Methylation Patterns of the IFN-γ Gene in Cervical Cancer Tissues

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Objective: To explore the relationship between methylation of interferon gamma (IFN-γ) gene and tumorigenesis in cervical cancer tissues, the biopsy specimens of cervical cancer and cervical intraepithelial neoplasia (CIN) (I-III) patients as well as normal controls were collected and analyzed. Methods: The methylation of the IFN-γ gene was verified by using methylation-specific PCR and DNA sequencing analysis, and the expression levels of IFN-γ mRNA were detected using quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). Results: The methylation rates of the IFN-γ gene were significantly higher in cervical cancer tissues (15/43, 34.9%) than those in CIN (3/23, 13.0% of CIN I; 6/39, 15.4% of CIN II/III) and normal cervical tissues (2/43, 4.7%) (P < 0.01). Furthermore, the mRNA expression of IFN-γ in cervical tumors with methylation (0.71 ± 0.13, n=8) was lower than that in those without methylation (1.58 ± 0.32, n=27) (P < 0.05). Likewise, the IFN-γ expression levels in CIN II/III tissues with methylation (0.87 ± 0.16, n=5) were significantly (P < 0.01) lower compared to those without methylation (2.12 ± 0.27, n=32). Conclusion: The hypermethylation of IFN-γ gene may be related with tumorigenesis of cervical cancer.

Cervical cancer is ranked as the second most common cancer in women worldwide, and continues to be a major public health problem. Immunological imbalance created by infiltrating inflammatory cells may contribute to cancer growth and spread of cervical carcinogenesis. The cytokine network of several common tumours is rich in inflammatory cytokines, growth factors, and chemokines. Now there are evidences that inflammatory cytokines and chemokines, which can be produced by the tumour cells and/or tumour-associated leucocytes and platelets, may contribute directly to malignant progression. Examples are tumour necrosis factor-alpha (TNF-α), IL-1 and 6, and Interferon gamma (IFN-γ). The local production of cytokines within the tumor microenvironment can prevent the effector’s response and cytokines also can mediate the activities of immune cells in the fight against malignant cells.

IFN-γ is a pleiotropic cytokine secreted by type-1 helper (Th1) T cells, cytotoxic T cells, and stimulated natural killer cells in response to antigenic stimulation and involved in activation of macrophages and endothelial cells. Production of IFN-γ is related to the induction of reaction in T lymphocytes, which contributes to enhance an immune response against malignant cells.

DNA methylation is characterized by the addition of methyl groups in cytosines within cytosine-phosphate-guanine (CpG) islands. Unmethylated islands are related with transcriptionally active regions, whereas methylated DNA recruits methyl-binding proteins that promote chromatin compaction and prevent the binding of transcription factors. Recently, evidence is emerging about the importance of epigenetic regulatory mechanisms in the control of inflammatory response. Changes in DNA methylation patterns, particularly in the promoter region of genes, can have profound effects on gene expression.

Epigenetic changes occur in cytokine genes in human cells, affecting the ability of the cell to express cytokines. Methylation profiling of cancer cells and studies of individual genes disclose that gene-specific hypomethylation occurs frequently in diverse cancers, e.g., colon cancer and breast cancer. Furthermore, hypomethylation of specific genes correlates well with increased transcription levels. Although CpG methylation in the IFN-γ promoter is considered a negative transcriptional regulator of IFN-γ production, such events have not been investigated in cervical tissues yet. Therefore, the aim of the present study was to evaluate the methylation status of the IFN-γ promoter region in cervical cancer tissues compared to normal and cervical intraepithelial neoplasia (CIN) tissues.
Methods

Clinical samples and DNA extraction. Human tissue samples were obtained from 148 patients (43 normal cervical tissues, 62 CIN tissues (CIN I: 23 cases; CIN II/III: 39 cases) and 43 cervical squamous carcinomas tissues) from January 2012 to October 2013. Cervical tissues were gained by trans-vaginal biopsies and then flash-frozen in liquid nitrogen and stored at -150 °C. All of these patients have been informed consent before collection of their samples, according to institutional guidelines. This protocol was approved by a regional ethics committee, in Tangshan of Hebei province, China. After detection, they underwent surgical resection of primary cervical cancer at the Department of Obstetrics and Gynecology in Tangshan workers hospital. The histological type and grade of tumor were classified on the basis of WHO criteria. The stage of each cancer was established according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria. These tissue samples for CIN diagnosis were performed by using micro-excision. All primary tumor tissues and control samples were diagnosed by HE-stained. The frozen samples were used for genomic DNA extraction from tissues using the standard Proteinase K treatment followed by phenol/chloroform extraction. The concentration of DNA was determined with a spectrophotometer.

Bisulfite Modification. Methylation pattern of tissues were assessed using DNA modification by bisulfite treatment similar to that reported by Goldenberg et al. With the bisulfite treatment, unmethylated cytosines of DNA were converted to uracil, whereas methylated cytosines remained unmodified. Extracted genomic DNA was modified using bisulfite conversion kit (EZ DNA Methylation-Gold Kit™, Zymo Research Corp.). The modified DNA was ready for immediate analysis or could be stored at or below -20 °C for later use. For long term storage, it should be stored at or below -70 °C. The volume of 1 μl eluted DNA was used for each PCR.

Methylation-specific PCR (MSP). Methylation-specific polymerase chain reaction are distinguished from the presence of methylation based on alterations produced after bisulfite treatment of DNA using specific primers for methylated or unmethylated DNA. All the samples were analyzed by MSP. The Primer sequences, annealing temperatures, and the expected product sizes were listed in Table 1.

Methylated and unmethylated PCR products were confirmed by sequencing of the MSP reactions of IFN-γ promoter methylation was 34.9% in cervical cancer, 13.0% in CIN I, 15.4% in CIN II/III, and 4.7% in normal cervical tissues. The methylation rates of the IFN-γ gene promoter were significantly higher in cervical cancer tissues than those in CIN and normal tissues (P = 0.003) (Table 2).

DNA Sequencing of MSP Products. The DNA sequencing was carried out in samples of the control, CIN I, CIN II/III and cervical cancer groups, respectively. Sequencing of the MSP products from the methylated control DNA showed the expected nucleotide changes. Representative results of bisulfite sequence analysis for IFN-γ gene promoter were shown in Fig. 1B. There was only one CpG site in the amplicon excluding the primer sites.

Transcriptional activation by IFN-γ methylation in the cervical tissues. The mRNA expressions of IFN-γ promoter methylation on transcriptional silencing in control, CIN I, CIN II/III, and cervical cancer tissues were detected by real-time RT-PCR. The loss of IFN-γ expression was present 1 of 43 (2.3%) in Control, 2 of 23 (8.7%) in CIN I, 2 of 39 (5.1%) in CIN II/III, and 8 of 43 (18.6%) in cancer, respectively (Tab. 3). IFN-γ mRNA levels in cervical tumors with methylation (0.71 ± 0.13, n=8) were significantly (P < 0.05) lower than those in cervical tumors unmethylated (1.58 ± 0.32, n=27), and IFN-γ mRNA levels in CIN II/III tissues with methylation (0.87 ± 0.16, n=5) were significantly (P < 0.01) lower than those unmethylated (2.12 ± 0.27, n=32) (Fig. 2).

Clinicopathological features and hypermethylation of IFN-γ in cervical cancer. Based on the methylation status, the results of

| Table 1 | Primer sequences, annealing temperatures, and PCR product sizes |
|---|---|---|
| Gene | Sequence (5’-3’) | Location | Annealing temperature (°C) | Product size (bp) |
| IFN-γ | UF | TGAAGCTTAATTACATTAGAGGTGA | -320 ~ -164 | 60 | 156 |
| | Ur | TAAACTCCCTAAATGTCTTAAACAT | | | |
| | Mt | AAGACTTAATTATTTTAGGGGGA | -317 ~ -164 | 59 | 154 |
| | Mr | TAAACTCCCTAAATGTCTTAAAGGAT | | | |

GenBank accession no.: J00219.

| Table 2 | The methylation of the IFN-γ gene in normal cervical tissue, CIN and cervical cancer |
|---|---|---|
| Group | Age (years old) | Methylation (n) | Methylation rate (%) |
|---|---|---|---|
| Control (n=43) | 42.67 ± 0.10.61 | 2 | 4.7 |
| CIN I (n=23) | 43.32 ± 8.54 | 3 | 13.0 |
| CIN II/III (n=39) | 42.97 ± 9.48 | 6 | 15.4 |
| Cancer (n=43) | 44.39 ± 10.83 | 15 | 34.9 |
| PValue | 0.367 | 0.003 |

1. ANOVA test, b. Fisher’s exact test. Compared with control group, *P < 0.05; compared with CIN I group, *P < 0.05; compared with CIN II/III group, *P < 0.05.
Multiple variable analysis of the IFN-\(\gamma\) promoter regions in cervical cancer tissues were shown in Table 4. The clinicopathological parameters were evaluated including nodal status, squamous carcinomas pathology classification and clinical stages. There were no obvious relationship between methylation of IFN-\(\gamma\) and the following prognostic factors—nodal status (\(P = 0.539\)), squamous carcinomas pathology classification (\(P = 0.237\)), and clinical stages (\(P = 0.396\)).

Discussion

In the present study, the methylation patterns of the IFN-\(\gamma\) gene in cervical tissues from normal controls, CIN and cervical cancer patients were detected. These data provided evidence that IFN-\(\gamma\) methylation was higher in cervical cancer tissue than in the normal and CIN groups, and DNA hypermethylation in the promoter region may influence gene activation during cervical tumorigenesis.

IFN-\(\gamma\) exhibits both antitumor and protumor activities\(^{18}\). The dual opposing functions of immunity formed conceptual basis for cancer immuno-surveillance, equilibrium and escape, also named cancer immunoediting\(^{19}\). In this process, many immune cells might interact with tumour cells from the earliest stages of transformation to the terminal phase of widespread metastasis, in which IFNs have been found to have distinct functions. Vijay Shankaran, et al\(^{20}\) found that lymphocytes and IFN-\(\gamma\) collaborate to protect against development of carcinogen-induced sarcomas and spontaneous epithelial carcinomas and also to select for tumor cells with reduced immunogenicity. Therefore, IFN-\(\gamma\) has been shown to be crucial components of the cancer-immunoediting process, and controls the immunogenicity of tumour cells, possibly as a result of the selective production of IFN-\(\gamma\) in the tumor microenvironment\(^{21}\).

IFN-\(\gamma\) is a major contributor to an effective Th1-type cellular immune response against HPV infection\(^{22}\), and nearly all cells constitutively express functional IFN receptors. Many studies shown that a wide complement of immune cells can be found in the cervical epithelium, including T cells (CD4\(^+\) and CD8\(^+\) T cells, major sources of IFN-\(\gamma\))\(^{23,24}\). Decreased expression of IFN-\(\gamma\) results in suppression of cell-mediated local immune responses and enhances persistent high-risk HPV infection of the uterine cervix, which may promote HPV invasion and tumor progression\(^{25,26}\). Reduced epithelial and sub-epithelial IFN-\(\gamma\), may play a role in the development and progression of HPV16 associated cervical pre-cancer\(^{27}\). The role of IFN-\(\gamma\) has been studied extensively and there was evidence that intrallesional IFN-\(\gamma\) might be a prognostic marker for clearance of high-risk HPV\(^{28}\).

The existence of a cancer-immunosurveillance process illustrate that suppression of tumour growth might be mediated by extrinsic

| Table 3 | Relationship between expression and methylation of IFN-\(\gamma\) in cervical tissues |
|-----------------------------|---------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| IFN-\(\gamma\) mRNA Expression | Methylation status | Control (n=43) | CIN I (n=23) | CIN II/III (n=39) | Cancer (n=43) |
| Present | Methylated | 1 | 1 | 5 | 8 |
|         | Unmethylated | 42 | 20 | 32 | 27 |
| Absent | Methylated | 1 | 2 | 1 | 7 |
|         | Unmethylated | 0 | 0 | 1 | 1 |
forces. Epigenetic modification of gene expression is a novel mechanism by which environmental exposures may influence disease expression through modification of promoter regions regulating gene transcription. Recent evidence indicates that methylation patterns changes could occur in cervical cancer. The interaction between DNA methylation and inflammation may have relevance for analyzing the effect of inflammation on tumor development. As a naive CD4+ T cell develops into a Th1 cell that secretes predominantly IFN-γ, the expression or permanent silencing of one cytokine gene is orchestrated using epigenetic mechanisms. Activated Ag-specific CD8 T cells exhibit rapid DNA demethylation at IFN-γ promoter and fail to exhibit increased DNA methylation at the IL-2 promoter and fail to acetylate histones at the IFN-γ locus. This study suggested that most cervical cancer samples exhibited hypermethylation of IFN-γ in contrast to control samples. This indicates that cervical tumorigenesis may be associated with DNA hypermethylation in the promoter region of the IFN-γ gene.

To verify the hypothesis of epigenetic regulation of IFN-γ production, the transcription of this gene in each group was evaluated according to the MSP status. Although loss of IFN-γ expression was present in 1 of 43 (2.3%) in Control, 2 of 23 (8.7%) in CIN I, 2 of 39 (5.1%) in CIN II/III, and 8 of 43 (18.6%) in cancer, the methylated samples with observed gene expression showed lower mRNA levels of IFN-γ than unmethylated samples in CIN II/III and cancer groups. There was only one methylated sample present in control and CIN I tissues respectively, and gene expressions were also low in control (0.77) and CIN I (0.62) tissues. These data would suggest that decreased production of IFN-γ via hypermethylation and silencing of its gene promoter could enhance Th2 differentiation and Th2 cytokine-directed inflammation and persistence infection of high-risk HPV, which could be second or/and third phase of cancer immunosurveillance process. Although we did not investigate the entire epigenome in this study, it is probable that epigenetic modification of multiple genes could also be associated with cervical tumorigenesis.

The methylation of IFN-γ had no obvious association with some prognostic variables including age, nodal status, squamous carcinoma pathology classification and clinical stages of cervical cancer. However, IFN-γ methylation demonstrated a significant correlation with the lower expression of IFN-γ.

The present study suggests that methylation induced silencing of IFN-γ gene may play an important role in the pathogenesis of cervical cancer. In future studies, methylation status of more cytokines should be further investigated to identify the relationships between their methylation status and cervical tumorigenesis. What’s more, the critical roles need to be recognized which are played by the immune system in cervical oncogenesis, progression and therapeutic response.

| Pathology classification | Patients with methylated IFN-γ [%] (n=15) | Patients with unmethylated IFN-γ [%] (n=28) | P value |
|--------------------------|---------------------------------------------|---------------------------------------------|---------|
| Nodal metastasis         | 12 (80.0)                                   | 20 (71.4)                                   | 0.539   |
| Negative                 | 3 (20.0)                                    | 8 (28.6)                                    |         |
| Squamous carcinomas      |                                             |                                             |         |
| Pathology classification |                                             |                                             |         |
| High                     | 7 (46.7)                                    | 7 (25.0)                                    | 0.237   |
| Middle                   | 3 (20.0)                                    | 12 (42.9)                                   |         |
| Low                      | 5 (33.3)                                    | 9 (32.1)                                    |         |
| Clinical stages (FIGO,2000) |                                         |                                             |         |
| I stage                  | 6 (40.0)                                    | 15 (53.6)                                   | 0.396   |
| II/III stage             | 9 (60.0)                                    | 13 (46.4)                                   |         |

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**Author contributions**

D.M. and C.Y.T. conceived, designed and carried out the experiments, analysed the experimental data and wrote the paper; X.L.H. and H.B.L. guided the experiments and prepared figures 1-2 and tables 1-4; Q.Z.L. co-performed clinical samples collections and DNA extraction experiments; T.T.L and Y.Y.Y. co-performed methylation-specific PCR experiments; O.L. supervised and directed the project. All authors discussed the results and commented on the manuscript and reviewed the manuscript.

**Additional information**

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Huibin Liu was omitted from the author list in the original version of this Article.

The Author Contribution Statement should read ‘D.M. and C.Y.J. conceived, designed and carried out the experiments, analysed the experimental data and wrote the paper; X.L.H. and H.B.L. guided the experiments and prepared figures 1-2 and tables 1-4; Q.Z.L. co-performed clinical samples collections and DNA extraction experiments; T.T.L. and Y.Y.Y. co-performed methylation-specific PCR experiments; O.L. supervised and directed the project. All authors discussed the results and commented on the manuscript and reviewed the manuscript.’

This has now been corrected in the PDF and HTML versions of the Article.