DOI: 10.4149/BLL_2017_033

Bratisl Med J 2017; 118 (3)

164 – 168

CLINICAL STUDY

Aberrant promoter hypermethylation of p16, survivin, and retinoblastoma in gastric cancer

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ABSTRACT

BACKGROUND: Gastric cancer is common and can be found throughout the world. Notoriously like other cancers, gastric cancer is a consequence of cellular regulation disorder. Epigenetics, including many oncogenes or tumour suppressor genes, can provide some clues for this kind of disorder. DNA methylation, especially promoter methylation, which causes the silence/decrease of tumour related genes, has become a focus in various tumour types. The aim of this study was to investigate promoter methylation of certain genes: p16, survivin, retinoblastoma (Rb), all of which have been regarded as genes related to gastric cancer.

MATERIAL AND METHODS: To detect the promoter methylation of p16, survivin, and Rb genes, peripheral blood samples from 106 gastric cancer patients as well as 18 healthy individuals were collected for Methylation-Specific Polymerase Chain Reaction (MSP) analysis.

RESULTS: According to the statistical analysis, positive methylation ratio of p16 was 72.6 % (77 cases), 6 % had methylated survivin (7 cases), and positive methylation ratio of Rb was only 17.9 % (19 cases). The differences of promoter methylation of these genes between the patients group and control group were observed: P (P16) = 5.097E-08, P (survivin) = 0.262, and P (Rb) = 0.187. In the control group, methylated p16 was found in only one case that also had a methylated Rb. However, Survivin could not be found methylated in control cases.

CONCLUSION: In this study, promoter methylation was observed for the p16 gene, which was considered an early potential marker in gastric cancer. This data supports that further investigations should study Rb and survivin as candidate markers for gastric cancer *(Tab. 2, Fig. 1, Ref. 35)*. Text in PDF *www.elis.sk.*

KEY WORDS: promoter hypermethylation, P16, survivin, Rb, MSP, gastric cancer.

List of abbreviations: Rb – Retinoblastoma MSP Methylation-Specific Polymerase Chain Reaction, INK4I – cyclin-dependent kinase 4 inhibitors, CDK4 – cell cycle-dependent protein kinase 4, CDK6 – cell cycle-dependent protein kinase 6, E-cadherin – epithelial cadherin, or CDH1 – cadherin 1, RUNX3 – human runt-related transcription factor 3, MGMT – O6-methylguanine-DNA methyltransferase, DAPK – Death-associated protein kinase, TFPI – tissue factor pathway inhibitor, SOCS1 – Suppressor of cytokine signalling 1, IAP – inhibitors of apoptosis

Introduction

Gastric cancer is found worldwide with 934,000 cases identified every year (13.86 incidences/1million individuals), with 42 % of cases in China, with an incidence and mortality rate higher than average by two times. Every 2–3 minutes one Chinese person dies because of gastric cancer (1).

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Only 7.5 % of early gastric cancer patients are diagnosed in China (2). Notoriously, like other cancers, gastric cancer is a consequence of cellular regulation disorder. Epigenetics, including oncogenes and tumour suppressor genes, provides some clues towards this disorder. DNA methylation, especially promoter methylation, which causes the silencing of tumour suppressor genes, has been studied for early gastric cancer diagnosis. The aim of this study was to investigate promoter methylation of certain genes, p16, survivin, retinoblastoma (Rb), all of which have been regarded as genes associated with gastric cancer.

Significant evidence suggests that cancer is caused both by epigenetic and genetic abnormalities (3). It was reported that there was a strong correlation between gene expression and methylation; however, only methylation in the promoter region was associated with gene silencing/decrease (4). Aberrant DNA hypermethylation of promoter regions occurs before a tumour is seen through the proliferation of a single cell (5). Thus, identification of promoter hypermethylation may be useful to diagnose the disease at early stages (Tab. 1).

P16 belongs to the INK4I (cyclin-dependent kinase 4 inhibitors) family, the most common human cancer suppressor gene, which encodes the cyclin kinase inhibitor. This inhibitor protein binds to and inhibits cell cycle-dependent protein kinases CDK4 and CDK6, reduces Rb phosphorylation, and leads cell through G1 phase, thereby inhibiting the proliferation of cells. Many re-

Tab. 1. Promoter hypermethylation in gastric tumours and healthy subjects.

	P16				Survivin				Rb			
	+	–	Total	p	+	–	Total	p	+	–	Total	p
Subject(n=106)	77	29	106	5.097E-08	7	99	106	0.262	19	87	106	0.187
Control(n=18)	1	17	18		0	18	18		1	17	18	
Tumour Stage												
Type I(n=22	15	7	22	0.903	2	20	22	0.882	3	19	22	0.731
Type II(n=10)	8	2	10		1	9	10		1	9	10	
Type III(n=24)	18	6	24		1	23	24		4	20	24	
Type IV(n=50)	36	14	50		3	47	50		11	39	50	
Total	77	29	106		7	99	106		19	87	106	
Age												
>60 (n=51)	33	10	43	0.434	2	41	43	0.503	7	39	46	0.525
≤60(n=55)	44	19	63		5	58	63		12	48	60	
Total	77	29	106		7	99	106		19	87	106	

searchers find that some mutation/deletion of P16 leads to abnormal cell growth and that methylation of p16 is often reported in many different cancer research studies.

Ambrosini of Yale University found the survivin gene in 1997 in a hybrid screening of the human genome library (7). Survivin is known to be a powerful inhibitor of apoptosis. Studies have shown that survivin can inhibit cell apoptosis and regulate cell cycle, angiogenesis, tumour invasion/metastasis, and drug and radiation resistance, to name but a few of its actions. Under pathological conditions, survivin is expressed in most malignant tumours, playing an important role in cancer pathogenesis. Survivin can be expressed largely in tumour tissue, but can be silenced in adult terminally differentiated tissues. It is expected to become a broad-spectrum marker for cancer diagnosis, treatment, and prognosis.

Retinoblastoma gene can be translated into a nuclear phosphoprotein (7). It is associated with cell proliferation, cell cycle regulation, and transcription regulation. Once the Rb gene is deleted or inactivated, a cell cycle disorder or some oncogene amplification will cause tumours. In the development of tumours, the Rb gene can be rearranged, deleted, or hypermethylated. It is related to Rb occurrence and blocks tumour growth by continued expression. Retinoblastoma gene hypermethylation can inhibit the transcription of suppressor genes and abnormalities in this gene occur in many tumours. Some research has reported that Rb in gastric cancer, acute myeloid leukemia, soft tissue sarcoma, liver cancer cell lines, and glioblastoma tumours was methylated.

Therefore, aberrant alteration in these three genes may result in cancer. In this study, the methylation status of the p16, survivin, and Rb genes were examined in blood samples taken from 106 patients with gastric cancer and 18 healthy individuals, subjected to Methylation-Specific Polymerase Chain Reaction (MSP), and studied for a correlation between the methylation status and then clinicopathological findings was evaluated.

Materials and methods

Peripheral blood samples of 106 gastric cancer patients and 18 healthy individuals were collected at the Hospital of Tumour,

Tab. 2. Primer sequences, PCR conditions and size of the PCR products.

Primer	Sequence of 5'-3'	PCR conditions	PCR products
p16-U-F	TTATTAGAGGGTGGGGTGGATTGT	94°C 25s/58°C 25s/72°C 25s/35 cycle	151 bp
p16-U-R	CAACCCCAAACCACAACCATAA		
p16-M-F	TTATTAGAGGGTGGGGCGGATCGC	94°C 25s/58°C 25s/72°C 25s/30 cycle	150bp
p16-M-R	GACCCCGAACCGCGACCGTAA		
Survivin-MF	GGCGGGAGGATTATAATTTTCG	94°C 30s/53°C 30s/72°C 20s/35cycles	164bp
Survivin-MR	CCGCCACCTCTACCAACG		
Survivin-UF	GGTGGGAGGATTATAATTTTTG	94°C 30s/56°C 30s/72°C 20s/35cycles	168bp
Survivin-UR	ACCACCACCACCTCTACCAACA		
Rb-MF	GGG AGT TTC GCG GAC GTG AC	94°C 30s/55°C 30s/72°C 20s/35cycles	152bp
Rb-MR	ACG TCG AAA CAC GCC CCG		
Rb-UF	GGG AGT TTT GTG GAT GTG AT	94°C 30s/66°C 30s/72°C 20s/35cycles	163bp
Rb-UR	ACA TCA AAA CAC ACC CCA		

p16-U-F: The forward primer sequence of the unmethylated sequence of p16 gene; p16-U-R: The reverse primer sequence of the unmethylated sequence of the p16 gene; p16-M-F: The forward primer sequence of the methylated sequence of p16 gene; p16-M-R: The reverse primer sequence of the methylated sequence of p16 gene; survivin-U-F: The forward primer sequence of the unmethylated sequence of survivin gene; survivin-U-R: The reverse primer sequence of the unmethylated sequence of survivin gene; survivin-M-F: The forward primer sequence of the methylated sequence of survivin gene; survivin-M-R: The reverse primer sequence of the methylated sequence of survivin gene; Rb-U-F: The forward primer sequence of the unmethylated sequence of Rb gene; Rb1-U-R: The reverse primer sequence of the unmethylated sequence of the Rb gene; Rb-M-F: The forward primer sequence of the methylated sequence of Rb gene; Rb-M-R: The reverse primer sequence of the methylated sequence of Rb gene; bp: Base pair(p16, ref. 36; survivin, ref. 37; Rb, Ref. 38)

Shandong in 2014. All peripheral blood samples were carried on ice when transported to the laboratory and all were processed immediately upon arrival.

We found that all gastric cancer patients had no evidence of other diseases. Clinicopathologic data were available for all 106 gastric cancer patients; Tumour stages and pathologic features of primary tumour were defined according to the criteria of Borrmann. The distribution of tumour stages was as follows: 22 cases were at Stage I, 10 at stage II, 24 at stage III, and 50 were at stage IV.

The study protocol was in adherence to the tenets of the Declaration of Helsinki and the procedure was done according to the ethics committee approval. Informed consent was obtained from all patients and healthy individuals after explaining the nature and possible consequences of the study. All patients signed a statement of consent to publish this report.

Genomic DNA was isolated from peripheral blood using the DNeasy Blood & Tissue Kit (Qiagen, USA) and was stored at –20 °C. DNA samples were measured at 260 nm with minidrop spectrophotometry (Eppendorf) and bisulfite modification reaction was conducted using 1 µg genomic DNA. All DNA samples were modified with the reagents provided in the EpiTect Bisulfite kit (Qiagen, USA) and the modification reaction lasted for approximately 2 hours. After the bisulfite treatment, converted DNA samples were stored at –20°C and could be used up to four weeks. The modified DNA samples were then subjected to MSP with methylation-specific primers of the p16, survivin, and Rb genes. We used methylated primers (M) to amplify methylated regions and unmethylated primers (U) to amplify unmethylated regions. Additionally, we used CpGenomeTM Universal Methylated DNA (Millipore, California, USA) as a positive control. Each PCR reaction mixture contained 2X PCR Mixer enzyme (22 mM Tris-HCl, pH 8.4, 55 mM KCl, 1.65 mM MgCl2, 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP, 2 U Taq), 0.4 pmol sense and antisense primers for target genes in a total volume of 20 µl. The primer sequences, PCR conditions, and product sizes are given in Table 2. After PCR amplification, the products were electrophoresed on a 1 % agarose gel (Sigma-Aldrich, Steinheim, Germany) and visualized under ultraviolet light after ethidium bromide staining.

Statistical analyses were done using SPSS 17.0 for Windows Evaluation Version (SPSS Inc.; Chicago, IL, USA). Methylation-Specific Polymerase Chain Reaction results were compared between the subject and control group by the $\chi 2$ test, $p < 0.05$ was considered as statistically significant.

Results

Blood samples of 106 patients, who were referred to the department of pathology (tumour hospital, Shandong) and 18 healthy individuals were examined for promoter methylation of p16, survivin, and Rb by MSP. Aberrant methylation of p16 was detected in 77 out of 106 (72.6 %) cancer patients and 1 out of 18 (5.5 %) healthy patients, respectively. In total, 6 % of them have methylated survivin (7 cases) and 17.9 % of them have methylated Rb (19 cases) (Fig. 1). For healthy individuals, no promoter methylation of survivin was found. One case of methylated Rb was found and it was the same patient that had the methylated p16. Among the patients and healthy individuals, the difference between the tumour stage and methylation of p16, survivin, and Rb genes were insignificant.

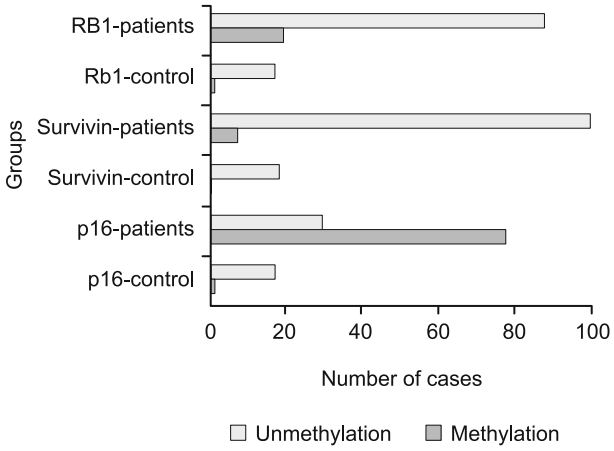

Fig. 1. Promoter hypermethylation of p16, Survivin and Rb genes in gastric cancer patients and healthy individuals.

Discussion

Gastric cancer, which has a high incidence in many countries, is one of the most fatal types of cancer, especially in China (Yang, 2006). Studies performed on migrants in the US showed that gastric cancer incidence decreases in descendants of Japanese migrants, suggesting that the environment plays an important part (8). Therefore, it might be important to determine the genetic alterations, such as epigenetic modifications, as a new parameter to estimate the cancer (9). Given the difficulty of procuring human cancer tissue, a key question in molecular medicine concerns the extent to which epigenetic signatures measured in more accessible tissues such as blood can serve as a surrogate marker. In spite of the fact that the diagnostic results with blood cannot have good correspondence to those markers in tissues (10), blood is still a good, convenient, and important material for cancer diagnostics. So far, just a few biomarkers have been well characterized in their involvement in gastric cancer development. E-cadherin (epithelial cadherin, or CDH1-cadherin 1), RUNX3 (human runt-related transcription factor 3), MGMT (O6-methylguanine-DNA methyltransferase), DAPK (Death-associated protein kinase), TFPI (tissue factor pathway inhibitor), RASSF1A (Ras association domain family 1 isoform A), and SOCS1 (Suppressor of cytokine signalling 1) were all investigated as potential early diagnostic biomarkers in gastric cancer by many different researchers (11, 12, 13, 14, 15, 16, 17).

We used gastric cancer patients as a subject group and examined the tumour related genes of p16, survivin, and Rb. Among the subject group, we found that 72.6 % have methylated p16 (77 cases), 6 % of them have methylated Survivin (7 cases), and 17.9 %

have methylated Rb (19 cases). In the control group, methylated p16 was only found in one case that also had a methylated Rb, but no cases of methylation on survivin was found in healthy patients. In the present study, we focused on determining if most of the gastric cancer patients had methylated p16 promoters because there were many researchers that supported this idea and our findings correlated with other researchers.

Kim et al (18) reported that methylation of p16 leads to low expression of p16 that contributed to tumour enlargement. The clinicopathological analysis also indicates that p16 methylation in blood of the patients with colorectal cancer was related with later Dukes' stage (19). In another study, it is claimed that p16 gene silencing contributes to the changes in the epigenetic landscape widely found in neoplasia (20).

Dysregulation of apoptosis, unwanted cells survival, or required cells death, result in several diseases, including cancer (21). The inhibitors of apoptosis (IAP) protein family are novel intracellular proteins that suppress the apoptosis by a variety of stimuli (22), which include survivin (23). Survivin is in most adult normal tissues undetectable, whereas its expression in human common tumours has been associated with increased aggressiveness and decreased patient survival (24, 25, 26, 27, 28), suggesting that apoptosis inhibition by survivin is an important predictive/prognostic parameter of poor outcome in human cancers and that survivin will be a diagnostic/therapeutic target in the development of malignant tumours. In our research, methylated survivin can be found in the subject group, but methylated survivin cannot be found in any healthy individual. Considerable evidence suggests that elevated expression of survivin may promote tumorigenesis, and in fact, survivin is highly expressed in human common cancers (24, 25, 27). In contrast, several recent studies have reported survivin gene expression in several normal tissues (29, 30, 31). Work that is more detailed should be done to investigate the exact role of survivin in different cancers as we also found methylated survivin in the subject group in which the expression of survivin should be silenced or decreased.

Retinoblastoma protein is involved in cell cycle regulation and its function/expression is often lost in various kinds of tumours. The consequences of Rb inactivation in tumours can be very different depending on the context and the type of cancer. Recent evidence indicates that Rb status correlates with a different therapeutic response according to the tumour type and the therapeutic agent. As Maimoona Sabir et al (32) found, Rb can be methylated in head and neck cancer and we can arrive at the conclusion: Rb methylation also plays a vital role in cancers, besides Rb phosphorylation.

We also studied the unmethylated promoter regions for these genes and observed that 90.6 % of patients have unmethylated p16 (96 cases; 18 cases of all 18 controls can be found with unmethylated p16, 100 %) and 23.58 % of them have unmethylated Rb (25 cases; 18 cases of all 18 controls can be found with unmethylated Rb, 100 %), 97.17 % of them have unmethylated survivin (103 cases; 18 cases of all 18 controls can be found with unmethylated survivin, 100 %). Similar results were obtained by the study of Virmani et al (33). They claimed that normal cells were found in tumour specimens. That is why unmethylated sequences can be detected in methylated tumour samples (34). Tumour tissue is a combination and has many cells with different genotypes. It also means that, through the tumorigenesis, many varied genetic alterations affect tumour cells in tumour tissue, a complex that houses many different cells. Thus, in tumour tissue, some cells may have methylated p16, while others may have unmethylated p16 because of the complicated structure of a tumour that has the combination of genetically different cells. Therefore, both methylated and unmethylated types of genes can be found in tumour tissue (34).

We found one case with methylated p16 in healthy individuals, but many other researchers reported that they could not find any methylated p16 in healthy individuals (19, 35). The possible reason is that the healthy controls were selected from the "patients" who went to our cancer hospital for early diagnosis. Some of them may have faced the cancer risk but we cannot diagnose it with current equipment. Such a result may also indicate that p16 is a prospective marker for early gastric cancer diagnosis and that p16 can contribute to the maintenance of Rb in unphosphorylated state, which inhibits cell cycle progression (36). More attention should be paid to p16 and Rb methylation as they might be a co-marker for early cancer diagnosis.

Conclusion

In our research, we just looked for the methylation status of p16, survivin, and Rb genes. We found some of them can be methylated, which can lead them to be inactive or less expressed, but we should also consider other possibilities, such as gene mutation or gene loss that can lead to the same result as methylation. More work should be done to further this line of research.

At the same time, the difference of survivin and Rb genes between subject and control groups is not significant. We found that some patients with unmethylated p16 could be found with methylated survivin or Rb. If we classify all aberrant methylated patients into one group, we can find a significant difference between subject and control groups. We suggest a combination of different genes for gastric cancer markers are used in future investigations.

In conclusion, the aim of this study was to investigate the relationship between gastric cancer and promoter methylation of p16, survivin, and Rb genes. We analysed methylation of p16, survivin, and Rb with MSP and found that gastric tumour have a significant relationship with the methylation of p16 genes. Of course, there are many reasons for the cause of gastric cancer, for example, mutation and gene loss, so a combination of different genes should be studied in future research to identify a combination of different genes that can be used as gastric diagnosis markers.

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Received December 1, 2016.
Accepted December 12, 2016.