EBV-infection in cardiac and non-cardiac gastric adenocarcinomas is associated with promoter methylation of *p16*, *p14* and *APC*, but not *hMLH1*

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Abstract. *Background:* Epstein–Barr virus (EBV)-associated gastric carcinomas (GC) constitute a distinct clinicopathological entity of gastric cancer. In order to determine underlying distinct aberrant promoter methylation we tested cardiac and non-cardiac GC with regard to the presence of EBV.

Methods: One hundred GC were tested by RNA-*in situ* hybridization for the presence of EBV by EBV-encoded small RNA (EBER). Aberrant promoter methylation was investigated by methylation-specific real-time PCR for *p16*, *p14*, *APC* and *hMLH1*. P16 protein expression was assessed by immunohistochemistry.

Results: In our selected study cohort, EBER-transcripts were detected in 19.6% (18/92) of GC. EBV-positive GC revealed significantly more often gene hypermethylation of *p16*, *p14* and *APC* ($p < 0.0001$, $p < 0.0001$ and $p = 0.02$, respectively) than EBV-negative GC. The majority of GC with p16 hypermethylation showed a p16 protein loss (22/28). In contrast, no correlation between the presence of EBV and *hMLH1* hypermethylation was found ($p = 0.7$). EBV-positive GC showed a trend towards non-cardiac location ($p = 0.06$) and lower stages (I/II) according to the WHO ($p = 0.05$).

Conclusions: Hypermethylation of tumor suppressor genes is significantly more frequent in EBV-associated GC compared to EBV-negative GC. Our data add new insights to the role of EBV in gastric carcinogenesis and underline that EBV-associated GC comprise a distinct molecular-pathologic as well as a distinct clinicopathological entity of GC.

Keywords: Gastric, cancer, cardia, EBV, methylation

1. Introduction

Diverse human malignancies have been linked to the Epstein–Barr virus (EBV), e.g., nasopharyngeal carcinoma, Burkitt's lymphoma and gastric carcinoma (GC) [30,37]. Approximately 10% of GC worldwide are associated with the presence of EBV which ren-

ders GC due to its high worldwide incidence to one of the most frequent EBV-associated human malignancies. During the recent years the underlying molecular mechanisms of EBV contributing to gastric carcinogenesis are more and more understood. Compared to EBV-negative GC, EBV-associated GC (EBVaGC) are characterized by distinct alterations on the molecular, transcriptional and translational level [3,5,14–17,28,30,33,39]. In addition, the expression of the BARF1 oncogene of EBV has been identified in EBV-associated GC and has been reported to impact, e.g., apoptotic or oncogenic gene expression [1,19,35,36,38]. EBV-positive GC have been shown to be associated with hypermethylation of the tumor suppressor genes p14, p16 and APC in Asian patients [6,16,20]. However, data on hypermethylation of these genes in Caucasian pa-

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tients are very sparse [34]. The tumor suppressor genes p16, p14 and APC have been reported to be hypermethylated in GC at varying frequencies (range 10–84%) [3,5,6,13,14,22,23]. The hypermethylation of the DNA mismatch repair gene hMLH1 often occurs in a setting of microsatellite instability and was found only in EBV-negative GC so far [16]. In the present study we related the methylational status of p16, p14, APC and hMLH1 to the presence of EBV in GC. Since cardiac and non-cardiac GC (located in body or antrum) are characterized by distinct molecular alterations [10], 50 carcinomas from each tumor localization were analyzed. Further, EBV-status was correlated to the available clinicopathological features [12,21,31,32], in order to determine if aberrant promoter hypermethylation can be added to the list of distinct molecular changes of EBV-associated GC possibly contributing to EBVaGC as distinct clinicopathological entity.

2. Methods

2.1. Case selection and tissue microarrays

From a large ongoing study [10,26], we selected 50 patients with a carcinoma of the gastric cardia and 50 carcinomas, located in the distal stomach, namely in the body and antrum. Cardiac GC were defined as type II and type III carcinomas from the esophago-gastric junction, according to the classification of Siewert and Stein [29]. Type I carcinomas (Barrett carcinomas) were excluded. All tumors were adeno-carcinomas, partly with signet ring histology. Details on application of WHO-classification and Lauren's classification have been described previously [24]. Characteristics of patients and carcinomas are summarized in Table 1.

2.2. EBER-RNA *in situ* hybridization

The EBV-status of the GC had been tested previously by EBER-RNA *in situ* hybridization (RISH) on tissue microarrays (TMA) [26]. The TMAs contained three 0.6 mm thick punches per tumor (Beecher Instruments, Silver Spring, MD, USA) and only for signet ring cell carcinomas six instead of three punches were used. One complete and tumor bearing core per case was regarded as sufficient for the evaluation of the EBV-status according to previous results that EBV-associated GC do not reveal heterogeneity with respect to EBER-transcript expression [38]. One of 50 cardiac carcinomas and 7 of 50 gastric carcinomas were excluded from further analyses due to technical reasons, e.g., loss of tissue cores during RISH. Thus, in 49 car-

Table 1
Characteristics of patients and carcinomas

		Cardiac ADC <i>n</i> = 49*	Non-cardiac ADC <i>n</i> = 43*
Age	Median	61	68
	Range	38–79	37–90
Gender	Male	44	27
	Female	5	16
Tumor (mm)	Median	61	71
	Range	20–125	13–185
pT	1	5	11
	2	25	25
	3	13	6
	4	6	1
pN	0	13	17
	1	17	20
	2	14	3
	3	5	3
Stage	I	13	21
	II	8	13
	III	19	5
	IV	9	4
Grading	1	1	2
	2	13	12
	3	35	29
WHO	ADC	44	29
	SRC	5	14
Lauren's	Intestinal	37	26
	Diffuse	1	9
	Mixed	11	8

Notes: * Only informative cases are listed. ADC – adenocarcinomas; SRC – signet ring cell carcinomas.

diac and 43 distal gastric carcinomas a specific result could be achieved.

2.3. Methylation specific PCR

Details of methylation specific real-time PCR (MSP) for APC, p16^{INK4A}, p14^{ARF}, hMLH1 and control gene MYOD1 have been published previously [3,7]. In brief, genomic DNA from formalin-fixed, paraffin-embedded carcinoma tissue and corresponding tumor-free gastric smooth muscle tissue which served as constitutive negative control, were prepared under light microscopic control as published before [2, 10,25]. For MSP, 1 µg of genomic DNA was modified using a CpG modification kit (CpG Genome™ DNA modification kit, Intergen, Purchase, NY, USA) according to the manufacturer's protocol. Real-time MSP was performed in a Lightcycler (Roche Diagnostics,

Table 2
Sets of primers and probes for the investigated gene loci and conditions of methylation-specific PCR (MSP)

Locus	Primer and probe set (5'FAM and 3'TAMRA)	MSP-Conditions
<i>APC</i>	F: GAA CCA AAA CGC TCC CCA T R: TTA TAT GTC GGT TAC GTG CGT TTA TAT Probe: CCC GTC GAA AAC CCG CCG ATT A	(1) 10 min 95°C, (2) 10 s 95°C, (3) 5 s 58°C, (4) 8 s 72°C, (5) → 60 cycles (2)–(4) (6) 30 s 40°C
<i>p16^{INK4A}</i>	F: TGG AGT TTT CGG TTG ATT GGT T R: AAC AAC GCC CGC ACC TCC T Probe: ACC CGA CCC CGA ACC GCG	(1) 10 min 95°C, (2) 10 s 95°C, (3) 5 s 58°C, (4) 8 s 72°C, (5) → 60 cycles (2)–(4) (6) 30 s 40°C
<i>p14^{ARF}</i>	F: ACG GGC GTT TTC GGT AGT T R: CCG AAC CTC CAA AAT CTC GA Probe: CGA CTC TAA ACC CTA CGC ACG CGA AA	(1) 10 min 95°C, (2) 10 s 95°C, (3) 5 s 60°C, (4) 8 s 72°C, (5) → 60 cycles (2)–(4) (6) 30 s 40°C
<i>hMLH1</i>	F: CGT TAT ATA TCG TTC GTA GTA TTC GTG TTT R: CTA TCG CCG CCT CAT CGT Probe: CGC GAC GTC AAA CGC CAC TAC G	(1) 10 min 95°C (2) 10 s 95°C (3) 5 s 61°C (4) 8 s 72°C (5) → 60 cycles (2)–(4) (6) 30 s 40°C
<i>MYOD1</i>	F: GGA TTT ATA TTT ATG TGG TGG GTG G R: CCA ACT CCA AAT CCC CTC TCT AT Probe: TCC CTT CCT ATT CCT AAA TCC AAC CTA AAT ACC T	(1) 10 min 95°C, (2) 10 s 95°C, (3) 5 s 61°C, (4) 8 s 72°C, (5) → 60 cycles (2)–(4) (6) 30 s 40°C

Mannheim, Germany) using the Taqman technology. We selected previously published primers and probes, and in addition established new primers and probes [8]. Primer sequences and MSP conditions are summarized in Table 2.

2.4. Immunohistochemistry

The p16 protein expression was investigated in all cases with p16 hypermethylation by immunohistochemistry as published before [11,25]. Briefly, slides from one representative tumor block per case were stained using a monoclonal p16-antibody (Clone 16P07; Neomarkers, Westinghouse, CA, USA) at a dilution of 1:50 according to a standard avidin-biotin-peroxidase protocol. In 4 of 32 cases non-specific positivity was observed and these cases therefore were excluded.

2.5. Statistics

Statistical analyses were performed by the program SAS, version 9.1 [27]. Using the X^2 -test p -values < 0.05 were considered as statistically significant. For analysis of age, patients were dichotomized before in two groups, younger or older than 60 years, respectively. For analysis of tumor diameter, patients were dichotomized before in two groups, less or more than 50 mm, respectively.

3. Results

Out of 92 GC, EBV-positive carcinomas ($n = 18$, Fig. 1) were significantly more often methylated

Fig. 1. EBV-positive gastric cancer in RNA *in situ* hybridization (RISH). (a) Moderately differentiated (G2); (b) poorly differentiated (G3).

than EBV-negative adenocarcinomas ($n = 74$): While 83.3% (15/18) of EBVaGC were methylated in p16, only 23.0% (17/74) of EBV-negative GC were methylated in p16 ($p < 0.0001$). The majority of EBVaGC (88.9%; 16/18) was also methylated in p14, but only a minority of EBV-negative GC (5.4%; 4/74) (p -value < 0.0001). Since 83.3% (15/18) of EBVaGC were methylated in APC, 54.1% of EBV-negative cases were positive for APC methylation ($p = 0.02$).

In contrast, no association between EBV-status and methylation of hMLH1 was detected ($p = 0.7$). Accordingly, hMLH1 was methylated in 6/18 (33.3%) of EBVaGC and 21/74 (28.4%) of EBV-negative GC.

The majority of carcinomas with p16 hypermethylation revealed a concomitant p16 protein loss (22/28, 78.6%).

12 of 43 (27.9%) non-cardiac GC were EBV-associated, while only 6 of 49 (12.3%) cardiac GC were EBV-positive, showing a trend towards more EBV-infections in the distal stomach ($p = 0.06$).

Further, we proved 15 of 58 (25.9%) carcinomas of a lower stage (I and II) to be EBV-positive, but only 3 of 34 carcinomas in stages III and IV (8.8%), although this correlation marginally failed to reach statistical significance ($p = 0.05$).

All results are summarized in Table 3.

4. Discussion

There are several indications, that aberrant methylation might be a central molecular mechanism of EBV-infection in gastric carcinoma cells. When viral DNA

enters a cell, it is often deactivated by methylation. This leads to repression of viral protein expression and escape of immune surveillance of cells with oncogenic potential. But the EBV-induced methylation also affects the adjacent host DNA [22] and can even occur genome-wide [6]. This may result in inactivated tumor suppressor genes.

Gene silencing by aberrant methylation (hypermethylation) of the tumor suppressor genes p16, p14, and APC and of hMLH1 are prevalent findings in GC [9, 10, 25]. In the present study, we proved that methylation of these tumor suppressor genes is important mechanism in EBVaGC. The actually epigenetic silencing is demonstrated by p16 protein loss in most methylated cases.

These results have been confirmed by other authors, so far mostly in Asian patients [6, 16, 20]. Similar to our results, Chong and co-workers [6] proved 88.2% of methylation in p14 and p16 each, which was significantly associated with EBV positivity [6]. These findings of high-density methylation strengthen the hypothesis that EBV-infection causes a de novo and maintenance of methylation [23].

Similar to our results, Kang and co-workers found multiple genes, e.g., APC and p16 being hypermethylated in a frequency $>90\%$ in EBVaGC, defining a so-called methylator phenotype [16]. EBV-positive GC show a concordant methylation of multiple genes very much like to microsatellite instable (MSI) GC, although in our and other studies EBVaGC usually show no MSI and vice versa [16]. In our study EBV-infection was not associated with hMLH1 hypermethylation, which is a marker for MSI. These results,

Table 3
Results in EBV-positive and negative gastric and cardiac carcinomas

		EBV positive <i>n</i> = 18 (%)	EBV negative <i>n</i> = 74 (%)	<i>p</i> -value
p16 methylation	Yes	15 (83.3)*	17 (23.0)*	<0.0001
	No	3 (16.7)	57 (77.0)	
p14 methylation	Yes	16 (88.9)	4 (5.4)	<0.0001
	No	2 (11.1)	70 (94.6)	
APC methylation	Yes	15 (83.3)	40 (54.1)	0.02
	No	3 (16.7)	35 (45.9)	
hMLH1 methylation	Yes	6 (33.3)	21 (28.4)	0.7
	No	12 (67.7)	53 (71.6)	
Location	Cardiac	6 (33.3)	43 (58.1)	0.06
	Non-cardiac	12 (67.7)	31 (41.9)	
Stage	I/II	15 (83.3)	43 (58.1)	0.05
	III/IV	3 (16.7)	31 (41.9)	
Grading	1/2	3 (16.7)	25 (33.8)	0.16
	3/4	15 (83.3)	49 (66.2)	
WHO	ADC	14 (77.8)	58 (78.4)	0.5
	SRC	4 (22.2)	16 (21.6)	
Lauren's	Intestinal	12 (67.7)	49 (66.2)	0.7
	Diffuse/mixed	6 (33.3)	25 (33.8)	
Tumor diameter	<50 mm	5 (27.8)	35 (47.3)	0.1
	>50 mm	13 (72.2)	39 (52.7)	
Age	<60 J	8 (44.4)	36 (48.6)	0.7
	>60 J	10 (55.6)	38 (51.4)	
Gender	Male	17 (94.4)	56 (75.7)	0.07
	Female	1 (5.6)	18 (24.3)	

Notes: * 22 of 28 informative cases with p16 hypermethylation show p16 protein loss.

which are corroborated by other investigators, indicate that EBV induces a malignant pathway, independent of MSI [22].

The different molecular pathways of EBV-positive GC are reflected by different genetic aberration. EBV-associated GC are characterized by distinct chromosomal aberrations and in addition distinct patterns of allelic losses, e.g., at the TP53 locus including p53 immunoreactivity, are associated with the EBV-status [33,39]. Furthermore, underlying the distinct molecular pathology, EBV-associated GC reveal a unique type of EBV-latency, which is mainly characterized by the expression of the viral oncogene BARF1 and the absence of LMP1 [4,18,38].

These molecular differences are also reflected by the finding that EBV-associated GC comprise a distinct clinicopathological entity: EBV-associated GC are linked to male predominance, lower age, intestinal histological type, proximal location and a favorable prognosis [31,32,38].

The purpose of our study was not to investigate the clinico-pathological parameters associated with the prevalence of EBV, but the impact of differences in promoter methylations with regard to EBV.

However, the identification of the tight association to aberrant methylation of p16, p14 and APC in EBVaGC in this study, which was not observed to that extent in EBV-negative GC, strongly underline together with the missing association to hMLH1 hypermethylation that EBVaGC comprise a distinct clinicopathological entity.

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