Elevated DNA polymerase iota (Poli) is involved in the acquisition of aggressive phenotypes of human esophageal squamous cell cancer

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Abstract: DNA polymerase iota (Poli) can repair several types of DNA damage but has extremely low fidelity. Previous studies have shown an aberrantly elevated Poli expression in human esophageal squamous cell cancer tissues. However, there were few reports describing the role of Poli in esophageal cancer progression. Based on Real-time PCR assay, we found Poli expression was up-regulated in esophageal cancer tissues compared to adjacent normal tissues and overexpression of Poli was correlated to lymph node metastasis. Clonogenic assay and transwell chamber assay showed that overexpression of Poli had higher clonogenic capability and invasive tendency in human esophageal squamous cell cancer cells. Expression of cyclin D1, an important cell cycle regulator, was found to be associated with that of Poli in tissue samples and cancer cells as analyzed by real-time PCR, immunohistochemistry, Western blotting and immunofluorescence assay. Flow cytometry analysis further showed that cell cycle distribution was altered in Poli overexpressing cells. These results indicated that expression of Poli correlates significantly with tumor proliferation and invasion. We conclude that Poli is involved in the degree of aggressiveness of human esophageal squamous cell cancer.

Keywords: Esophageal cancer, DNA polymerase iota, cyclin D1, proliferation, metastasis

Introduction

DNA polymerase iota (Poli, hRAD30B), encoding an 80KD protein, is a conserved Y family DNA polymerase that participates in translesion DNA synthesis (TLS). Poli was found to be a remarkably error-prone human DNA polymerase when replicating undamaged DNA [1]. The error-prone character of Poli may lead to accumulation of DNA mutations thereby affecting genomic stability. Several studies reported up-regulation of Poli expression in human uveal melanoma, breast cancer cells and bladder cancer [2-4] (4). However, down-regulation of Poli expression was also found in human stomach, lung and colorectal cancers [5]. Several lines of evidence supported the Poli gene as a candidate in the mouse pulmonary adenoma resistance 2 locus (PAR2) responsible for higher tumor susceptibility [6]. While the controversial results may imply that Poli expression pattern is tissue-specific, the relationship between Poli and cancer progression has not been reported in any tissue types. Furthermore, the role of Poli in esophageal cancer progression has not been elucidated. Because of the error-prone DNA replication features of Poli, dysregulation of Poli may contribute to the acquisition of mutated phenotype that, along with the defective cell cycle control or disruption of other genome stability pathways, could facilitate or accelerate tumor progression. Hence, Poli may be involved in the acquisition of aggressive phenotypes of human esophageal squamous cell cancer.

Malignant nature of cancer is due to uncontrolled cell proliferation and invasive potential.
DNA polymerase iota and esophageal squamous cell cancer

Cyclin D1 is known as a key cell cycle regulator that contributes to cancer cell proliferation. Recent studies also revealed that cyclin D1 plays an essential role in cellular adhesion and migration. Cyclin D1 deficiency conferred a dramatic morphological phenotype that overrides the significant CSF-1-regulated morphological changes observed in WT macrophages [7-9]. Cyclin D1 stabilized p27Kip1, thereby inhibiting the RhoA-induced Rho-associated protein kinase and myosin light chain kinase, and promoting cell migration [8-10]. Cyclin D1/p21 signaling axis was also found to be related to tumor growth initiation and local tumor cell invasion [11].

We have previously reported that the mRNA expression of Polι was 7.2-fold elevated in human esophageal cancer tissues compared with normal controls [12]. However, the role of Polι in esophageal cancer progression remains unknown. In this study, we analyzed the expression of Polι in esophageal cancer tissues and adjacent tissues, as well as its association with clinicopathological parameters. We further elucidated the role of Polι in esophageal cancer progression and its underlying mechanisms.

**Materials and methods**

**Tissue samples**

68 human esophageal squamous cell cancer tissues and 48 adjacent tissues used in this study were obtained from patients who had not received chemotherapy and radiation therapy before surgery in 2008 at the Gastrointestinal Center, Jiangbin Hospital (Zhenjiang, China), and were immediately frozen and stored in -80°C refrigerator. All the tissues used for scientific research were collected only after signing informed consent from the patients. The study was approved by the Institutional Ethics Committee of Jiangbin Hospital. Histological features and immunohistochemical conclusions were microscopically evaluated by two pathologists according to the classification of the World Health Organization [13].

**RNA extraction and real-time PCR assay**

Total RNA from frozen tissues was extracted using Trizol (Life Technologies, Grand Island, NY, USA) and cDNA was synthesized from total RNA using an oligo (dT)12 primer and Superscript II (Life Technologies, Grand Island, NY, USA). The SYBR green dye (Life Technologies, Grand Island, NY, USA) was used in real-time PCR reactions with a Real-Time PCR System (ABI PLUS ONE, Life Technologies, Grand Island, NY, USA). The sequences of the primers were shown in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was analyzed for normalization of real-time PCR data.

**Immunohistochemistry**

The formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated in graded alcohols series. Tissue sections were washed in distilled water and PBS, and treated with 0.03% hydrogen peroxide for 5 min to block endogenous peroxidase activity. Then the sections were incubated with anti-Polι antibody (Proteintech, Chicago, IL, USA) and anti-Cyclin D1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:400 for 60 min, washed in PBS, and incubated with labeled HRP-conjugated anti-rabbit antibody (Beyotime, Haimen, China) for 30 min. The sections were washed and incubated with diaminobenzene for 10 min, followed by counterstaining with hematoxylin, dehydration and mounting. Scoring was performed blind without clinical data. Positive cell rates of 0-10, 11-25, 26-50, 51-75, and > 75% were scored 0, 1, 2, 3 and 4, respectively. The staining intensity was graded no staining (score 0); pale yellow staining (score 1); buffy staining (score 2); strongly brown staining (score 3). For Polι, the final score was defined low expression level (≤ 4 score) and high expression level (> 4 score). For Cyclin D1, the

<p>| Table 1. Primer sequences for real-time PCR analysis |</p>
<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-GAAGATGGTATGGGATTTC-3'</td>
</tr>
<tr>
<td>Poli</td>
<td>5'-TCAACCTTTCTCCCTTGAA-3'</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>5'-GAAGCGGTCCAGTAGTCTCAT-3'</td>
</tr>
<tr>
<td>MMP7</td>
<td>5'-GCAACATGTGGCACCACATC-3'</td>
</tr>
<tr>
<td>MMP9</td>
<td>5'-GACAGTAGTGTCCGTTAAG-3'</td>
</tr>
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<table>
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<tbody>
<tr>
<td>5'-GAAGATGGTATGGGATTTC-3'</td>
<td>5'-TCAACCTTTCTCCCTTGAA-3'</td>
</tr>
<tr>
<td>5'-GAAGCGGTCCAGTAGTCTCAT-3'</td>
<td>5'-GCAACATGTGGCACCACATC-3'</td>
</tr>
<tr>
<td>5'-GACAGTAGTGTCCGTTAAG-3'</td>
<td>5'-GACAGTAGTGTCCGTTAAG-3'</td>
</tr>
</tbody>
</table>

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final score was defined low expression level (< 2 score) and high expression level (≥ 2 score).

Cell culture

Human esophageal cancer cell lines ECA-109 and TE-1 were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM with 10% FBS at 37°C with 5% CO₂.

Construction of Polι expression vector

The cDNA encoding human full-length Polι gene was amplified by PCR using the following primers: Forward, 5'-TTTGGATCCATGGAGAAGCGGTTGGTG-3'; Reverse, 5'-GCCCTCGAGTTATTTATGTCCAATGTGG-3'. The PCR product was cloned into the pcDNA-3.1 vector (Life Technologies, Grand Island, NY, USA). The cloned fragment was verified by DNA sequencing. The pcDNA-3.1 vector served as control.

Western blotting

The cells were lysed with RIPA (Beyotime, Haimen, China) and the protein concentration was measured by Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Protein (60 μg) was resolved by electrophoresis in a 10% SDS-PAGE gel, transferred onto PVDF membrane (Millipore, Billerica, MA, USA) and blocked with 5% skimmed milk. Membranes were incubated with antibodies against Polι (Abcam, Cambridge, MA, USA), Cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Proteintech, Chicago, IL, USA) respectively, and then incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies (Beyotime, Haimen, China). Protein bands were visualized using ECL solution (Beyotime, Haimen, China).

Clonogenic assay

Approximately 1,000 cells were cultured in a 6-well plate for 14 days. The colony was defined to consist of at least 50 cells. Colonies were fixed with glutaraldehyde, stained with crystal violet and counted under a microscope.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 1% Triton X-100 in PBS for 5 min. Samples were then blocked with blocking solution (PBS containing 10% BSA and 1% triton-X-100) at 37°C for 60 min and incubated overnight at 4°C with the Polι antibody (Proteintech, Chicago, IL, USA). This was followed by 3 washes with PBS and then incubated with secondary Rhodamine-labeled goat anti-rabbit antibody (KPL, Gaithersburg, MD, USA) for 1 hour at room temperature. Cells were visualized using a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan).

Cell-cycle analysis

Cell cycle was analyzed as previously described [14]. In brief, cells were permeabilized by ethanol and treated with RNase A. Propidium iodide (Beyotime, Haimen, China) was applied to stain cellular DNA. Samples (1 × 10⁵ cells/well) were analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA).

Transwell chamber assay

Invasive ability of cells was evaluated in 24-well transwell chambers (Corning, Corning, NY, USA). The polycarbonate filters containing 8 mm pores were covered with 30 μl of Matrigel (BD Biosciences, Bedford, MA, USA) at 5 mg/ml. 1% BSA was used for blocking at 37°C for 1 h. Cells (10⁶/ml) were placed in upper chamber in serum-free culture medium. Medium with 10% FBS (600 μl) was added in lower chamber. After 48 h incubation, lower surface of the filter were fixed in ethanol and stained with hematoxylin-eosin. Cells invaded were counted in 5 random microscopic fields at a magnification of × 200 (DMIL LED, Leica, Wetzlar, Germany).

Statistical analysis

Statistical significance for two experimental groups was analyzed using Mann-Whitney U test or Student’s t-test. For more than two groups, a Kruskal-Wallis H test was adopted. Correlation analysis of mRNA expression was analyzed using Pearson test. Correlation analysis between IHC staining was analyzed using Chi-square test. All tests were two-sided. The data were considered significant if P < 0.05.

Results

Elevated DNA polymerase iota (Polι) in human esophageal cancer

To compare expression differences between cancer and adjacent tissues, reverse transcrip-
tase PCR and real-time PCR were used to evaluate the mRNA levels of Polι expression in 68 human esophageal squamous cell cancer tissues and 48 adjacent tissues. As shown in Figure 1A and 1B, the expression of Polι in esophageal squamous cell cancer tissues was significantly higher than in adjacent tissues (P < 0.05). This observation is consistent with our recent report showing that Polι is overexpressed in esophageal cancer as analyzed by immunohistochemistry and PCR [12].

**Poli expression was correlated with lymph node metastasis and clinical stages**

To evaluate whether Polι expression was related to clinicopathological features, the characteristics of 68 esophageal squamous cell cancer patients included in this study were described in Table 2. As Mann-Whitney U test showed, Polι expression was positively correlated with lymph node metastasis (Figure 1C, P < 0.05). Furthermore, the tumors at higher clinical stages (IIb and III) showed higher Polι expression than that of the lower stages (I and IIa) (Figure 1D, P < 0.05). In contrast, pT, as well as ages, gender, and histological grade were found no statistical differences among the groups of patients. Collectively, these findings indicated that overexpression of Polι in esophageal cancer is associated with lymph node metastasis and tumor progression.

**Elevated Polι expression promoted cancer cell clonal formation and invasion**

To understand whether overexpression of Polι in esophageal squamous cancer cells promote tumor progression, the human Polι expression vector pcDNA3.1-Polι was transfected into TE-1 and ECA-109 cells to up-regulate Polι expression. Clonogenic assay demonstrated a higher...
clonal formation efficiency in pcDNA3.1-Polι-transfected cells compared with control cells (Figure 2A, P < 0.05). The transwell chamber assay was used to explore the relationship between high Polι expression and cell invasion. As shown in Figure 2B, cells transfected with pcDNA3.1-Polι were more invasive than the control vector-transfected cells in both cell lines (Figure 2B, P < 0.05). Together these results indicated that cells with higher Polι expression exhibit more aggressive phenotypes, consistent with our observations obtained from cancer tissue samples.

**Overexpression of Polι enhanced Cyclin D1 expression in esophageal squamous cancer cells**

To characterize the molecular mechanism underlying the induction of cell proliferation and invasion by overexpression of Polι, we examined expression of several genes related to tumor metastasis using real-time PCR assay in TE-1 and ECA-109 cells. We found that there was no difference of MMP1, MMP2, MMP7, MMP9, E-cadherin, VEGFA, VEGFB and VEGFC expression between pcDNA3.1-Polι-transfected cells and control vector-transfected cells (data not shown). However, overexpression of Polι increased the expression of Cyclin D1 at both mRNA and protein levels in human esophageal squamous cancer cells (Figure 3A and 3B). Immunofluorescence staining showed that Polι was mainly located in nucleus while Cyclin D1 was expressed in both nucleus and cytoplasm (Figure 3C). Therefore, Polι may accelerate cell cycle progression by up-regulating Cyclin D1 expression.

**Overexpression of Polι increased the S phase of esophageal squamous cancer cells**

To test whether overexpression of Polι alters cell cycle progression, a cell cycle analysis was performed using flow cytometry. Detailed analysis showed that overexpression of Polι increased the proportion of S phase from 20% to 35% in TE-1 (P=0.006) cells and 22% to 41% in ECA-109 (P=0.002) cells. This was accomplished by a significant depletion of G2/M phase from 26% to 15% in TE-1 (P=0.003) cells and 27% to 7% in ECA-109 (P=0.003) cells. G1 phase was found no statistical difference between Polι overexpression and control cells in both cell lines (Figure 4). This observation support the conclusion that overexpression of Polι accelerates cell cycle progression.

**Correlation between expression of Polι and Cyclin D1 in esophageal squamous cell cancer tissues**

To verify the findings from our cell culture experiments, expression of MMP7 and MMP9 was determined in 48 esophageal squamous cell cancer tissues by real-time PCR. We found no correlation between expression levels of Polι and MMP7 or MMP9 (Figure 5). However, a significant association was found between expression levels of Polι and Cyclin D1 (Figure 6A, r=0.833, P < 0.001). Furthermore, we analyzed protein expression of Cyclin D1 and Polι using IHC staining. As shown in Figure 6B, the expression of Cyclin D1 was mostly in agreement with that of Polι. The expression of Cyclin D1 was positively associated with elevated Polι expression as analyzed by Chi-square test (r=0.55, P < 0.05). Taken together, these data, along with our observations from cell culture experiments, suggested that Polι regulates Cyclin D1 expression in esophageal squamous cell cancer.

**Table 2. Relationship between clinicopathological parameters and TNM stages in oesophageal squamous cell carcinoma (n=68)**

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>Relative Polι expression (relative to GAPDH)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 60</td>
<td>28</td>
<td>1.587 (19.557)</td>
</tr>
<tr>
<td>≥ 60</td>
<td>40</td>
<td>1.206 (4.264)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>50</td>
<td>1.293 (5.613)</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>1.495 (7.805)</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>9</td>
<td>0.941 (60.491)</td>
</tr>
<tr>
<td>Moderately</td>
<td>33</td>
<td>1.315 (9.435)</td>
</tr>
<tr>
<td>Well</td>
<td>26</td>
<td>1.587 (8.747)</td>
</tr>
<tr>
<td>pT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1-2</td>
<td>25</td>
<td>1.810 (9.648)</td>
</tr>
<tr>
<td>pT3-4</td>
<td>43</td>
<td>1.237 (5.103)</td>
</tr>
<tr>
<td>pN (-)</td>
<td>28</td>
<td>1.133 (1.016)</td>
</tr>
<tr>
<td>pN (+)</td>
<td>40</td>
<td>2.361 (19.337)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-IIa</td>
<td>26</td>
<td>1.128 (0.940)</td>
</tr>
<tr>
<td>llb-III</td>
<td>42</td>
<td>2.541 (17.338)</td>
</tr>
</tbody>
</table>


Discussion

Esophageal squamous cell carcinoma stands the eighth place in incidence and sixth place in cancer-related deaths worldwide. According to recent reports, three hundred thousand people died of esophageal cancer each year with 70% cases occurred in China [15, 16]. Multiple factors contribute to tumorigenesis such as genetic alterations, epigenetic modifications and dysregulation of key molecules [17]. Genetic alterations can be repaired by the DNA repair
system involving base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and translesion DNA synthesis (TLS). Polι is known to participate in TLS and has the lowest fidelity with T-G misincorporation at a frequency of approximately $6.7 \times 10^{-1}$. Recent studies showed that TLS across a particular DNA lesion may involve different TLS polymerases including Polι [18]. Despite its error-prone characters, the involvement of Polι in cancer development had not been clarified. Previous controversial reports [2-6] implied that Polι may act in a tissue-specific manner.

Previously, we have reported that mRNA expression of Polι was significantly up-regulated in human esophageal cancer tissues compared with normal controls [12]. In this study, overexpression of Polι in esophageal squamous cell cancer tissues was confirmed at both mRNA and protein levels, indicating the possible involvement of Polι in esophageal cancer progression. In addition, Polι expression is significantly correlated with pN and clinical stages. Thus, Polι expression may have prognostic potential for esophageal cancer outcome. Our findings were consistent with a previous report in which overexpression of Polι was positively correlated with the clinical tumor grade in bladder cancer [2].

The epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells and is essential in the initiation of metastasis for cancer progression [19]. These processes involve various factors such as cell adhesion molecules, enzymes and cytokines such as matrix metalloproteinases (MMPs), E-cadherin, and vascular endothelial growth factor (VEGF) [20]. However, we found no evidence from both tissue culture and tissue sample studies to support the association of MMP1, MMP2, MMP7, MMP9, E-cadherin, VEGFA, VEGFB, or VEGFC with Polι during esophageal cancer progression. Hence it is highly likely that other cellular mechanisms relating to tumor metastasis are involved in Polι's action in esophageal cancer. In this context, the correlation between Polι and cyclin D1 expression was found in esophageal cancer tissues and further confirmed by in vitro experiments, suggesting that Polι and cyclin D may act in synergy to promote esophageal cancer progression.

Cyclin D1 functions as a regulatory subunit of CDK4 or CDK6 and controls cell cycle progression. It is apparent that cyclin D1 promotes cell growth and acts as an oncogene [21]. However, results from several studies supported the concept that the oncogenic effects of cyclin D1 may not be simply attributed to an enhanced tumor proliferation. Studies with large samples (over 100 patients) indicated...
Figure 4. Cell-cycle analysis after transfection of the Polι expression vector in TE-1 and ECA-109 cells. Polι overexpression cells had higher percentage of S phase in both TE-1 and ECA-109 cell lines and lower percentage of G2/M phase (**, P < 0.01, Student’s t-test, n=3).
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that cyclin D1 is likely an independent esophageal squamous cancer prognostic factor [22, 23]. In bladder cancer and oral squamous cell carcinoma, cyclin D1 was found to be correlated with lymphoid node metastasis [24, 25]. The results from our clonogenic assay and transwell assay indicated that overexpression of Polι promotes esophageal cancer cell proliferation and invasion. Flow cytometry studies further showed an S-phase accumulation in Polι overexpressing cells. Given that overexpression of Polι also enhanced cyclin D1 expression as determined by western blot analysis, it is logical to conclude that cyclin D1 mediates Polι’s action in promoting esophageal cancer growth and metastasis. This is consistent with previous findings showing that cyclin D1 could interact with p27^Kip1, in mammary epithelial cells or cooperate with p21 in breast cancer cells, which plays an essential role in regulating cellular adhesion and migration [9, 11].

Translesion synthesis by DNA polymerases was an important mechanism by which cancer cells could tolerate DNA damages and help bypass cell cycle arrest [26]. As a post-replication repair DNA polymerase, Polι has been shown to be accumulated in cells entering S-phase with DNA damage [27, 28]. Hence overexpression of Polι may repair certain DNA damages and help the fulfillment of DNA replication, which promotes cell cycle progression. This is in agreement with our observations that overexpression of Polι promotes cell proliferation and metastasis. However, the molecular mechanism of how Polι might interact with cyclin D1 to regulate this process requires further investigation.

Figure 5. No correlation between Polι and MMP7 or MMP9 expression in 48 esophageal squamous cancer tissues analyzed by real-time PCR (P > 0.05, Pearson test, n=48).

Figure 6. Expression of Polι was correlated with expression of cyclin D1 in human esophageal cancer tissues. A. Real-time PCR assay of the expression of Polι and cyclin D1 (r=0.833, P < 0.001, Pearson test, n=48). B. Immunohistochemical staining of Polι and cyclin D1 (r=0.55, P < 0.05, Chi-square test, n=48).
In summary, Polι expression was significantly up-regulated in esophageal squamous cell cancer tissues and was positively correlated with lymph node metastasis and clinical stages. Polι overexpression contributed to cancer proliferation and metastasis at least in part through induction of cyclin D1. These findings illustrated a role of Polι in human esophageal cancer progression, which may serve as a therapeutic target for esophageal cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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