Research Article

Methylation and Expression of Retinoblastoma and Transforming Growth Factor-β1 Genes in Epstein-Barr Virus-Associated and -Negative Gastric Carcinomas

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Background. Retinoblastoma (RB) and transforming growth factor-β1 (TGF-β1) are important tumor-related factors. Methods. A series of 30 EBV-associated gastric carcinoma (EBVaGC) and 38 matched EBV-negative gastric carcinoma (EBVnGC) tissues were examined for the promoter methylation of RB by methylation-specific PCR (MSP) method. The expression of RB and TGF-β1 in gastric carcinoma tissues was detected by immunohistochemistry. Results. The methylation rate of RB gene in EBVaGC and EBVnGC was 80.0% (24/30) and 50.0% (19/38), respectively. The difference of RB methylation rate between EBVaGC and EBVnGC was significant (χ² = 6.490, P = 0.011). There was no significant difference for RB expression between EBVaGC and EBVnGC (43.3%, 13/30) and EBVnGC (63.2%, 24/38), and also for TGF-β1 between EBVaGC (56.7%, 17/30) and EBVnGC (63.2%, 24/38). RB methylation was not inversely correlated with RB expression in gastric carcinoma tissues (χ² = 2.943, P = 0.086, r = 0.208). RB methylation, loss expression of RB, and TGF-β1 expression were significantly associated with tumor invasion and lymph node metastasis (P < 0.05), but was not associated with sex, age, histological subtype (differentiation status) and tumor location. Conclusions. Methylation of RB is a common event in gastric carcinomas and EBV induces methylation of RB in EBVaGC, which may contribute to the development of gastric carcinomas. EBV has no significant effect on induction of TGF-β1 expression. Detection of RB methylation, RB expression, and TGF-β1 expression may be helpful to judge the status of tumor invasion and lymph node metastasis in gastric carcinomas.

1. Introduction

Gastric carcinoma is the second leading cause of cancer-related death worldwide [1]. Epstein-Barr virus (EBV) is a tumor-related herpes virus associated with the transformation of various types of cells, such as lymphoid, dendritic, smooth muscle, and epithelial cells [2]. EBV-associated gastric carcinoma (EBVaGC) is characterized by the monoclonal growth of EBV-infected epithelial cells, and the entity was recognized by Imai in 1994 [3]. EBVaGC is distributed worldwide with an annual incidence of more than 90,000 patients (10% of total gastric carcinoma (GC)) [4]. Following infection, EBV remains in a latent state in EBVaGC, which is classified as latency I. Compared with EBV-negative gastric carcinoma (EBVnGC), EBVaGC has unique clinical and pathological features, such as a younger age of incidence, high incidence in men than in women, and more diffuse than intestinal types [5–7], suggestive of a particular oncogenic mechanism of EBVaGC.

Epigenetic alterations, including methylation of CpG dinucleotides in promoters and changes in chromatin structure, can affect gene expression without modifying the underlying in genetic sequences. Aberrant methylation of promoters in tumor-related genes is now regarded as one...
of the major mechanisms in the development of gastric carcinoma [8]. Tumor-related genes p16, p14, E-cadherin, PTEN (phosphatase and tensin homolog deleted on chromosome ten), RASSFIA (Ras association domain family 1A), GSTP1 (Glutathione S-transferase pi 1), MGMT (O(6)-methylguanine-DNA-methyltransferase), and MINT2 (Munc18-1-interacting protein 2) are hypermethylated in EBVaGC [9–11], suggesting that EBV-related aberrant methylation may play an important role in development of EBVaGC.

Retinoblastoma (RB) and transforming growth factor-β1 (TGF-β1) are important regulatory factors in cell growth and differentiation, whose abnormal transcription or expression are closely associated with tumor occurrence and development. RB was the first successfully cloned human tumor suppressor gene (TSG). Its inactivation may result in cell proliferation leading to tumorigenesis [12]. TGF-β1 is a multifunctional cytokine and triggers an intracellular signal transduction protein to regulate numerous developmental and homoeostatic processes via regulation of gene induction. It plays a dual regulatory role in cell proliferation and differentiation. In the early stage of cancer, TGF-β1 can inhibit cell proliferation through arrest in the G1 phase and be regarded as a tumor suppressor; in the late stages, TGF-β1 becomes a tumor promoting factor by stimulating angiogenesis, cell spread, immune suppression, and synthesis of extracellular matrix [13–16]. It has been reported that EBV latent membrane protein 1 (LMP1) has a resistant to the TGF-β1-mediated growth inhibition in EBV-positive gastric carcinoma cell lines (GT38 and GT39) and indicated that TGF-β1 may be a key factor for EBV reactivation and selective growth of EBV-infected epithelial cells in vivo [17, 18]. However, the LMP1 expression is absent in EBVaGC tissues [19]. The identified role of TGF-β1 in EBVaGC has not been understood well and needs further research. The absence of RB expression and overexpression of TGF-β1 have been found in gastric carcinomas [20, 21], and the mutations and methylation of RB gene in gastric carcinomas were also reported in the literature [22, 23]. Mukherjee et al. [24] found TGF-β1 treatment in late G(1) acutely blocks S-phase entry, this acute block by requiring the function of RB and loss of RB abrogates late-G(1) arrest by TGF-β1, suggesting a novel role for RB in mediating this effect of TGF-β1 late-G(1) arrest through direct interaction with and control of the MCM helicase. However, there is no report about the expression and promoter methylation status of RB and TGF-β1 in EBVaGC and EBVnGC to our knowledge. In this study, we examined RB methylation status, RB and TGF-β1 protein expression in EBVaGC and matched EBVnGC. The aim of the study is to understand the relationship among EBV, RB and TGF-β1 and their role in gastric carcinoma tumorigenesis.

2. Materials and Methods

2.1. Patients and Tissue Samples. Fresh and paraffin-embedded gastric carcinoma tissues were obtained from 1678 gastric carcinoma patients in Shangdong Province, China from 2001 to 2009. The positivity of EBV in GC tissues was determined by EBV-encoded small RNA 1 in situ hybridization, as described previously [25]. The clinical features (gender, age, pathologic grade, location, invasion and lymph node metastasis) matched 30 EBVaGC and 38 EBVnGC samples were chosen for study. The study was approved by the Medical Ethics Committee at the Medical College of Qingdao University, China, and informed consent was received from all patients.

2.2. DNA Extraction. DNA was extracted from fresh tumor tissues using the standard method with proteinase K digestion and phenol-chloroform purification. The QIAamp DNAFFPE Tissue kit (QIAGEN GmbH, Hilden, Germany) was used to extract the DNA from paraffin-embedded tumor tissues.

2.3. Immunohistochemistry (IHC). Paraffin sections were deparaffinized and hydrated as per routine. Rabbit anti-human polyclonal antibody TGF-β1 and mouse antihuman monoclonal antibody RB (ZSGB-Bio) were diluted to 1:50. The reagents (PV9000 and DAB) were obtained from ZSGB-Bio and staining was performed as per protocol. PBS (phosphate buffer saline) was used in replacement of primary antibody as a blank control. The section was considered as expressing the protein if cellular staining ≥5%, following the methods described previously [26, 27].

2.4. Bisulfite Treatment of Genomic DNA and Methylation-Specific PCR (MSP). 5 μg DNA was denatured in 33.3 μL of 0.3 mol/L NaOH at 37°C for 15 minutes. Denatured DNA was mixed directly with 333 μL of bisulfite solution and treated in darkness. The bisulfite solution was prepared as either 2.4 mol/L sodium metabisulfite (pH 5.0–5.2) (Sigma S-1516, St. Louis, MO, USA)/0.5 mmol/L hydroquinone (Sigma H-7148) for a 4-hour treatment [28]. DNA was desalted and purified using the QIAEX Gel Extraction system (QIAGEN, Cat. no.20021). DNA was then treated with 0.3 mol/L NaOH at 37°C for 15 minutes and precipitated with 3 mol/L ammonium acetate (pH 7.0) and 1 mol/L sodium acetate (pH 5.2). Recovered DNA was dissolved in 100 μL of TE buffer (pH 8.0) and stored at −20°C.

RB promoter methylation status was determined using MSP. In this method, bisulfite treatment converts unmethylated cytosine to uracil, but does not affect the methylated cytosine. Thus, PCR primers can be designed that anneal selectively to methylated or unmethylated DNA after bisulfite conversion. The sequences of the unmethylated DNA and methylated DNA-specific primers are listed in Table 1. The primer U/I/UR pair was designed specifically for amplification of the bisulfite-converted unmethylated promoter, while the MF/MR primer pair was designed specifically for the amplification of the bisulfite-converted methylated promoter. MSP results determined whether the samples are methylated or unmethylated. If there is M primers amplified band, the sample was considered to be in the methylation status. One microliter of bisulfite-treated DNA (around 25 ng) was amplified with 1.5 mmol/L MgCl2 and
2.5. Statistical Analysis. RB promoter methylation status, RB expression and TGF-β1 expression between EBVaGC and EBVnGC was compared using the Chi-square test. The correlation between promoter methylation and the protein expression was analyzed by Paired fourfold table Chi-square test. The association of clinical features with RB promoter methylation, RB expression, and TGF-β1 expression was compared by chi-square test. P < 0.05 was considered to be statistically significant.

3. Results

3.1. Comparison of Clinicopathological Data between EBVaGC and EBVnGC Patients. 102 of 1678 (6.1%) cases of gastric carcinoma were EBV positive. 30 EBVaGC and 38 EBVnGC tumor tissues with matching clinical parameters were chosen for methylation detection. The clinical and pathological data are listed in Table 2. The two kinds of gastric carcinomas were similar in gender, age, pathologic grade, location, invasion, and lymph node metastasis.

3.2. The Promoter Methylation Status of RB Gene in EBVaGC and EBVnGC. Promoter methylation of the RB gene was detected by MSP (Figure 1(a)). In total, 43/68 (63.2%) cases of gastric carcinomas demonstrated RB promoter methylation. The difference in the percent of positive methylation bands detected by MSP for RB was statistically different between EBVaGC (24/30, 80%) and EBVnGC (19/38, 50.0%) (P = 0.011).

The association of clinicopathological parameters of 68 cases with RB gene methylation status was studied. There was no relationship of RB gene methylation status with patient age, gender, pathologic types, and tumor location. However, the methylation status was associated with the depth of tumor invasion (P = 0.036) and lymph node metastasis (P = 0.012), (Table 3).

3.3. The Promoter Methylation Status of RB Gene in GC and Corresponding Adjacent Normal Gastric Tissues. The promoter methylation of RB gene was detected in EBVaGC and EBVnGC corresponding adjacent normal gastric tissues (Figure 1(b)). The percent of positive methylation bands by MSP for RB in gastric carcinoma and corresponding adjacent normal gastric tissues was 63.2% (43/68) and 39.7% (27/68); the difference was significant (P = 0.006).

3.4. The Protein Expression of RB and TGF-β1. RB and TGF-β1 protein expression was detected by IHC, shown in Figures 2(a) and 2(b). The percent of positive protein expression by IHC for RB was 43.3% (13/30), lower than in EBVaGC (63.2%, 24/38), but not significantly different (P = 0.103). There was not obvious difference of TGF-β1 protein expression between EBVaGC (56.7%, 17/30) and EBVnGC (63.2%, 24/38) (P = 0.587) (Table 4).

The correlation of RB protein expression with RB promoter methylation was studied. There were 23 negative

| Primers | Sequence | Product size (bp) | Annealing temp (0°C) | Genomic position |
|---------|----------|------------------|----------------------|-----------------|
| RBMF    | 5′GGGAGTTTCGCGGGAGTGAC3′ | 163 | 60 | −61 to 102 |
| RBMR    | 5′ACGTCGAACACCCGGG3′ | 163 | 58 | −61 to 102 |
| RBUF    | 5′GGGAGTGGCGTGAGTGAC3′ | 163 | 60 | −61 to 102 |
| RBUR    | 5′ACATCAAAACACCCCGA3′ | 163 | 58 | −61 to 102 |

| Table 1: List of primers used in MSP. |

| Age (yr)   | EBVaGC (n = 30) | EBVnGC (n = 38) | χ²  | P   |
|------------|----------------|----------------|-----|-----|
| <50        | 18             | 19             | 0.676 | 0.411 |
| ≥50        | 12             | 19             |       |     |
| Gender     |                |                |     |     |
| Male       | 27             | 31             | 0.494 |     |
| Female     | 3              | 7              |       |     |
| Pathologic grade |            |                |     |     |
| Poorly differentiated | 28 | 32 | 0.288 |
| Well-moderately differentiated | 2 | 6 |     |
| Location   |                |                |     |     |
| Gastric cardia | 7             | 7              | 0.738 |     |
| Gastric body | 13            | 15             |       |     |
| Antrum     | 10             | 16             |       |     |
| Depth of invasion |         |                |     |     |
| Invasion to serosa and invasion through serosa | 22 | 24 | 0.793 | 0.373 |
| Not invading serosa | 8  | 14 |       |     |
| Lymph node metastasis |     |                |     |     |
| Positive   | 17             | 21             | 0.134 | 0.908 |
| Negative   | 13             | 17             |       |     |

Table 2: Comparison of clinicopathological data between EBVaGC and EBVnGC patients.
$RB$ protein expression cases in 43 $RB$ gene methylated gastric carcinoma (53.5%), which was higher than in $RB$ gene unmethylated gastric carcinoma (32.0%, 8/25), but without reverse correlation ($P = 0.09$, $r = 0.21$), (Table 5).

The association between $RB$ expression and clinico-pathological parameters is shown in Table 6. There was no relationship between $RB$ protein expression and patients' age, gender, pathological grade, and tumor location, but it was related with the depth of tumor invasion ($P = 0.04$) and lymph node metastasis ($P = 0.02$).

The association between $TGF-\beta 1$ expression and clinico-pathological parameters is shown in Table 6. There was
Table 3: Correlation of methylation status of RB gene with clinicopathological data of gastric carcinoma patients.

|                        | n   | Methylated (n) | Unmethylated (n) | \( \chi^2 \) | P    |
|------------------------|-----|----------------|------------------|--------|------|
| EBV infection          |     |                |                  |         |      |
| EBVaGC                 | 30  | 24             | 6                | 6.490  | 0.011|
| EBVnGC                 | 38  | 19             | 19               |         |      |
| Age (yr)               |     |                |                  |         |      |
| <50                    | 37  | 22             | 15               | 0.498  | 0.481|
| ≥50                    | 31  | 21             | 10               |         |      |
| Gender                 |     |                |                  |         |      |
| Male                   | 58  | 36             | 22               | —      | 0.835|
| Female                 | 10  | 7              | 3                |         |      |
| Pathologic grade       |     |                |                  |         |      |
| Poorly differentiated  | 60  | 40             | 20               |         |      |
| Well-moderately differentiated | 8  | 3              | 5                | 0.216  |
| Location               |     |                |                  |         |      |
| Gastric cardia         | 14  | 10             | 4                |         |      |
| Gastric body           | 28  | 20             | 8                | 3.172  | 0.205|
| Antrum                 | 26  | 13             | 13               |         |      |
| Depth of invasion      |     |                |                  |         |      |
| Invasion to serosa and invasion through serosa | 46 | 33             | 13               | 4.423  | 0.036|
| Not invading serosa    | 22  | 10             | 12               |         |      |
| Lymph node metastasis  |     |                |                  |         |      |
| Positive               | 38  | 29             | 9                | 6.339  | 0.012|
| Negative               | 30  | 14             | 16               |         |      |

Table 4: Comparisons of the expression of RB and TGF-β1 between EBVaGC and EBVnGC.

|         | n   | RB expression | TGF-β1 expression |
|---------|-----|---------------|-------------------|
|         |     | Positive      | Negative          | Positive      | Negative |
| EBVaGC  | 30  | 13            | 17               | 17            | 13       |
| EBVnGC  | 38  | 24            | 14               | 24            | 14       |
| \( \chi^2 \) | 2.656 | 0.295   |
| P       | 0.103 | 0.587          |

Table 5: Correlation of methylation status of RB gene with its protein expression.

| Protein expression | Total |
|--------------------|-------|
| Positive           | 20    |
| Negative           | 23    |
| Total              | 43    |

no relationship between TGF-β1 protein expression and patients’ age, gender, pathological types, and tumor location, but there was positive association between TGF-β1 protein expression and the depth of tumor invasion \( (P = 0.02) \) and lymph node metastasis \( (P = 0.002) \).

4. Discussion

In this study, the percent of EBVaGC with positive methylation bands by MSP for RB was significantly higher than that of EBVnGC, indicating that EBV may induce RB promoter methylation during infection. Previous studies showed that EBVaGC had higher methylation frequency and promoter CpG island methylation density than EBVnGC in some TSGs, such as p16, E-cadherin and p73, and the methylation status was reverse correlated with protein expression \([4, 10, 29]\). These results indicate that methylation and silence of TSGs induced by EBV may be an important oncogenic mechanism for the development of EBVaGC. Only a few studies detected the methylation of RB in gastric carcinomas. Zhao et al. \([30]\) found that the percent of positive methylation bands for RB gene was 44.6% \((45/101)\), similar to our study of 30 EBVnGC (50%), but less than that of 30 EBVaGC cases (80%), which provides further support that EBV induces RB gene methylation in EBVaGC.

Promoter CpG island methylation is considered an important mechanism of TSG inactivation. In the present study, 23 of 43 \((53.3\%)\) methylated gastric carcinoma tissues lost RB protein expression, which was higher than that in unmethylated gastric carcinoma tissues \((32.0\%, 8/25)\), but RB promoter methylation was not reversely correlated with RB protein expression \( (P = 0.086) \). This phenomenon was also found between p16INK4 gene methylation status and expression in meningiomas by Tse et al. \([31]\). The possible explanations include: (1) gene methylation in gastric carcinoma tissue is heterogeneous; (2) gene methylation may occur in only one allele of cancer cells, while the other allele remains unmethylation. The above reasons may also explain the existence of methylated and unmethylated gene...
bands by MSP. Increasing of CpG island methylation density is a dynamic process, and only the methylation density increases to a certain extent, it results in the complete loss of the expression. The RB promoter methylation could result the decrease or loss of protein expression. Thus, lacking reverse correlation between RB promoter methylation and protein expression was not contradictory. Because of the limitation of major disadvantage of MSP, the methylation status of single CpG site in primer binding sequences is not be detected [32]. The correlation between RB promoter methylation dynamic change and protein expression need further study. These reasons above can also be used to explain why there wasn’t a significant difference in RB protein expression between EBVaGC and EBVnGC, even though RB promoter methylation of EBVaGC was significantly higher than EBVnGC. If the RB gene promoter methylation and its protein expression were negatively correlated, RB protein expression in EBVaGC should have been significantly lower than that in EBVnGC. In this study, the percent of EBVaGC and EBVnGC that were positive by IHC for RB were 43.3% and 63.2%, respectively. Although no significant difference was found of the positive rate of RB protein expression between EBVaGC and EBVnGC, the relatively lower expression rate in EBVaGC also suggests that EBV-induced RB promoter methylation could lead to inhibition of RB protein expression to some extent. Moreover, the promoter methylation of RB gene was also detected in GC and corresponding adjacent normal gastric tissues and the difference was significant, which confirmed that RB promoter methylation is involved with the development of GC.

Similar to the result of RB protein expression, the positive rate of TGF-β1 protein expression was 56.7% (17/30) and 63.2% (24/38) in EBVaGC and EBVnGC, respectively, without significant difference (P = 0.404), suggesting that EBV is not related to the TGF-β1 expression in EBVaGC. Kim et al. [33] examined the association of EBV with RB and p53 protein expression in classic Hodgkin lymphoma and found that EBV wasn’t associated with RB and p53 protein expression. Xu et al. [34] found TGF-β1 level in the serum of nasopharyngeal cancer patients was significantly higher than that in normal persons, and also the advanced stage was higher than the early stage, and recurrent tumors was higher than primary tumors, which indicating that serum TGF-β1 can be used for diagnosis and judgment for prognosis of NPC, and EBV infection can induce the synthesis and release of TGF-β1. This result was different from our result of TGF-β1 in EBVaGC.

Previous studies showed that TGF-β1 expression rate and expression level were higher in gastric carcinoma tissues than that in normal tissues, and the TGF-β1 expression were associated with gastric invasion, metastases, and prognosis [35–37]. In the advanced cancer, TGF-β1 can provide the microenvironment suitable for tumor growth, invasion, and metastases by stimulating angiogenesis, cell spread, immune suppression, and synthesis of extracellular matrix. In gastric carcinoma, RB protein loss was also found to be associated with metastases and prognosis [38–41]. The RB protein expression rate was 40% ~ 90% [38–40, 42, 43] and TGF-β1 protein expression rate was 22.8% ~ 71% [35–37, 44] in previous studies. In the present study, RB protein loss and TGF-β1 protein wasn’t associated with patient age, gender.
pathologic types, and tumor location, but associated with the depth of tumor invasion and lymph node metastasis. At the same time, we also confirmed that RB promoter methylation was associated with tumor invasion and lymph node metastasis, indicating that RB promoter methylation, RB and TGF-β1 protein expression can be as clinical reference index for judgement of gastric carcinoma invasion and metastasis.

5. Conclusion

Our study showed that Aberrant RB promoter methylation was common in gastric carcinoma. EBV could induce RB gene methylation and affect the gene expression in EBVaGC development. EBV has no significant effect on TGF-β1 expression.

Abbreviations

GC: Gastric carcinoma
EBV: Epstein-Barr virus
EBVaGC: Epstein-Barr virus-associated gastric carcinoma
EBVnGC: EBV-negative gastric carcinoma
TSG: Tumor suppressor gene
RB: Retinoblastoma
TGF-β1: Transforming growth factor-β1.

Conflict of Interests

None of the authors has any conflicts of interests.

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