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Polo-like kinase and survivin are esophageal tumor-specific promoters ^{☆,☆☆}

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14 Abstract

15 For developing successful cancer gene therapy strategies, tumor-specific gene delivery is essential. In this study, we used esophageal
 16 cancer (EC) cells to identify and evaluate esophageal tumor-specific gene promoters. Four genes (polo-like kinase-1/PLK, survivin/
 17 BIRC5, karyopherin α 2/KPNA2, and pituitary tumor transforming gene protein 1/PTTG1) were identified by a microarray analysis
 18 as highly expressed in EC cell lines vs. five normal organ tissues (liver, lung, kidney, brain, and heart). By quantitative RT-PCR, the
 19 average mRNA expression levels of these four genes in 20 primary ECs were 2.7-fold (PLK), 6.1-fold (survivin), 2.6-fold (KPNA2),
 20 and 2.4-fold (PTTG1) higher than that of each gene in 24 different normal organs. By dual luciferase assay, the promoter activity of
 21 PLK and survivin in EC cell lines was 18.9-fold and 28.5-fold higher, respectively, than in normal lung and renal cells. The promoters
 22 of PLK and survivin could be useful tools for developing EC-specific gene therapy vectors.

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24 **Keywords:** Esophageal cancer; Gene therapy; Tumor-specific promoter; Microarray

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^{☆☆} **Abbreviations:** EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma; RT-PCR, reverse-transcriptase polymerase chain reaction; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; PLK, polo-like kinase; PTTG1, pituitary tumor transforming gene 1; KPNA, karyopherin α 2; SAM, significance analysis of microarray; TNF, tumor necrosis factor; TSP, tumor-specific promoter; HNBEC, human bronchial epithelial cells; HNRCEC, human normal renal cortical epithelial cells.

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Esophageal cancer (EC) has a very aggressive behavior and tends to recur after surgery and to spread systematically, despite recent surgical and chemo-radiotherapeutic advances; moreover, EC ranks as the eighth most common malignancy and the sixth most frequent cause of cancer death worldwide [1]. Survival of esophageal cancer patients remains poor, being approximately 10% for squamous cell carcinoma (ESCC) and 20% for adenocarcinoma (EAC) [2]. Thus, other therapeutic methods with the capability of systematic application are needed. Gene therapy is one such therapeutic approach.

At the experimental level, a number of gene therapy approaches have been applied to esophageal cancer, and results have been promising. Gene transfer of p16 [3], p53 [4], p21 [5], and p14 [6], and gene knock-down of cyclin D1 [7], c-myc [8], and VEGF [9] induced cell growth 41

42 inhibition or apoptosis in esophageal cancer cells. However, these therapeutic approaches to esophageal cancer have not been applied to the clinic as much as in other malignancies. Among 1132 approved clinical trials worldwide (<http://www.wiley.co.uk/genetherapy/clinical/>), only four gene therapy clinical trials have been approved for EC. In the U.S.A., only one gene therapy protocol (0208-549) transferring tumor necrosis factor (TNF) gene by adenovirus vector is registered in a NIH gene transfer protocol (<http://www4.od.nih.gov/oba/rac/PROTOCOL.pdf>). In Japan, a protocol transferring wild-type p53 gene into ESCC using adenovirus vector has reached a clinical phase [10]. The other two protocols are genetic immunotherapies in United Kingdom and Belgium.

56 In gene therapy for any type of malignancy, tumor specificity is of utmost importance. For other cancers, investigators have utilized tumor-specific promoters (TSP), such as Cox-2 [11], CXCR4 [12], Survivin [13], CEA [14], and others, for transcriptional targeting of cancer genes. However, to our knowledge, systematic screening for TSPs in esophageal cancer has not yet been reported. Thus, the aim of the current study was to systematically identify genes that are expressed specifically in esophageal cancer cells as candidate TSPs in an esophageal cancer-specific expressing vector.

67 Materials and methods

68 *Clinical tissue specimens.* ESCC tumor samples were obtained from 10 patients with primary ESCC who underwent surgery at Akita University Hospital, Akita, Japan. In addition, 10 Barrett's esophagus and 10 EAC tumor samples were obtained from patients who underwent upper gastrointestinal endoscopy at the Baltimore VA Medical Center, Baltimore, MD. Other clinical tissue specimens of normal organs listed in Table 1 were obtained from the University of Maryland Tissue Bank Core Facility, Baltimore, MD.

76 *Cell lines and cell culture.* Eight ESCC cell lines used in this study were established at Kyoto University [15]. The EAC cell lines, SEG-1, FLO-1, and BIC-1, were kindly provided by Dr. David Beer, Department of Surgery, University of Michigan [16]. The EAC cell line OE33 was purchased from the European Collection of Cell Culture (ECACC). All EC cell lines were maintained in RPMI 1640 and Ham's F12 (Invitrogen, Carlsbad, CA) mixed (1:1) medium containing 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Two normal tissue-derived cell lines, human normal bronchial epithelial cells (HNBEC) and human normal renal cortical epithelial cells (HNRCEC), were purchased from Cambrex (Walkersville, MD) and maintained according to the manufacturer's protocol.

88 *RNA.* Total RNA from cell lines and clinical tissue specimens was extracted using Trizol reagent (Invitrogen, CA). For microarray analysis, mRNAs of 12 EC cell lines were purified from total RNA using Oligotex (Qiagen, Valencia, CA). Five mRNAs from human normal liver, lung, kidney, heart, and brain were purchased from the Clontech library (Takara Bio Company, Mountain view, CA).

94 *Microarray analysis.* The aim of the current study was to identify esophageal TSPs. Thus, we first needed to select differentially expressed genes in esophageal cancer cells. If EC tissue specimens were used, it would have been difficult to eliminate from considered genes expressed in cancer stromal or other nonepithelial cells. Therefore, in this study, microarray analysis was performed on EC cell lines (ESCC: KYSE30, 70, 110, 140, 170, 220, 520, and 770, EAC: OE33, BIC-1, FLO-1, and SEG-1) vs. normal organs (lung, kidney, liver, heart, and brain). Our in-house

cDNA microarray chip (8192 clones per slide) and experimental procedures were previously described [16]. In brief, 2 µg of mRNA prepared from reference cells and EC cell lines or normal organs was labeled using incorporation of Cy3- or Cy5-labeled dCTP, random primers, and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting labeled probes were purified with a Microcon microcentrifuge filter device (Millipore, MA) and recovered in a volume of 25 µl. Each slide was incubated in 35 µl of hybridization solution containing Cy3- and Cy5-labeled target, 1 µl of 50× Denhardt's blocking solution (Sigma, St. Louis, MO), 20 µg of Human COT 1-DNA (Roche Diagnostics Corp., Indianapolis, IN), 10 µg of yeast tRNA (Roche Diagnostics Corp.), and 8–10 µg of poly(A) (Roche Diagnostics Corp.) in 2.24× SSC/0.25% SDS under a 40×22-mm coverslip at 65 °C overnight. On the next day, each hybridized slide was washed with SSC solution and scanned using a GenePix 4000A dual-laser slide scanning system (Axon) at wavelengths corresponding to each probe's unique fluorescence (635 and 532 nm for Cy5 and Cy3, respectively). The obtained data were normalized using a previously described method [17]. To obtain powerful promoters, genes with low signal in EC cells (less than 1000 in 1–65536 scanning range of microarray slide) were eliminated before statistical analysis. Differentially expressed genes were identified by SAM software (Stanford University, <http://www-stat.stanford.edu/~tibs/SAM/>).

124 *Quantitative RT-PCR.* The samples used for verification of gene expression levels are listed in Table 1. One-step Quantitative RT-PCR (qRT-PCR) using CYBR Green dye was performed on an iCycler real-time PCR machine (Bio-Rad, Hercules, CA). For qRT-PCR, each amplicon spanned an exon–exon boundary in order to exclude genomic DNA contamination. We obtained exon–exon boundary information on genes of interest from the Ensembl Genome Server of the Sanger Centre (<http://www.ensembl.org/>). RNA sequence and exon–exon boundary information was placed into qPCR primer design software (PrimerExpress version 1.5, Applied Biosystems). The primer sequences used in qRT-PCR were as follows: Sense, 5'-CTTCGTGTTTCGTGGTGTGGA-3', Anti-sense, 5'-GCCAAGCACAATTTGCCGTA-3' for PLK, Sense, 5'-TTCAAGAAGTGGCCCTTCTTG-3', Antisense, 5'-CAACCGGACGAATGCTTTT-3' for survivin, Sense, 5'-TAATACACCAGCTGCCCGTCT-3', Antisense, 5'-TTGACCTCTATTCTGCGACGC-3' for KPNA2, and Sense, 5'-ACCCCTCAAACAAAAACAGCC-3', Antisense, 5'-GGCAGGAACAGAGCTTTTTGC-3' for PTTG1. Oligonucleotides for qRT-PCR primers were chemically synthesized by IDT(IA). qRT-PCR mix contained 12.5 µl of SYBR Green Quantitect PCR mastermix (Qiagen, CA), 1 µl of each forward and reverse primer (10 µM), 0.25 µl of reverse transcriptase, 10 ng of tRNA, and H₂O up to 25 µl total volume. PCR conditions were as follows: 50 °C for 30 min (reverse transcription), 95 °C for 15 min (polymerase activation), and 50 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. After the PCR steps, to check the specificity of the PCR, a melting curve analysis was performed as follows: 95 °C for 1 min (denature), 55 °C for 1 min (annealing), and 0.5 °C increment for 30 s up to 95 °C. In each qRT-PCR run, serial dilutions of a single reference total RNA derived from HT-29 were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve. qRT-PCRs of each sample were run in triplicate; the mean value of three reactions was defined as a representative expression of the sample. βActin amplification was used to normalize quantitative data. The formula for normalization was: ratio of sample to reference RNA = Gene(s)/Gene(r)/(βActin(s)/βActin(r)), where Gene(s) and Gene(r) were expression levels of each gene of interest in the sample and reference RNA, respectively, and βActin(s) and βActin(r) were βActin mRNA expression levels in the sample and reference.

161 *Cloning of promoter regions.* The promoter region of each selected gene was predicted by a web-based promoter prediction engine (<http://www.genomatix.de/>). PCR primers for amplification of promoter regions were designed at approximately 600 bps from the transcriptional start, which overlaps most predicted promoter regions. Primer sequences used for promoter cloning were as follows: Sense, 5'-TACCGAGCTCTGGAAATTCAGTAGCGAGGA-3', Antisense, 5'-TACCGTAGCC TGCAGACCTCGATCCGAG-3' for PLK (position –617 to –16 from the translational start site), Sense, 5'-TAC

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Table 1
Tissues used in RT-PCR study

Tissue	#	Type	Source
Adrenal gland	61	Pool	C
Bladder	3	Ind.	UMD
Brain (whole)	1		C
Breast	2	Ind.	UMD
Colon	4	Ind.	UMD
Fetal brain	21	Pool	C
Fetal liver	63	Pool	C
Heart	10	Pool	C
Kidney	1		C
Kidney	4	Ind.	UMD
Liver	1		C
Liver	2	Ind.	UMD
Lung	2	Ind.	UMD
Lung (whole)	3	Pool	C
Placenta	8	Pool	C
Prostate	32	Pool	C
Salivary gland	24	Pool	C
Skeletal muscle	7	Pool	C
Small intestine	5	Pool	C
Spinal cord	49	Pool	C
Spleen	14	Pool	C
Stomach	1		UMD
Testis	45	Pool	C
Thymus	3	Pool	C
Thyroid	65	Pool	C
Trachea	2	Pool	C
Uterus	3	Pool	C
Normal esophagus	6	Ind.	UMD
Barrett's esophagus	5	Ind.	UMD
ESCC	10	Ind.	UMD
EAD	10	Ind.	UMD

Pool, pooled total RNA; Ind., individual total RNA; C, Clontech library (Takara Bio Company, CA); UMD, University of Maryland.

170 CGAGCTCGTCTGGAGTAGATGCTTTTTGCAG-3', Antisense, 5'-T
171 ACCGCTAGCGGGTCCCGGATTCAAAT-3' for survivin (-625 to
172 -25), Sense, 5'-TACCGAGCTCGCGAGTACTTTGGGTGTCATAA
173 TA-3', Antisense, 5'-TACCGCTAGCTCGACTCAGCTCAAAGACCG
174 T-3' for KPNA2 (-711 to -107), and Sense, 5'-TACCGAGCT
175 CGTCAGTACCTCAGTCCATGCAGC-3', Antisense, 5'-TACCGCTA
176 GCCACACAAAAACAAGAGCTAAACAG-3' for PTTG1 (-730 to
177 -100). The underlined sequences indicate the *SacI* and *NheI* recognition
178 sites. PCR-amplified fragments were inserted into the *SacI*-*NheI* site of the
179 pGL3-basic vector (Promega, Madison, WI). The sequences inserted into
180 the pGL3-basic vector were confirmed by BigDye sequencing reagent
181 (Applied Biosystems, CA) with a 96-capillary auto-sequencer (Spectru-
182 uMedix, PA).

183 **Promoter assay.** One hundred thousand cells of each cell line were
184 placed into a 24-well plate at 24 h before transfection. To form a lipid/
185 DNA complex, 0.2 ng each of pGL3-Basic with promoter and pRL-TK
186 vector, and 0.8 μ l of TransIT-Keratinocyte Transfection reagent (Mirus,
187 WI) were mixed in 50 μ l Opti-MEM medium (Invitrogen, CA) and incu-
188 bated at room temperature for 20 min. This lipid/DNA complex was
189 added into 500 μ l of culture media of the cells prepared in 24-well plate.
190 Twenty-four hours after transfection, cells were harvested by 75 μ l of
191 passive cell lysis buffer (Promega, WI). The dual luciferase reporter assay
192 was performed by Dual-Glo luciferase Assay kit (Promega, WI). The
193 intensity of luminescence was measured by a TD-20/20 luminometer
194 (Turnerbiosystems, Sunnyvale, CA). pRL-TK vector was used as internal
195 control, and data were normalized by *Renilla* luciferase luminescence
196 intensity.

197 **Statistical analysis and software.** The normalization procedure for
198 microarray data was performed using STATISTICA ver. 6.1 (Statsoft,

OK). Differentially expressed genes were identified by SAM software
(Stanford University). Student's *t* test was performed by STATISTICA,
and *p*-values less than 0.05 were considered significant.

Results

Microarray analysis

SAM software calculates a score for each gene by mean
expression difference divided by variance of gene expression.
This sometimes makes a high score for genes with low fold
change but low variance. Therefore, the SAM gene list was
sorted by SAM score and fold-change to select genes with
a high SAM score and high fold-change. The top 15 gene list
is shown in Table 2. Four genes (polo-like kinase 1/PLK, sur-
vivin/BIRC5, karyopherin α 2/KPNA2, and pituitary tumor
transforming gene protein 1/PTTG1) overlapped in the two
gene lists sorted by SAM score and fold-change. These four
genes were selected for further study.

Quantitative RT-PCR

To validate the microarray data, one-step qRT-PCR
was performed using EC clinical tissue samples and various
normal organ tissues (Table 1). For the four genes, the
average mRNA expression levels in EC tumors were 2.7-
(PLK), 6.1- (survivin), 2.6- (KPNA2), and 2.4- (PTTG1)
fold higher than in normal organs excluding testis. The
qRT-PCR data were normalized by the housekeeping gene,
 β actin. However, our data suggested that testis had rela-
tively low β actin expression. This finding is one possible
explanation why the expression of the four genes in testis
was estimated as extremely high. The average mRNA
expression of each gene in EC was significantly higher than
that in normal organs or Barrett's esophagus tissues. How-
ever, no significant difference in gene expression was found
between normal organs and Barrett's esophagus.

Promoter assay

mRNA expression level is not always associated with
promoter activity, because mRNA expression level is con-
trolled by both mRNA transcription and mRNA degrada-
tion. Therefore, the promoter activity of each gene was
measured using various EC cell lines and normal cells.
The average promoter activities of the four genes in EC
cells were 18.9 (PLK), 28.5 (survivin), 5.4 (KPNA2), and
2.1 (PTTG1)-fold higher than in normal lung and kidney
cells. This difference was not statistically significant,
because KYSE110 showed extremely high promoter activ-
ity of these four genes, and this finding made the variance
of the EC group data larger. If, however, KYSE110 was
excluded from statistical analysis, the differences in pro-
moter activity between EC cells and normal cells reached
statistical significance for PLK ($p = 0.025$), survivin
($p = 0.001$), and KPNA2 ($p = 0.027$). KPNA2 and PTTG1
had moderate promoter activity even in normal cells.

Table 2
Microarray analysis

Gene name (top 15 genes sorted by SAM's score)	Score	Fold
<i>(A)</i>		
Polo (<i>Drosophila</i>)-like kinase (PLK)	10.03	9.120
Cdc6-related protein (HsCDC6)	9.37	5.270
Protein regulator of cytokinesis 1 (PRC1)	8.63	7.370
Cyclin-selective ubiquitin carrier protein	8.48	8.400
Thymidine kinase 1, soluble	8.46	7.910
Baculoviral IAP repeat-containing 5 (survivin) (BIRC5)	8.40	13.800
Chaperonin containing t-complex polypeptide 1	8.34	6.130
Karyopherin α 2 (KPNA2)	8.25	9.810
IMAGE:122194 3'	8.15	4.210
Mitotic checkpoint protein kinase BUB1B (BUB1B)	8.11	4.750
Chromosome segregation 1-like (CSE1L)	8.04	5.560
Pituitary tumor transforming gene protein 1 (PTTG1)	8.02	9.800
Topoisomerase (DNA) II α (170kD) (TOP2A)	7.98	8.170
Flap endonuclease-1	7.96	5.030
Cyclin-dependent kinase 4 (CDK4)	7.89	6.670
Gene name (top 15 genes sorted by fold change)	Score	Fold
<i>(B)</i>		
Keratin 6A (KRT6A)	1.99	26.85
Keratin 13 (KRT13)	1.31	19.22
Keratin 5 (KRT5)	0.78	17.85
S100 calcium-binding protein A2 (S100A2)	2.26	17.21
AIM1, aurora, and IPL1-like midbody-associated protein kinase-1	3.76	14.11
Baculoviral IAP repeat-containing 5 (survivin) (BIRC5)	8.40	13.80
Keratin 14 (KRT14)	1.59	12.26
Hexokinase 1 (HK1)	2.48	11.29
Karyopherin α 2 (KPNA2)	8.25	9.81
Pituitary tumor transforming gene protein 1 (PTTG1)	8.02	9.80
Proliferating cell nuclear antigen (PCNA)	6.61	9.76
CDC28 protein kinase 2 (CKS2)	7.08	9.60
Keratin 19 (KRT19)	4.04	9.45
Polo (<i>Drosophila</i>)-like kinase (PLK)	10.03	9.12
14-3-3 sigma protein	2.91	9.11

(A) Top-15 genes highly expressed in EC, sorted by SAM's score. (B) Top-15 genes sorted by fold-change. Four genes (PLK, survivin, KPNA2, and PTTG1) were overlapped in both tables.

249 Discussion

250 In the current study, we first utilized cDNA microarray
251 analysis to identify genes expressed specifically in EC cells.
252 In order to establish an effective gene therapy strategies,
253 side effects of a proposed transgene should be minimized
254 in normal cells, particularly cells of vital organs. Thus, in
255 the current study, mRNAs of liver, lung, kidney, heart,
256 and brain were used to screen out gene in the initial micro-
257 array analysis.

258 This microarray investigation identified four genes,
259 PLK, survivin, KPNA2, and PTTG1 (Table 2). PLK is a
260 serine–threonine kinase and one of the most important reg-
261 ulators of mitotic progression in mammalian cells [18]. The
262 elevation of PLK expression is positively correlated with a
263 broad range of human tumors [19,20]. Ninety-seven per-
264 cent of esophageal cancers overexpress the PLK gene
265 [21]. Survivin is a member of the inhibitor of apoptosis pro-
266 tein (IAP) family, which has been implicated in both the
267 control of cell division and the inhibition of apoptosis
268 [22]. Survivin is overexpressed in esophageal cancer [23]
269 as well as other malignancies and is associated with EC

patients' prognosis [23]. p53 interacts specifically with
PTTG1 both in vitro and in vivo; this interaction blocks
the specific binding of p53 to DNA and inhibits its tran-
scriptional activity [24]. PTTG1 also inhibits the ability
of p53 to induce cell death. In esophageal cancer, PTTG1
overexpression was associated with lymph node metastasis
and a poor prognosis [24]. Thus, it is reasonable to assume
that these three genes were found in our initial gene filtra-
tion step because of their functional role in cancer. On the
other hand, the role of KPNA2, a nuclear transport protein
[25], in malignant tumors has been mostly unknown. The
expression of KPNA2 in esophageal cancers has not yet
been reported.

All four genes selected by microarray analysis were high-
ly expressed in clinical tissue specimens of EC tumors, com-
pared with other normal organ-derived cells (Fig. 1).
However, in the qRT-PCR analysis, fold changes of
mRNA expression of the four genes were less than the esti-
mation from microarray results. The mRNA derived from
cancer cells was diluted by mRNA from stromal cells. This
result could explain why the fold-change from qRT-PCR
was lower than in the microarray analysis.

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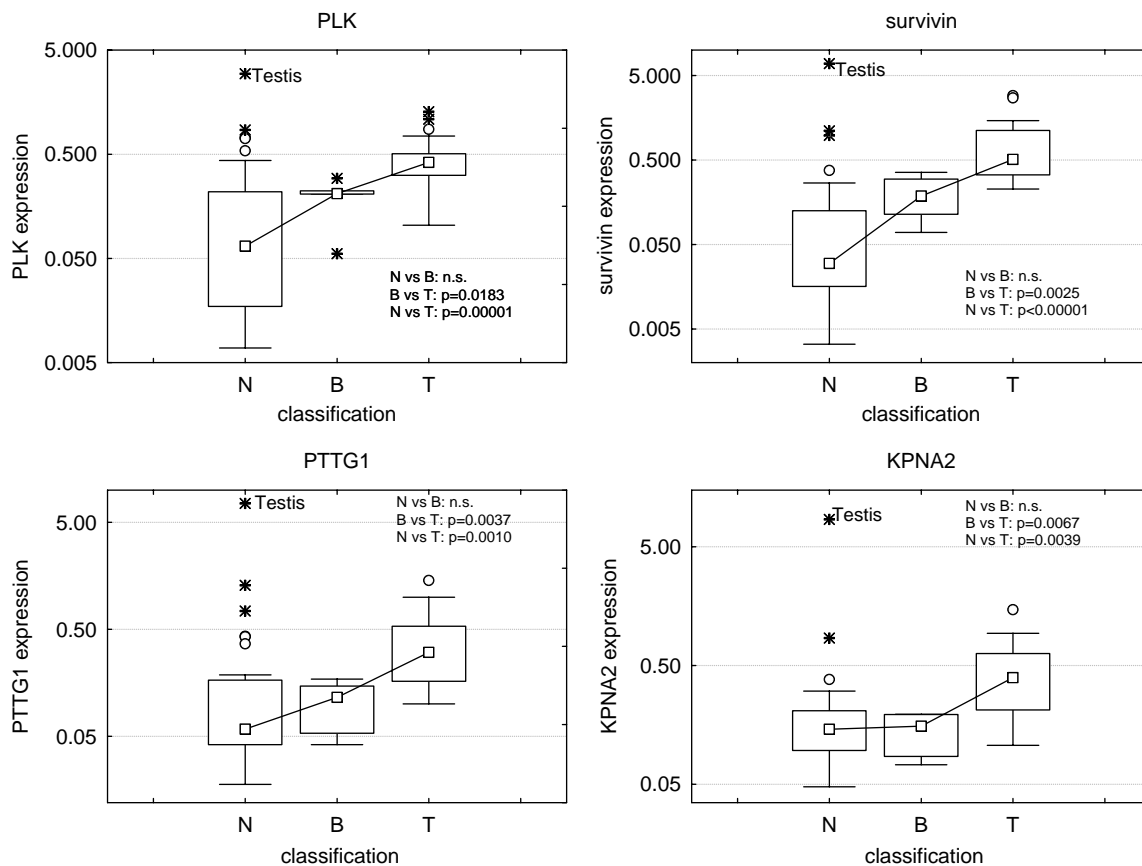


Fig. 1. Quantitative RT-PCR. The mRNA expression levels of the four genes were measured in 23 different kinds of normal organs (N), 10 Barrett's esophagus (B), and 10 esophageal tumors (five ESCCs and five EACs, T). The average mRNA expression levels of the four genes in EC tumors were 2.7 (PLK)-, 6.1 (survivin)-, 2.6 (KPNA2)-, and 2.4 (PTTG1)-fold higher than in the normal organ cells excluding the testis that is expressing these four genes at extremely high levels.

292 The promoter assay showed that survivin and PLK promoters were strong and highly EC-specific. In contrast to
 293 these two promoters, KPNA2 and PTTG1 promoters
 294 had moderate levels of promoter activity, even in normal
 295 cells. In the initial screening and the validation analysis,
 296 normal clinical tissues were used in which normal cells were
 297 not proliferating extensively. On the other hand, normal
 298 cells for promoter assays are growing continuously in pri-
 299 mary culture medium. This difference of cell cycle propor-
 300 tions could be a reason why PKNA2 and PTTG1 had low
 302 expression levels in normal tissue cells, but had moderate
 303 promoter activity in normal primary culture cells. Thus,
 304 survivin and PLK promoters were superior to KPNA2
 305 and PTTG1 promoters as EC-specific promoters for gene
 306 therapy vectors (see Fig. 2).

307 Previous extensive studies have identified the promoter
 308 regions and transcriptional factor-binding sites of the
 309 PLK [26], survivin [27,28], and PTTG1 [29,30] genes. In
 310 the current study, the promoter region of each gene was pre-
 311 dicted by a web-based promoter prediction engine ([http://](http://www.genomatix.de/)
 312 www.genomatix.de/). Next, an approximately 600 bp frag-
 313 ment covering the predicted promoter region was cloned.
 314 The promoter regions cloned in this study included core
 315 promoter regions and most of the important transcription

316 factor-binding sites for PLK (TRE, SP1, c-myc, E2F, 316
 317 CCAAT-box, and CDE/CHR elements) [26] and survivin 317
 318 (TCF4, SP1, p53, and CDE/CHR elements) [28,31]. The 318
 319 PTTG1 promoter region cloned in this study covered most 319
 320 of the critical transcription factor-binding elements of the 320
 321 gene, however, it did not include a TCF4-binding motif that 321
 322 was recently identified –1096 bps upstream from the trans- 322
 323 lational start site of PTTG1 [29]. Because a dominant-neg- 323
 324 ative TCF4 did not affect the basal level of PTTG1 324
 325 transcription [29], normal lung and kidney cells would have 325
 326 a moderate level of PTTG1 transcription, even if the TCF4- 326
 327 binding site was included. A detailed promoter analysis of 327
 328 the KPNA2 gene has not been reported yet. 328

329 For targeting cancer cells, the promoters of PLK and 329
 330 survivin could be useful in two ways. The first targeting 330
 331 strategy would be to express the transgene specifically in 331
 332 EC cancer cells. The transgene controlled by the EC-specif- 332
 333 ic promoter would be expressed in EC cells, but not in nor- 333
 334 mal cells. This would minimize the side effects of the 334
 335 transgene in normal cells. For example, a CEA-driven gene 335
 336 therapy vector expressed the suicide gene (HSV-tk) strictly 336
 337 in tumor cells [14]. The other strategy would be to control 337
 338 the replication of virus vector. If the replication-related 338
 339 virus genes were placed downstream of the EC-specific 339

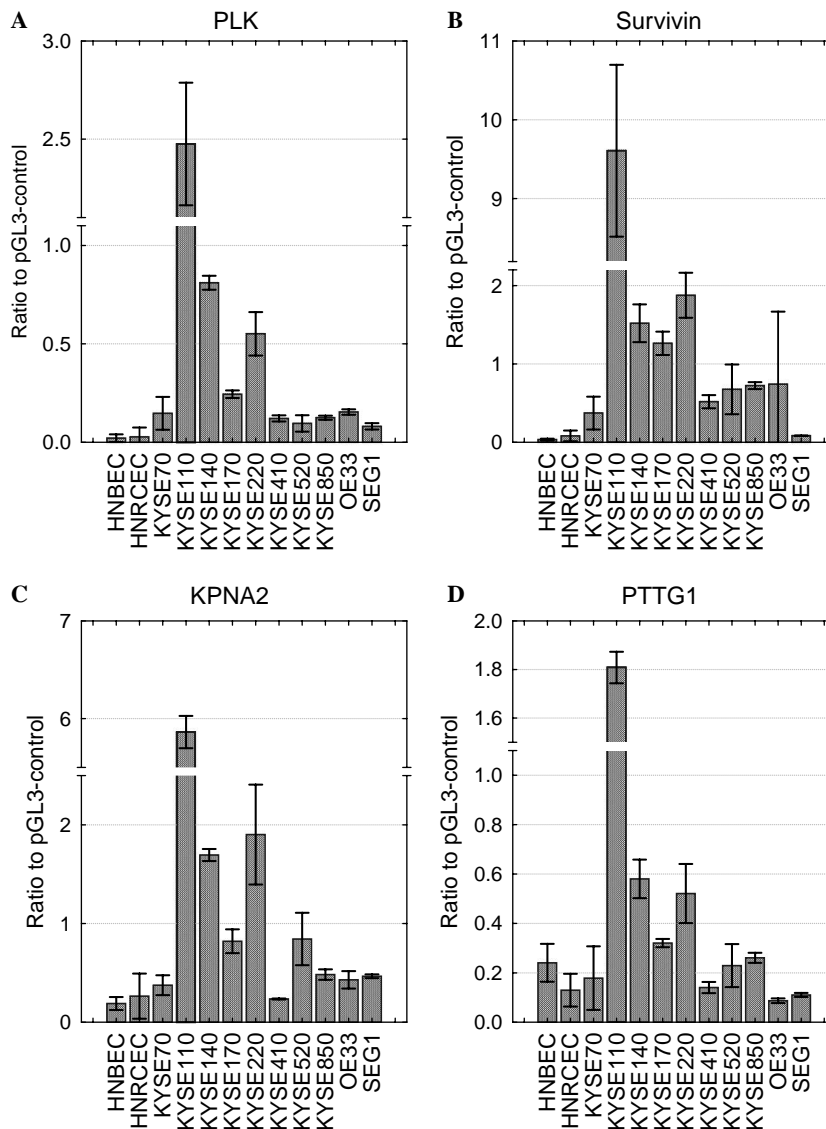


Fig. 2. Promoter assay. PLK, survivin, KPNA2, and PTTG1 promoters were cloned into the 5' upstream region of the *firefly* luciferase gene of pGL3-Basic. The *firefly* luciferase intensity was normalized by the *Renilla* luciferase activity of the internal control vector, pRL-TK. The promoter activities of the four genes was shown as a ratio to that of the pGL3-control vector. The bars and error lines represent the mean promoter activity of three independent measurements and 95% confidence interval, respectively. (A) PLK promoter activity, (B) survivin promoter activity, (C) KPNA2 promoter activity, and (D) PTTG1 promoter activity. HNBECE, human normal bronchial epithelial cells; HNRCEC, human normal renal cortical epithelial cells (Cambrex, MD); KYSEs, esophageal squamous cell carcinoma cell lines; others, esophageal adenocarcinoma cell lines.

340 promoter, the virus vector would replicate specifically in
 341 EC cells. This would maximize the transgene delivery into
 342 EC cells. Indeed, a recent study [13] utilized the survivin
 343 promoter to regulate the expression of adenoviral early
 344 region 1A (E1A) and successfully restricted the replication
 345 of the adenovirus vector in tumor tissues.

346 On the other hand, EC-specific expressed genes as well
 347 as their promoters could also be useful as targets of gene
 348 knock-down strategies. Knock-down of PLK by siRNA
 349 induced apoptosis of cancer cells [32] and prevented blad-
 350 der cancer cell growth in vivo [33]. Moreover, silencing of
 351 survivin by siRNA also induced apoptosis and attenuated
 352 tumor cell growth in vitro and in vivo [34].

353 To date, various kinds of TSP have been identified and
 354 evaluated. In the current study, the PLK and survivin

355 promoters were selected from approximately 8000 genes
 356 on our microarray chip. Many investigators have utilized
 357 the survivin promoter, whereas the PLK promoter has
 358 not been recognized as a TSP, and would thus represent
 359 a novel TSP candidate.

360 In conclusion, the promoters of PLK and survivin are
 361 esophageal tumor-specific promoters and could serve as
 362 useful tools in the establishment of a transcriptional target-
 363 ing strategy for esophageal cancer gene therapy.

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