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Aberrant methylation of the PTPRO gene in peripheral blood as a potential biomarker in esophageal squamous cell carcinoma patients

Yan-Jie You^{a,c,e}, Yu-Ping Chen^d, Xiaoxuan Zheng^e, Stephen J. Meltzer^{f,g}, and Hao Zhang^{a,b,c,e,*}

^aDepartment of Integrative Chinese and Western Medicine, Cancer Hospital of Shantou University Medical College, Shantou, People's Republic of China ^bTumor Tissue Bank, Cancer Hospital of Shantou University Medical College, Shantou, People's Republic of China ^cOncological Research Lab, Cancer Hospital of Shantou University Medical College, Shantou, People's Republic of China ^dDepartment of Surgery, Cancer Hospital of Shantou University Medical College, Shantou, People's Republic of China ^eCancer Research Center, Medical College of Shantou University, Shantou, People's Republic of China ^fDivision of Gastroenterology, Departments of Medicine and Oncology, the Johns Hopkins University School of Medicine, Baltimore, Maryland ^gSidney Kimmel Comprehensive Cancer Center, the Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

Inactivation of protein tyrosine phosphatase receptor-type O (PTPRO), a new member of the PTP family, by hypermethylation has been described in several forms of cancers. We evaluated PTPRO hypermethylation as a potential epigenetic biomarker in esophageal squamous cell carcinoma (ESCC). PTPRO hypermethylation was observed in 27 (75%) of 36 primary tumors and correlated significantly with depth of invasion (T-stage, $P = 0.013$). Among matched peripheral blood samples from ESCC patients, 13 (36.1%) of 36 had detectable methylated PTPRO in plasma, and 15 (41.7%) of 36 had it in the buffy coat. No methylated PTPRO was observed in normal peripheral blood samples from 10 healthy individuals. In addition, demethylation by 5-aza-dC treatment led to gene reactivation in PTPRO-methylated and -silenced ESCC cell lines. This is the first report for detection of PTPRO as a non-invasive biomarker for solid tumors in peripheral blood. Our findings suggest that hypermethylated PTPRO occurs frequently in ESCC. Its detection in the peripheral blood of ESCC patients illustrates its potential clinical application as an epigenetic biomarker for noninvasive diagnosis and disease monitoring.

Keywords

PTPRO; DNA methylation; epigenetics; Cancer surveillance and screening

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*Corresponding author address: Hao Zhang, Cancer Research Center, Medical College of Shantou University, 22 Xinling-Road, Shantou 515041, People's Republic of China. Tel.: 86-754-8900406; Fax: 86-754-8900406; haozhang@stu.edu.cn (H Zhang).

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None Declared

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1. Introduction

Esophageal carcinoma ranks sixth as a cause of cancer mortality worldwide [1]. Its most common histological subtype, esophageal squamous cell carcinoma (ESCC), is more prevalent and accounts for > 90% of the esophageal carcinoma in Asian countries [2]. Although surgical techniques and preoperative management have improved, the prognosis for patients with ESCC remains poor. Detection and treatment of primary and recurrent ESCCs in their early stages is associated with a significantly improved outcome. However, a key obstacle to improving ESCC patient survival is a lack of sensitive early detection methods. There is no accurate clinical method for ESCC screening. Radiological imaging and endoscopic biopsy histological examination are too expensive and invasive to be useful for population-based screening. In addition, these procedures are impractical as repeat serial monitoring modalities for post-therapy residual or recurrent tumor. Thus, there is an urgent need to develop biomarkers for noninvasive diagnosis and disease monitoring of ESCC, which might help to improve early diagnosis and therapy of patients with this refractory cancer.

Tyrosine phosphorylation, which is reciprocally controlled by tyrosine kinases and protein-tyrosine phosphatases (PTPs), is important in all cancers including ESCC. It has long been well-known that oncogenic activation of tyrosine kinases is a common feature in cancer [3]. PTPs, which catalyze the reverse reaction, often therefore function as tumor suppressors. However, compared with tyrosine kinases, our understanding of the role of PTPs in ESCC remains largely unknown, while such knowledge could provide new therapeutic targets and diagnostic markers in this type of cancer. Protein tyrosine phosphatase receptor-type O (PTPRO), a member of the receptor-type PTP family, has exhibited characteristics of a tumor suppressor gene in multiple cancer types [4]. PTPRO is a type III protein tyrosine phosphatase consisting of a single intracellular catalytic domain, a transmembrane region, and an extended extracellular domain with eight repeated fibronectin type III-like motifs [5]. A truncated isoform of PTPRO (PTPROt) is predominantly expressed in hematopoietic cells. Ectopic expression of PTPRO(t) in cancer cells results in reduced proliferation, delayed progression through the cell cycle, and increased susceptibility to apoptosis [6; 7; 8]. It has been shown that PTPRO expression is co-regulated by E2F1 and miR-17-92 [9]. One recent study also revealed that PTPRO is also a target of the Wnt signaling pathway and may function as a novel receptor for Wnt [10].

Epigenetic silencing of tumor suppressor genes by aberrant promoter methylation plays a significant role in the initiation and development of cancers, and the presence of methylated tumor suppressor genes in tissue biopsy or body fluids can serve as tumor-specific markers [11]. Previous studies demonstrated methylation-mediated down-regulation of PTPRO expression in rat hepatocellular carcinomas, human chronic lymphocytic leukemia, human lung cancer and some breast cancer cell lines [5; 12; 13; 14]. Nevertheless, to our knowledge, the methylation status of PTPRO has not been studied in ESCC. Therefore, we evaluated of aberrant methylation of the PTPRO gene as a potential epigenetic event and biomarker in ESCC. In the current study, we investigated the methylation status of PTPRO in ESCC to define the frequency of this epigenetic aberration by (1) examining the methylation status of PTPRO in primary ESCC tumors and matched peripheral blood samples; (2) testing whether promoter methylation mediated the silenced expression of this gene in ESCC cell lines; and (3) evaluating the potential association between promoter methylation status of PTPRO and clinicopathological parameters.

2. Materials and Methods

2.1 Clinical specimens

Primary tumor specimens and matched preoperative peripheral blood samples from 36 ESCC patients were obtained from the Cancer Hospital of Shantou University Medical College. None of these patients had received any preoperative treatment, such as chemotherapy or radiotherapy. Tumor samples were separated and immediately frozen in liquid nitrogen for subsequent store at -80 °C until use. All tumors were histopathologically confirmed, and their clinical features were classified based on the TNM system of the International Union Against Cancer [15]. Corresponding adjacent non-cancerous tissues were also obtained from surgical resections. Peripheral venous blood samples from ESCC patients were collected in EDTA-containing tubes and immediately centrifuged at 2500 × g for 15 min to prepare plasma. The buffy coat fraction was also collected to assess the presence of circulating tumor cells in the peripheral blood. The plasma and buffy coat samples were stored at - 80 °C until further processing. Peripheral blood samples from an additional 10 healthy volunteers were employed as normal controls. Approval for use of human tissues and/or information was obtained from the Committee for Ethical Review of Research involving Human Subjects at Shantou University. All patients had signed informed consent forms for sample collection before surgery.

2.2. Cell lines and culture conditions

Four ESCC cell lines used for this study, denoted EC109 [16], HKESC-2, TE-1 and TE-12, were routinely cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 10 mmol/L glutamine, 100 units/mL penicillin (Sigma, St. Louis, MO), and 100 µg/mL streptomycin (Sigma) in 25-cm² tissue flasks at 37 °C in a humidified atmosphere containing 5% CO₂. The NE-2 immortalized esophageal epithelial cell line was cultured in a 1:1 mix of medium including Epilife (Cascade Biologics, Portland, OR) and dKSFM (Gibco). HKESC-2 and NE2 cells were kindly provided by Dr. S.W. Tsao (University of Hong Kong, Hong Kong) [17; 18], and TE-1 and TE-12 cells were provided by Dr. X.C. Xu (UT M.D. Anderson Cancer Center, USA).

2.3. DNA extraction and bisulfite modification

Genomic DNA from ESCC cell lines, primary tumors, plasma and buffy coat samples was extracted using a ZR Genomic DNA II Kit (Zymo Research, Orange, CA). 1µg of DNA from each sample was subjected to bisulfite modification through the use of an EZ DNA Methylation-Gold Kit (Zymo Research) following the manufacturer's instructions.

2.4. Methylation-specific PCR analysis

Methylation-specific PCR (MSP) analysis was performed on bisulfite-converted DNA to investigate methylation status. The sequences of PCR primers specific for methylated and unmethylated alleles of PTPRO and the sizes of expected PCR products are listed in Table 1. Each MSP reaction was carried out with 100 ng of bisulfite-modified DNA and 2U of Platinum Taq DNA polymerase (Invitrogen) in a final volume of 50 µl. A touch-down PCR amplification was conducted. Briefly, after an initial incubation at 94 °C for 3 min, 20 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C ($\Delta -0.4$ °C per cycle) for 1 min, and annealing at 72 °C for 30 sec were performed, followed by an additional 15 cycles of 30 sec of denaturing at 94 °C, 15 sec of annealing at 50.4 °C, and 30 sec of extension at 72 °C. MSP products were then analyzed by 2% agarose gel electrophoresis.

2.5. RNA extraction and reverse transcription PCR analysis

Total cellular RNA was extracted from ESCC cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA). The first-strand cDNA was prepared from 1 µg of total RNA using an M-MLV cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Semi-quantitative PCR for PTPRO expression was performed. Briefly, each reaction was prepared in a final volume of 25 µl of PCR mixture containing 1X PCR buffer, 2 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 0.2 mM of each primer, and 1.5 units of Platinum Taq DNA polymerase (Invitrogen). PCR conditions consisted of 35 cycles of 94 °C for 30 s, 54.5 °C for 30 s, and 72 °C for 45 s, and the resulting products were separated on 2% agarose gels and stained with ethidium bromide. DNA visualized under UV light was imaged using Bio-rad Quantity One software. β-actin transcripts in each sample were also amplified as internal controls to normalize the quantities of specific products. Gene-specific primers used for amplification of PTPRO and β-actin are listed in Table 1. Each primer was located in a different exon to avoid amplification of contaminating genomic DNA.

2.6. Demethylation treatment with 5-aza-dC

2×10⁵ cells were seeded in a 6-well plate and cultured for 24 h, then incubated with a final concentration of 20 µM 5-aza-dC (Sigma) for 72 h. Both medium and drug were replaced every 24 h. RT-PCR was performed to confirm the PTPRO expression.

2.7. Statistical analyses

All statistical analyses were performed using the statistical software package SPSS 17.0. Associations with clinical parameters were analyzed by Fisher's exact test. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Frequent methylation of PTPRO in primary tumors and peripheral blood samples of ESCC patients

Among the 36 primary ESCC tumor specimens investigated, 27 (75%) showed PTPRO hypermethylation. PTPRO promoter methylation was not observed in the majority of adjacent normal tissues. Representative MSP results in primary tumors are shown in Fig. 1. We further explored the feasibility of detecting PTPRO methylation in the plasma and buffy coat samples of matched peripheral blood from ESCC patients. Fig. 2 illustrates representative MSP results. Among 36 matched peripheral blood samples, PTPRO was aberrantly methylated in 13 (36.1%) patients in plasma, as well as in 15 (41.7%) patients in buffy coat, *i.e.*, 5 patients in plasma but not in buffy coat, 7 patients in buffy coat but not in plasma, and 8 patients in both plasma and buffy coat. Methylated PTPRO was only observed in plasma or buffy coat from patients whose corresponding primary tumors were also methylated. The results of PTPRO methylation status in primary tumors, plasma and buffy coat samples are summarized in Table 2. Additionally, no methylation of PTPRO was observed in any normal control peripheral blood samples from 10 healthy individuals, either in plasma or in buffy coat.

3.2. Clinicopathological significance of PTPRO methylation in primary tumors and peripheral blood samples

Statistical analyses of potential correlations of PTPRO methylation in primary ESCC tumors and matching peripheral blood samples with clinicopathological parameters is shown in Table 3. Methylation of PTPRO was more frequent in primary advanced-stage ESCC tumors (T3/T4) than in earlier T stages (T1/T2) (*P* = 0.013). No statistically significant correlation was observed between PTPRO methylation in primary tumors and patient age, gender,

tumor location, degree of differentiation, lymph node metastasis, pathologic stage, smoking or alcohol consumption. Additionally, statistical analyses revealed no significant correlations between methylation of PTPRO in plasma or buffy coat, and any of the common clinical or pathological parameters.

3.3. Silenced PTPRO expression correlates inversely with methylation status

The expression status of PTPRO was examined in four human ESCC cell lines and in an immortalized esophageal cell line by RT-PCR. PTPRO mRNA levels were high in HKESC-2 and NE-2 cells, but absent in three other ESCC cell lines (EC109, TE-1 and TE-12) (Fig. 3A). These cell lines thus provided a panel of PTPRO-expressing and -nonexpressing cells for subsequent analyses to define mechanisms of silencing of PTPRO expression. Subsequently, promoter methylation status was analyzed by MSP to examine whether promoter hypermethylation was associated with downregulated expression of PTPRO in ESCC cell lines. These experiments showed that in cell lines (EC-109, TE-1 and TE-12) that lacked PTPRO expression, PTPRO was methylated, whereas in cell lines expressing PTPRO (HKESC-2 and NE-2), PTPRO was unmethylated (Fig. 3B).

3.4. Re-expression of PTPRO after treatment with 5-aza-dC

To determine whether promoter hypermethylation was responsible for PTPRO inactivation, three ESCC cell lines (EC109, TE-1 and TE-12) with hypermethylated PTPRO and silenced PTPRO expression were subjected to 5-aza-dC treatment at a final concentration of 20 mM. After 72 h exposure of cells to this demethylating agent, restored PTPRO expression was observed in all three cell lines (Fig. 4A). Concomitantly, 5-aza-dC treatment increased unmethylated PTPRO alleles as determined by MSP (Fig. 4B). These data confirm that hypermethylation plays an important role in causing transcriptional silencing of PTPRO in ESCC cells.

4. Discussion

To our knowledge, this is the first report for detection of PTPRO hypermethylation as a non-invasive biomarker for solid tumors in peripheral blood. Moreover, this is also the first report of a protein-tyrosine phosphatase as a non-invasive biomarker for ESCC. In this study, promoter methylation of the PTPRO gene was found in three of four ESCC cell lines and 27 of 36 (75%) primary human ESCC specimens. This result is consistent with a previous report of PTPRO hypermethylation in human lung cancer [6]. This high frequency of methylation in primary tumors suggests that PTPRO is a common target for epigenetic silencing by methylation in ESCC, and that its methylation may be involved in ESC tumorigenesis. In PTPRO expression-silenced cell lines, expression was dramatically restored by treatment with the demethylating agent 5-aza-dC, confirming that DNA methylation is a major mechanism regulating PTPRO expression and that aberrant methylation of the PTPRO promoter is directly responsible for transcriptional inactivation of its expression in ESCC cell lines.

In cancer, oncogenic activation of tyrosine kinases is a common feature, whereas PTPs work as a counterbalance to tyrosine kinases in the regulation of tyrosine phosphorylation. To our knowledge, the current finding is the first analysis of PTPs in ESCC. Meanwhile, these results extend the study of PTPRO to two new categories of malignancy: squamous cell carcinomas and digestive tract cancers.

A key obstacle to improving ESCC patient survival is the lack of a suitable early detection method. Thus, there is an urgent need to develop novel markers of ESCC to facilitate cancer diagnosis. Promoter hypermethylation of tumor suppressor genes is not only an important

epigenetic event in the development and progression of many human cancers, but it also constitutes a potentially sensitive biomarker for the detection of diseases at early stages by using a trace amount of sample compared with the detection of protein and RNA [19; 20; 21]. Tumor markers for screening and monitoring applications with clinical value need to exhibit both high sensitivity and high specificity. Specimen collection should ideally be non-invasive and easily repeatable for monitoring residual or recurrent cancer after treatment. Since hypermethylated DNA may serve as a potential molecular tumor marker due to its high specificity in differentiating cancer from normal tissues, detection of aberrant methylation of tumor suppressor genes in the bodily fluids of cancer patients is attracting increasing attention, for example, in the detection of prostate and cervical cancer from urine, head and neck squamous cell carcinoma by saliva, lung cancer by sputum and bronchial lavage, ovarian cancer by peritoneal fluid, and nasopharyngeal carcinoma by mouth and throat fluid, or nasopharyngeal swab and plasma/serum or blood cells from peripheral blood [22-28]. Among these sample types, the most simple and broadly applicable strategy is to obtain peripheral blood samples from patients. Methylated DNA in plasma/serum is assumed to reflect abnormal DNA being disseminated from apoptotic or necrotic tumor cells [29]. It has been suggested that higher levels and larger quantities of tumor DNA are present in plasma than in serum because DNA released from normal peripheral blood nucleated cells (PBNCs) during the clotting process could reduce the fractional concentration of tumor DNA in serum [30; 31]. In contrast, the presence of detectable methylated promoter DNA in blood cells has been shown to indicate the presence of circulating cancer cells during the process of distant metastasis, the major cause of mortality in most cancers. PTPRO methylation indeed occurred only in the B-cell population of a subset of patients with CLL, but not in normal B or T lymphocytes, indicating that methylated PTPRO in blood cells is cancer-specific [13]. Consequently, we preferred to investigate PTPRO methylation in peripheral blood samples with plasma and buffy coat in the current study.

Our data support a high sensitivity and specificity of methylated PTPRO in peripheral blood samples, consistent with previous reports on other tumor suppressor genes [21; 32]. Identical alterations were detectable in the plasma or buffy coat of 20 (74.1%) of 27 patients positive for methylated PTPRO in the primary tumors. Notably, PTPRO methylation detectability was higher in buffy coat than in plasma, consistent with the previous reports [33; 34]. Such a high correlation of detecting PTPRO methylation when comparing primary tumors and matching peripheral blood further confirmed that peripheral blood samples could potentially be used to assist the detection and diagnosis of ESCC. Moreover, high specificity was indicated by the absence of methylated PTPRO in plasma or buffy coat from either ESCC patients without primary tumor methylation or any of the normal control peripheral blood samples.

Statistical analysis of our data demonstrated that a high frequency of PTPRO hypermethylation in primary tumors significantly correlated with T stage, indicating that PTPRO methylation may be involved in invasion of ESCC. Because all ESCC patients in this study were followed for up to 2 years, we could not evaluate the prognostic value of PTPRO methylation. However, methylation status of PTPRO in peripheral blood samples showed no significant correlation with clinicopathological factors. There are numerous potential explanations for this result: *e.g.*, the quantity of tumor DNA and tumor cells released into circulation is subject to specific physiological and histological characteristics of each tumor sample, and the quality and quantity of extracted DNA from plasma/serum and buffy coats depends on time of collection, methods of pretreatment, and other factors. Given that collection of peripheral blood can be easily conducted as a screening procedure without the necessity for complicated and invasive endoscopic biopsy, detection of methylated DNA in peripheral blood could be developed as a promising tumor marker in screening for

primary ESCC within high-risk populations, as well as in detection of minimal residual tumors.

In summary, we have detected methylation of the PTPRO promoter in primary tumors, plasma and buffy coat samples from patients with ESCC. Because this epigenetic tumor suppressor gene alteration is ubiquitous in ESCC, it represents a novel approach to early diagnosis and monitoring of this deadly disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Representative MSP results of the PTPTO gene in primary ESCC tumors. Numbers on top, sample number. M, methylated; U, unmethylated.

Fig. 2. Representative MSP result of the PTPTO gene in plasma and buffy coat samples of one ESCC patient (No.4) and one normal healthy individual. M, methylated; U, unmethylated.

Fig. 3.

Expression and methylation analysis of PTPRO in ESCC cell lines

A. Expression of PTPRO in human ESCC cell lines. Total RNA isolated from ESCC cell lines was subjected to RT-PCR analysis with PTPRO-specific primers. Immortalized esophageal epithelial cell line NE2 was used as a normal control and β -actin was used as an internal loading control. B. MSP analysis of PTPRO methylation status in ESCC cell lines. NE2 was used as a normal control. M, methylated; U, unmethylated.

Fig. 4.

Recovered expression of PTPRO by treatment of 5-aza-dC

A. Human ESCC cell lines were treated with 5-aza-dC for 72 h. Total RNA extracted from cells was subjected to RT-PCR to amplify PTPRO mRNA. β -actin was used as an internal loading control. B. MSP analysis of PTPRO methylation status in ESCC cell line with or without treatment of 5-aza-dC. M, methylated; U, unmethylated.

Table 1

PCR primer sequences

Primers	Sequences	Product size
PTPRO-forward	5'-CTCCACCCAAATCACTCTTCGCAG-3'	268 bp
PTPRO-reverse	5'-ACCATTGTTGAGACGGCTATGAACG-3'	
β -actin-forward	5'-GAACCCCAAGGCCAACC GCGAGA-3'	149 bp
β -actin-reverse	5'-TGACCCCGTCACCGGAGTCCATC-3'	
MSP-methylated-forward	5'-CGTTTTTGGAGGATTCGGGC-3'	170 bp
MSP-methylated-reverse	5'-AAAACACGACTACGCTAACG-3'	
MSP-unmethylated-forward	5'-ATGTTTTTGGAGGATTTGGGT-3'	201 bp
MSP-unmethylated-reverse	5'-ATACCCCATCACTACACAAACA-3'	

Table 2

Methylation patterns of the PTPRO genes in primary tumors, plasma and buffy coat samples from 36 ESCC patients

Patient no.	Primary tumor	Plasma	Buffy coat
1	○	○	○
2	○	○	○
3	○	○	○
4	●	●	●
5	●	●	●
6	○	○	○
7	○	○	○
8	●	○	○
9	●	○	●
10	○	○	○
11	●	○	●
12	●	●	●
13	●	○	●
14	●	○	○
15	○	○	○
16	●	●	○
17	●	○	○
18	●	●	●
19	●	●	○
20	●	○	●
21	●	○	○
22	●	●	●
23	●	●	●
24	●	○	●
25	●	○	○
26	●	○	○
27	●	●	○
28	●	●	●
29	○	○	○
30	●	○	○
31	●	●	●
32	●	●	○
33	●	○	●
34	●	○	●
35	●	●	○
36	○	○	○

● methylated;

○ unmethylated.

Table 3

Correlation of PTPRO methylation with clinicopathological characteristics

Variables	PTPRO methylation									
	Primary tumors					Plasma				
	M (%)	U (%)	P	M (%)	U (%)	P	M (%)	U (%)	P	M (%)
Total (N = 36)	27 (75)	9 (25)		13 (36)	21 (64)		15 (42)	21 (58)		20 (56)
Gender										
Male (24)	17 (71)	7 (29)	0.685	8 (33)	16 (67)	0.72	10 (42)	14 (58)	1	13 (54)
Female (12)	10 (83)	2 (17)		5 (42)	7 (58)		5 (42)	7 (58)		7 (58)
Age (years)										
< 60 (20)	15 (75)	5 (25)	1	6 (30)	14 (70)	0.493	9 (45)	11 (55)	0.741	10 (50)
≥ 60 (16)	12 (75)	4 (25)		7 (44)	9 (56)		6 (37)	10 (63)		10 (63)
Location of tumors										
Upper (3)	3 (100)	0 (0)	0.465	2 (67)	1 (33)	0.554	1 (33)	2 (67)	0.875	2 (67)
Middle (27)	19 (70)	8 (30)		8 (30)	19 (70)		11 (41)	16 (59)		9 (33)
Lower (6)	5 (83)	1 (17)		3 (50)	3 (50)		3 (50)	3 (50)		4 (67)
Lesion length										
< 5 cm (10)	8 (80)	2 (20)	1	2 (20)	8 (80)	0.27	6 (60)	4 (40)	0.26	6 (60)
≥ 5 cm (26)	19 (73)	7 (27)		11 (42)	15 (58)		9 (35)	17 (65)		14 (54)
Histology										
Well (8)	5 (63)	3 (37)	0.431	2 (25)	6 (75)	0.382	3 (37)	5 (63)	0.67	3 (37)
Moderately (18)	14 (78)	4 (22)		6 (33)	12 (67)		7 (39)	11 (61)		11 (61)
Poorly (9)	8 (89)	1 (11)		5 (56)	4 (44)		5 (56)	4 (44)		6 (67)
Depth of the tumors										
T ₁ /T ₂ (8)	3 (37)	5 (63)	0.013	1 (13)	7 (87)	0.213	2 (25)	6 (75)	0.424	2 (25)
T ₃ /T ₄ (28)	24 (86)	4 (14)		12 (43)	16 (57)		13 (46)	15 (54)		18 (64)
Lymphnode metastasis										
Positive (20)	15 (75)	5 (25)	1	6 (30)	14 (70)	0.493	9 (45)	11 (55)	0.741	11 (55)
Negative (16)	12 (75)	4 (25)		7 (44)	9 (56)		6 (37)	10 (63)		9 (56)
Stage of tumors										
II (19)	13 (68)	6 (32)	0.451	5 (26)	12 (74)	0.502	8 (42)	11 (58)	1	9 (47)
										10 (53)

Vairables	PTPRO methylation											
	Primary tumors			Plasma			Buffy coat			Plasma / buffy coat		
	M (%)	U (%)	P	M (%)	U (%)	P	M (%)	U (%)	P	M (%)	U (%)	P
III (17)	14 (82)	3 (18)		8 (47)	11 (53)		7 (41)	10 (59)		11 (65)	6 (35)	
Smoking status												
Never (21)	14 (67)	7 (33)	0.252	6 (29)	15 (71)	0.31	7 (33)	14 (67)	0.31	10 (48)	11 (52)	0.32
Current (15)	13 (87)	2 (13)		7 (47)	8 (53)		8 (53)	7 (47)		10 (67)	5 (33)	
Alcohol drinking status												
Never (13)	9 (69)	4 (31)	0.693	3 (23)	10 (77)	0.292	4 (31)	9 (69)	0.484	5 (38)	8 (62)	0.169
Current (23)	18 (78)	5 (22)		10 (43)	13(57)		11 (48)	12 (52)		15 (65)	8(35)	

Note: M, methylated; U, unmethylated. Data on histology had 1 sample with a special ESCC pathological style.