staging, degree of tumor differentiation, infiltration and lymph node metastasis in patients with cervical squamous cell carcinoma.

Materials and methods

Sampling source and processing

The cancerous cervical sampling was collected from 40 patients diagnosed with primary cervical squamous cell carcinoma in the Department of Pathology, the Fourth Affiliated Hospital of Harbin Medical University between January 2014 and January 2016. All patients were aged between 30 and 72 years. The cervical tissue specimen was fixed in neutral formalin solution and paraffin embedded. No patients received preoperative radiotherapy or chemotherapy. According to the standard criteria for cervical cancer staging proposed by The International Federation of Gynecology and Obstetrics (FIGO) in 1971, 4 patients were diagnosed with primary cervical cancer, 21 with stage I, 15 with stage II, 12 with highly-differentiated cervical cancer, 17 with moderately-differentiated cervical cancer and 7 with lowly-differentiated cervical cancer. Ten cases presented with seromembranous infiltration and 11 with lymph node metastasis.

Agents

LRP1B kit, HPV16/18 probe and DAB agent were purchased from ZSGB-bio Company (Beijing, China). All experimental procedures were performed strictly according to the manufacturers' instructions.

MRNA sequence of target genes of human LRP1B: (1) 5'-AATTG CCAAC AGCTG AATTG TCAGT ATAAA TGTAC-3'; (2) 5'-CATAC TCTGG ATTTT ATTTA TAATG AAGAT ATGAT-3'; (3) 5'-AGATG TTGAA TGGAT GGGAT GAACC GGACA AGGAT-3'.

Paraffin section

The specimen was collected, fixed in 4% paraformaldehyde, Paraffin-embedded specimens were first deparaffinized in xylene and ethanol before rehydration in water, 4-μm serial section and dried.

In situ hybridization

Paraffin-embedded specimens were first deparaffinized in xylene and ethanol before rehydration in water, quenched for 30 min with 3% H_2O_2 in methanol. After a wash with phosphate buffered saline (PBS), antigen retrieval was performed by incubation in citrate buffer (0.01 M, pH=6.0) for 60 min at 60°C. In situ hybridization was performed after pepsin digestion. Specimens were then cooled slowly and washed with PBS. Sections were blocked with BlockAce (BlockAce; DS Pharma Biomedical Co., Ltd., Osaka, Japan.) for 30 min at room temperature before applying DAB. Finally, slides were then rinsed in tap water, counterstained with hematoxylin, washed and mounted. Using oligonucleotide probe and highly-sensitive labeling techniques, LRP1B and HPV probes labeled with digoxin were prepared for subsequent in situ hybridization.

Evaluation outcomes

Yellow-brownish granules were considered as positive cells, which were mainly distributed in the cytoplasm, occasionally stained in the nucleus or both the cytoplasm and nucleus simultaneously. Five visual fields were randomly selected under the microscope (×400), and 100 cells were chosen for each visual field. The positive intensity and cell quantity were measured to evaluate the staining outcomes. Immunoreactive score (IRS) was calculated according to the equation: IRS=SI (staining intensity) × PP (percentage of positive specimen). Based upon the IRS, no staining was deemed as 0 point, slight staining as 1 point, moderate staining as 2 points and strong staining as 3 points. The percentage of positive cells less than 10% was considered as 0 point, 10% to
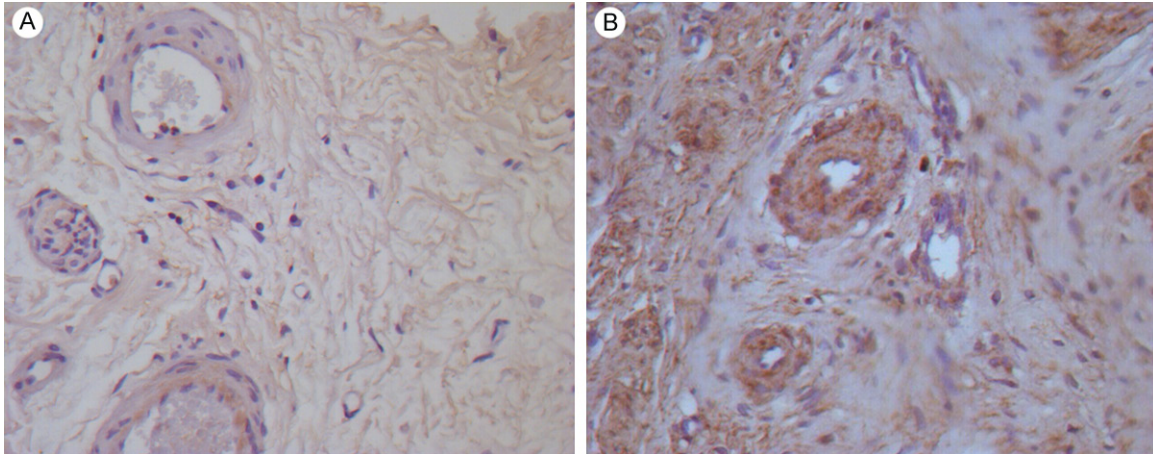
Figure 1. A. Expression of LRP1B in normal cervical tissues (×400); B. Expression of LRP1B in cervical squamous cell cancer tissue (×400).

Table 1. Expression of LRP1B in cervical squamous cell cancer and normal cervix

Histological classification	No. of cases	LRP1B			X^2 value	P
		Positive	Negative	Negative rate (%)		
Cervical squamous cell carcinoma tissue	40	22	18	45.00	5.28	0.022
Normal cervical tissue	20	17	3	15.00		

Table 2. Expression of LRP1B and HPV16/18 in cervical squamous cell cancer

HPV16/18 E6	LRP1B		Total
	+	-	
+	12	16	28
-	10	2	12
Total	22	18	40

50% as 1 point, 51% to 75% as 2 points and higher than 75% as 3 points. Immunoreactive score (IRS) was calculated according to the equation: IRS=SI (staining intensity) × PP (percentage of positive specimen). The IRS for each sample was calculated and classified. IRS ranging from 0 to 3 points was deemed as low expression or loss of expression, which was marked as (-), and IRS higher than 4 points was considered as normal expression level, which was labeled as (+).

Statistical analysis

The 2-sample t-test and Welch's t-test were used to compare for parametric and nonparametric data. Tests for no correlation were performed to correlate independent factors, such as age, body weight with dependent variables.

A *P* value of less than 0.05 was considered as statistical significance. All data were expressed as mean ± standard deviation (SD).

Results

Comparison of expression levels of LRP1B in cervical squamous cell carcinoma and normal cervical tissues

Under the light microscope, LRP1B expression was mainly distributed in the cytoplasm, as illustrated in **Figure 1**. Among 40 cases of cervical squamous cell carcinoma, LRP1B mRNA was positively expressed in 18 (45%) cases. In the control group, the positive rate of LRP1B expression was calculated as 85% (17/20). The positive rate of LRP1B expression significantly differed between two groups (P<0.05), as illustrated in **Table 1**.

Correlation between HPV16/18 E6 and LRP1B protein expressed in cervical squamous cell carcinoma tissue

Among 28 cases positive for HPV16/18E6 protein expression, 16 were negative for LRP1B protein expression. Of 12 patients with negative expression of HPV16/18 E6 protein, merely

Table 3. Correlation between clinopathological parameters and expression of LRP1B in cervical squamous cell cancer

Clinicopathological characteristics		No. of cases	LRP1B			X^2 value	P
			Positive	Negative	Negative rate (%)		
Age	≤50	26	15	11	42.30	0.22	0.641
	>50	14	7	7	42.90		
FIGO classification	Primary carcinoma	4	3	1	25.00	4.65	0.098
	Stage I	21	14	7	33.33		
	Stage II	15	5	10	66.67		
Histological classification	High differentiation	12	5	7	58.33	0.9	0.64
	Moderate differentiation	17	10	7	41.18		
	Low differentiation	7	4	3	42.86		
Infiltration depth	No serous membrane infiltration	30	20	10	33.33	4.85	0.028
	Serous membrane infiltration	10	2	8	80.00		
Lymph node metastasis	No	29	20	9	31.25	6.15	0.012
	Yes	11	2	9	87.50		

2 cases were negative for LRP1B protein expression. Significant correlation was observed between the expression status of HPV16/18 E6 protein and LRP1B protein (P<0.05), as illustrated in **Table 2**.

Expression profile of LRP1B in cervical squamous cell carcinoma tissues

No statistical significance was noted between the expression of LRP1B and age, degree of tumor differentiation or clinical staging. In terms of the degree of tumor infiltration, the negative expression rate of LRP1B in patients with seromembranous infiltration was calculated as 80%, significantly higher compared with 33.33% in those without seromembranous infiltration (P<0.05). Regarding the lymph node metastasis, the negative expression rate of LRP1B in patients with lymph node metastasis was counted as 87.5%, significantly higher compared with 31.25% in their counterparts without lymph node metastasis with statistical significance (P<0.05), as illustrated in **Table 3**.

Discussion

The main types of cervical cancer include squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma begins in the thin, flat cells that line with the cervix. Adenocarcinoma begins in cervical cells that form mucus and other fluids. Long-lasting infections with certain types of HPV can cause almost all cases of cervical cancer. The risk of cervical cancer in HPV patients is significantly higher compared with the correlation between smoking and the incidence of lung cancer. Vaccines that protect against infection with these types of HPV can greatly reduce the risk of cervical cancer. Having a Pap test to check for abnormal cells in the cervix or a test to check for HPV can identify those cells which potentially become malignant. These cells can be treated before transforming into malignancy. Cervical cancer can usually be cured if it is found and treated in the early stages. There are multiple types of HPV, and many do not cause problems. HPV infection usually self-heals without any intervention within a few months after acquisition, and approximately 90% restores within 2 years. A small proportion of infections with certain types of HPV can persist and progress to malignant tumors. Cervical cancer is the most common HPV-related disease [7-9]. Nearly all cases of cervical cancer can be attributable to HPV infection. Though previous data on anogenital cancers other than cervical cancer are limited, there is an increasing mass of evidence linking HPV with multiple types of cancers, such as the anus, vulva, vagina and penis. Although these cancers are less frequent than cervical cancer, their association with HPV makes them potentially preventable using similar primary prevention strategies as those for cervical cancer. Non-cancer causing types of HPV, especially types 6 and 11, can lead to genital warts and respiratory papillomatosis. Although these conditions rarely result in mortality, they may cause the incidence of severe diseases.

LRP1B gene was isolated from the non-small cell lung cancer for the first time in 2000, which was linked with the potential function of tumor suppression [10]. Currently, the potential role of LRP1B gene as a tumor suppressor gene is still being validated by accumulated evidence. Due to multiple exons and large proteins encoded by LRP1B gene, it is a challenging task to explore the exact function of LRP1B. In this study, the expression levels of LRP1B gene in the cervical squamous cell carcinoma and normal cervical tissues were quantitatively measured by using *in situ* hybridization technique. Moreover, the relationship between the LRP1B expression and clinicopathological characteristics was equally investigated in cervical squamous cell carcinoma patients, thereby providing a novel perspective for analyzing the function of LRP1B gene.

Previous investigations have demonstrated that LRP1B is capable of negatively regulating the urokinase-type plasminogen activator (uPA) system through binding with the site of uPA [11]. The uPA system has been proven to change the activity of cellular basement membrane, promote the degradation of extracellular matrix and accelerate the migration, infiltration and transition of tumor cells [12]. Sheng et al. [13] have quantitatively detected the uPA content in both the plasma and tissues of 42 cervical cancer patients and 10 controls with benign cervical tumors and concluded that uPA content might offer certain reference value for estimating the infiltration degree, lymph node metastasis and clinical prognosis of patients diagnosed with cervical cancer. In addition, uPA system can also destroy the balance between pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF). PEDF, also known as serpin F1 (SERPINF1), is a multifunctional secreted protein that has anti-angiogenic, anti-tumorigenic, and neurotrophic functions. First identified in vertebrates, PEDF with a size of 50 kDa protein is being researched as a therapeutic candidate for treatment of such conditions as choroidal neovascularization, heart disease, and cancer. In humans, pigment epithelium-derived factor is encoded by the SERPINF1 gene and widely expressed in human eyeball, nervous system, skeleton and endothelial cells, *etc*. Previous investigations have demonstrated that PEDF or VEGF was abnormally expressed in patients diagnosed with malignant tumors, such as liver cancer, renal carcinoma and laryngeal carcinoma [14].

In this present investigation, the expression level of LRP1B in cervical squamous cell carcinoma specimen was significantly lower than that in normal cervical tissues with statistical significance, suggesting the intimate correlation between down-regulated expression of LRP1B and the incidence of cervical squamous cell carcinoma. The positive rate of HPV16/18E6 in patients with negative LRP1B was significantly higher compared with that in their counterparts positive for LRP1B, indicating that LRP1B and HPV probably play a coordinating role in the occurrence of cervical squamous cell carcinoma. Meantime, no intimate correlation was observed between the low expression of LRP1B, age, clinical staging and histological classification in patients diagnosed with cervical squamous cell carcinoma, whereas the abnormal expression of LRP1B was significantly associated with degree of tumor infiltration and lymph node metastasis, suggesting that the metastasis of cervical squamous cell carcinoma is intimately correlated with abnormal expression of LRP1B.

Taken together, LRP1B probably plays a potential role in the incidence and progression of cervical squamous cell carcinoma. It may serve as a potential molecular target for the clinical diagnosis, treatment and prognosis of cervical cancer for clinicians.

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Disclosure of conflict of interest

None.

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